TANZANIA VETERINARY ASSOCIATION

Volume 35

CO-SPONSORED BY SOKOINE UNIVERSITY OF AGRICULTURE

PROCEEDING OF THE 35 SCIENTIFIC CONFERENCE OF THE TANZANIA VETERINARY ASSOCIATION HELD AT AICC ARUSHA, TANZANIA ON THE 5^{TH} *TO* 7^{TH} DECEMBER, 2017

Volume 35 2017 ISSN 0856 - 1451

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Instruction to authors are provided on the inside of the back cover of every issue of the Tanzania Veterinary Journal

Publishers: Tanzanina Veterinary Association.

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TANZANIANIAN VETERINARY JOURNAL

Tanzanian Veterinary Journal is the official Journal of Tanzania Veterinary Association which publishes Editorial Comments, Policy and Professional Matters, Field and Clinical Reports, Animal Health, Animal Production, Non-traditional Livestock production and Utilization, Wildlife Captive and Free Range Management and Health, pastures, Environment, Education and Training, Scientific Abstracts, Book Reviews, Letters to Editor, Announcements, Conference/symposium calenders, Leisure and Obituary.

TANZANIA VETERINARY ASSOCIATION PROCEEDINGS

VOLUME 35

CONTENTS

Molecular detection of tilapia lake virus (TiLV) genome in Nile tilapia (*Oreochromis niloticus*) from Lake Victoria

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SUMMARY

Tilapia lake virus (TiLV) is an emerging pathogen of Tilapiines associated with high mortalities of wild and farmed tilapia posing great threat to the fishery industry worldwide. The virus has been reported in Israel, Ecuador, Colombia, Thailand, Egypt, Taiwan, India and Malaysia. In this study, a reverse transcription polymerase chain reaction (RT-PCR) assay was developed and used to detect TiLV genome in Nile tilapia from Lake Victoria. Nile tilapia samples were collected from the Tanzanian (108 fish) and Ugandan (83 fish) parts of Lake Victoria in 2015 and 2016, respectively. Samples were screened for TiLV by using RT-PCR and the PCR products were sequenced. The findings show that out of the 191 fish examined, 28 had PCR products showing the presence of TiLV genome. The TiLV nucleic acids were detected in the spleen (10.99%, N=191), head kidney (7.69%, N=65), heart (3.45%, N=29) and liver (0.71%, N=140) samples while no PCR amplification was detected in the brain by the developed RT-PCR method. Generally, the findings show that the lymphoid organs, mainly comprising of the head kidney and spleen had the highest number of samples with positive nucleic acids for TiLV followed by heart samples. On the contrary, the liver and brain that have previously been shown to be target organs during acute infection either did not have or had the lowest level of TiLV nucleic acids detected in the present study. All the 28 sequences retrieved had an average length of 768 bp. A blast analysis on NCBI showed that all sequences obtained were homologous to TiLV segment-2 sequences obtained from previous outbreaks in Israel and Thailand. To our knowledge, this is the first detection of TiLV subclinical infections in Nile tilapia in Lake Victoria, a none-outbreak area.

Keywords: Lake Victoria, Nile tilapia, PCR, phylogenetic, surveillance, tilapia lake virus

INTRODUCTION

Tilapia lake virus (TiLV), also known as syncytial hepatitis of tilapia-SHT, was first identified and shown to cause mortalities in Nile tilapia (*Oreochromis niloticus*) in 2012 in Israel by KoVax inc. (Personal communication), following summer mortalities in tilapia fish farms in Israel. Soon after it was reported that the same virus was present in tilapia fish in the Sea of Galilee in Israel and again that this virus was causing disease and mortalities in Nile tilapia (Eyngor *et al.*, 2014b). It has since been associated with outbreaks in Colombia, Ecuador, Egypt, Israel, and Thailand (Kembou Tsofack *et al.*, 2017, Bacharach *et al.*, 2016a, Del-Pozo *et al.*, 2017b, Nicholson *et al.*, 2017, Surachetpong *et al.*, 2017, Fathi *et al.*, 2017). Based on motif alignment of its segment-1 with the PB1 segment of influenza A, B and C, the etiological agent has been characterized as an orthomyxo-like virus (Bacharach *et al.*, 2016b). Classification by the international committee of virus taxonomy (ICTV) puts TiLV as a single new species known as *Tilapia tilapinevirus* in the new genus Tilapinevirus (Adams *et al.*, 2017). It is made of 10 segments unlike other orthomyxoviruses such as influenza that are made up of eight segments (Palese and Schulman, 1976). The length of the total viral genome is about 10,323 kp (Bacharach *et al.*, 2016b, Bacharach *et al.*, 2016a, Del-Pozo *et al.*, 2017b, Eyngor *et al.*, 2014a). *In-vitro* studies show that the virus grows well at 23-30°C in vitro. Studies carried out this far show that mortalities occur at temperatures above 25°C as shown from the summer die-offs associated with TiLV in Egypt and Israel (Fathi *et al.*, 2017). TiLV has so far been reported in Nile tilapia (Egypt, Thailand) (Fathi *et al.*, 2017, Surachetpong *et al.*, 2017, Nicholson *et al.*, 2017), red tilapia (Thailand) *(Surachetpong et al.*, 2017) and the hybrid tilapia *O. niloticus* x *O aureus* (Israel

) (Bacharach *et al*., 2016b) suggesting that the range of the tilapines susceptible to TiLV could be wider.

Lake Victoria is the world's second largest freshwater lake covering a surface area of 68,000km^2 shared by three countries in East Africa namely Kenya (6%), Uganda (45%) and Tanzania (49%). By the 1960s, it was habitat for several fish species dominated by the tilapiine cichlids such as *O. esculantus* and *O. variabilis* and home to more than 200 haplochromine cichilds (Kudhongania and Cordone, 1974, Ogutu-Ohwayo, 1990, Goudswaard and Witte, 1997, Goudswaard *et al.*, 2002). Nile tilapia and Nile perch (*Lates niloticus*) were introduced in the 1950s to replace the declining tilapine species, which led to disappearance of >50% of the indigenous fish species in Lake Victoria (Ogutu-Ohwayo, 1990). Since then, Nile tilapia and Nile perch species continued to increase although by 2002, the Nile perch population began to decline giving way for the Nile tilapia to become the most dominant fish species in Lake Victoria (Njiru *et al.*, 2012, Ogutu-Ohwayo, 1994, Witte *et* fulfill the Koch's postulates by establishing the disease-causal factor relationship, virus isolation, culture and reinfection is not ideal for surveillance programs especially in situations with high number of samples. This is because the culture and reinfection approach is not only expensive, but could take long to generate results. Hence, there is urgent need for rapid diagnostic tests suitable for surveillance programs in order to expedite the process of establishing the distribution of TiLV. Moreover, developing surveillance diagnostic tools would pave way to designing appropriate disease control measures aimed at preventing the spread of the virus in the aquaculture industry. The aim of this study was twofold: 1) To develop and optimize a PCR-based method for the detection of TiLV and 2) to investigate the possible existence of TiLV in Nile tilapia found in Lake Victoria.

MATERIALS AND METHODS

Sample collection and study sites

Nile tilapia samples were collected from the Ugandan and Tanzanian parts of Lake Victoria in 2016 and 2015, respectively. For Ugandan samples, 83 fish were collected from 14 sampling sites and transported to Makerere University on ice in cool boxes. Among these, seven sites were from cage-farmed fish while the other seven were from wild fish (Table 2). Sampling for the wild sites was done in areas at least 20 – 50 km into the Lake away from the shore with minimum distances of 20 km apart while the cage farms were within 10 km from the *al*., 1991). The recent introduction of cage farming of Nile tilapia further increases its dominance on Lake Victoria. The emergence of viral diseases such as Tilapia lake virus disease (TiLVD) poses a significant threat to the expansion of Nile tilapia production, which has tremendously increased to become one of the leading cultured fish species in the world in the last decade. Furthermore, the rapid rate at which the disease is being reported to cause outbreaks in different continents across the world (Nicholson *et al*., 2017, Surachetpong *et al*., 2017, Fathi *et al*., 2017) calls for development of rapid diagnostic tools for prompt virus identification to pave way for the design of timely disease control strategies. Thus far, diagnosis of reported outbreaks has mainly been based on virus isolation, characterization, culture followed by re-infection in susceptible fish to demonstrate the characteristic syncytial hepatitis and other pathological lesions in susceptible fish (Tsofack *et al*., 2016; Del-Pozo *et al*., 2017a; Tattiyapong *et al*., 2017). Although these steps

Lake shore. Fish were dissected and processed at the Faculty of Veterinary Medicine of Makerere University. All tissues collected were stored in RNAlater and stored at 4°C for 24hrs followed by -80°C until transfer to the Norwegian University of Life Sciences (NMBU) in Oslo, Norway. In Tanzania, a total of 216 samples were collected from 108 wild fish at four sampling sites (Table 2). Two sampling sites approximately 20 km apart were sampled in Maganga beach area and another two sites in the Mchongomani area separated by approximately 25 km apart. Dissections were carried out at the Fisheries Education and Training Authority (FETA) laboratory in Mwanza and the tissues collected were stored in RNAlater at -20 °C for five days. Thereafter, all samples were transported to the College of Veterinary Medicine and Biomedical Sciences at Sokoine University of Agriculture in Morogoro where they were stored at -80°C until shipment to NMBU. Overall, a total of 442 organs from 191 fish were collected from Lake Victoria as summarized in Table 2.

Virus propagation and cell culture

Tilapia cell cultures were generated from hybrid *Oreochromis niloticus* X Oreochromis aureus. In brief, caudal fins were removed from euthanized 30g fish. Fish were then bathed in 1% sodium hypochlorite solution for 1 min, and then rinsed in 70% ethyl alcohol. Fins were washed three times in phosphate buffer saline (PBS) containing 10% penicillin streptomycin and 2.5% nystatin. The fins

were transferred to Petri dishes, extensively minced pieces of approximately 1mm3 were placed in dry 50 mL culture flasks (Nunc, Denmark). After 24 hours incubation at room temperature, the clumps adhering to the flasks were covered with Leibovitz (L-15) medium (Sigma) supplemented with 10% FBS (Biological Industries, Israel), 1% nystatin and 2.5% penicillin streptomycin. Cells were maintained at 28°C in a CO2 free environment. At 10–14 days incubation cells grew out from the tissue to form a monolayer around each clump. The monolayer cultures were trypsinized and transferred into new flasks with fresh medium. The cells have been passed for over 100 times to form a stable cell line and are referred to as Tilapia Fin Cells - TFC#10.

The virus used as a positive control in this study was provided by KoVax Vaccine Company in Israel. Virus isolation from sick fish was performed as follows: sick fish showing symptoms of apathy, reduced appetite and mortality were collected and frozen at -80°C. Kidney, spleen, intestine, gills and brains were collected and homogenized in PBS. The homogenate was filtered through a 0.2µm filter (Sartorius). Filtered homogenates were used to inoculate naïve TFC#10 cultures, incubated at 28°C and monitored daily. Cytopathic effect (CPE) appeared at 4-7 days post inoculation. Once extensive CPE was evident virus suspension was harvested, aliquoted and stored at -80°C for further use.

For the negative control cells, PBS only was used instead of the virus for adsorption. After seven days of incubation, suspensions for both virus infected and non-infected cells were harvested and used for RNA extraction as described below.

RNA extraction and cDNA synthesis

Extraction of total RNA from the 442 samples was carried out using a combination of the Trizol® (GIBCO, Life Technologies) and RNAeasy Mini kit (Qiagen, Hilden, Germany) techniques as previously described (Munang'andu *et al*., 2013, Munang'andu *et al*., 2012, Munang'andu *et al*., 2013). Briefly, approximately 30 mg of tissue was homogenized in 1mL Trizol followed by centrifugation at 12,000g for 10min at 4°C. Thereafter, the supernatant was transferred into an Eppendorf tube followed by addition of 0.2 mL chloroform to each sample. After vortexing for 15s, samples were left for 5min at room temperature followed by spinning at 12,000g for 15min. The aqueous phase was transferred into another Eppendorf tube. After adding 0.6 mL of 70% ethanol, the tubes were vortexed and the with scissors, and semi-dry small tissue contents were transfered to RNeasy spin columns. Thereafter, the Qiagen protocol was used based on the manufacturer's guidelines (Qiagen, Hilden, Germany). RNA quantification was carried out using a spectrophotometer (NanoDrop® ND-1000, Thermo Scientific Inc). The synthesis of cDNA was carried out in 20µl reaction volumes using the Transcriptor First Strand cDNA Synthesis Kit that has an integrated step for the removal of contaminated genomic DNA (Qiagen). The final cDNA was stored at −80°C until use.

Preparation of the negative control samples was done by extracting RNA and cDNA synthesis from the non-infected TFC#10 cells while RNA and cDNA synthesized from infected cells were used to prepare the virus positive controls. In addition, a second negative control was prepared by extracting RNA from headkidney, spleen and liver samples collected from six fish of the 15th Generation of Nile tilapia cultured by the GIFT project cultured at the NMBU followed by cDNA synthesis. The cDNA prepared from the GIFT fish samples was pooled for use as negative control from a population not previously exposed to TiLV. Hence, the negative control samples used in this study were designated as TCF#10 cells and GIFT tissue.

Optimization of the polymerase chain reaction test

A total of 10 primers (Table 1) were designed targeting the 10 segments of the TiLV genome. For PCR optimization, each primer pair was tested against two TiLV positive controls designated as 2V and 5V, two GIFT tissue negative controls designated as 4T and 5T, two TCF#10 cells negative control namely 2C and 3C, and one sterile water negative control (NC). The objective of using two replicates for each control sample was to compare the reproducibility of the PCR products generated after amplification between duplicates. Further, the purpose of using two negative controls (TCF#10 cells and pooled GIFT tissue cDNA) was to compare the reliability of a continuous cell-line and host tissue derived negative control during PCR optimization. Hence, each primer was tested against a total of seven samples in order to identify primers that only detect viral cDNA in order to reduce the chances of producing unspecific PCR products. All PCR reactions for amplification of the segment 1-10 genes were carried out using the Q5 High-Fidelity DNA Polymerase (New England BioLabs inc.). After gel electrophoresis analyses, only primers showing bands in the TiLV positive controls without bands in the TFC#10 cells and GIFT tissue

negative controls were selected for use in the screening of Nile tilapia samples for the presence of TiLV in the next step.

Table 1: Primer sequences

Segment	Primer sequence	Length (bp)	Tm (°C)
Segment-1	FWD-CCTCATTCCTCGTTGTGTAAGT REV-AGGAGTTGCTGTTGGGTTATAG	1000	62
Segment-2	FWD-GTCCAGGGCGGTATGTATTG REV-CTTACGGCTGACAAGTCTCTAAG	834	62
Segment-3	FWD- GTCGAGGCATTCCAGAAGTAAG REV- GAGCTAAGGGAACGGCTATTG	834	62
Segment-4	FWD-GCCTACTTCGTTGCCTATCTC REV-GCCCAATGGTTCCCATATCT	524	62
Sgement-5	FWD-CAACTCTTAGCCTCCGGAATAC REV-CGTTCTGCACTGGGTTACA	696	62
Segment-6	FWD-CCCACACGACAGGACATATAG REV- GAGTTGGCTTAGGGTGATAAGA	948	62
Segment-7	FWD-TCCTTTAGGGATTGGCACTAAC REV-TTCCATCGACTGCTCCTAGA	486	62
Segment-8	FWD-CTTAAGGGCCATCCTGTCATC REV-TGGCTCAAATCCCAACACTAA	476	62
Segment-9	FWD-GATATCCTCCACATGACCCTTC REV-GTACGTCACTTTGTGCCATTAC	261	62
Segment-10	FWD-TCCTCTCTGTCCCTTCTGTT REV-CAGGATGAGTGTGGCAGATTAT	276	62

Screening and sequencing of Nile tilapia samples from Lake Victoria

Once the PCR optimization process was completed, the selected primers were used to screen Nile tilapia samples from Lake Victoria for the presence of TiLV. As shown in Table 2, a total of 442 samples were examined from different organs including heart, liver, brain, head, kidney and spleen. All the 28 PCR products obtained from the screening of Nile tilapia samples shown in Table 2 were extracted and purified using the QIAquick Gel extraction kit according to manufacturer's instruction (Qiagen, Hilden, Germany). Amplification of the TiLV segment-2 genes was done using the Q5 High-Fidelity DNA Polymerase as described above. PCR products were then separated by using 1.5% Agarose gel electrophoresis and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing was done on a commercial basis by GATC Biotech (https://www.gatc-biotech.com). The CLC Workbench 6.0 (www.clcbio.com) and Mega7 software (Kumar et al., 2016) were used for sequence alignment and phylogenetic tree analyses. Phylogenetic trees were inferred by the Maximum Likelihood method, bootstrapped 1000 times based on the JTT+G matrix-based model (Jones *et al*., 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 36 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 270 positions in the final dataset.

Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). The results obtained for the different groups and organs were analyzed statistically using Fisher's exact test using GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA.

RESULTS

Polymerase chain reaction optimization

Figure 1 shows that PCR products obtained for segments 1, 3, 6, 8, 9 and 10 had strong bands in the TiLV positive controls (lanes 2V and 5V) and faint bands in the GIFT tissue (lanes 4T and 5T) and no bands were detected in TCF#10 cells (lanes 2C and 3C) and sterile water (Lane N). Although the presence of strong bands in the virus positive controls show that these primers detected the viral cDNA, the presence of faint bands in the GIFT tissue negative controls show that they produced unspecific amplifications. In addition, there were no PCR products for segment-4 for the positive and negative controls while PCR products for segment 7 had weak bands in the virus control and no bands were seen in the negative controls (Figure 1). However, PCR products for segment-2 were only detected in the virus controls (2V and 5V) and no bands were detected in the GIFT fish (lane 4T and 5T), TCF#10 cells (lanes 2C and 3C) and sterile water (lane N) negative controls. Therefore, the presence of clear bands in the TiLV positive controls (lanes 2V and 5V) and the absence of PCR products in the negative control was indicative that segment-2 primers were only able to detect TiLV cDNA, but not unspecified amplifications. Therefore, segment-2 primers were selected for the screening of Nile tilapia samples from Lake Victoria in the next step based on their ability to only detect viral cDNA and not host DNA, while primers for other segments were considered less suitable because either they gave some levels of unspecific amplifications in the controls or failed to detect the viral cDNA. Finally, the GIFT fish negative control was more reliable at detecting unspecific amplifications (Fig. 1, lanes 4T and 5T) compared to the TFC#10 cells negative control (lanes 2C and 3C) that showed absence of unspecific amplifications for primers tested during the PCR optimization process.

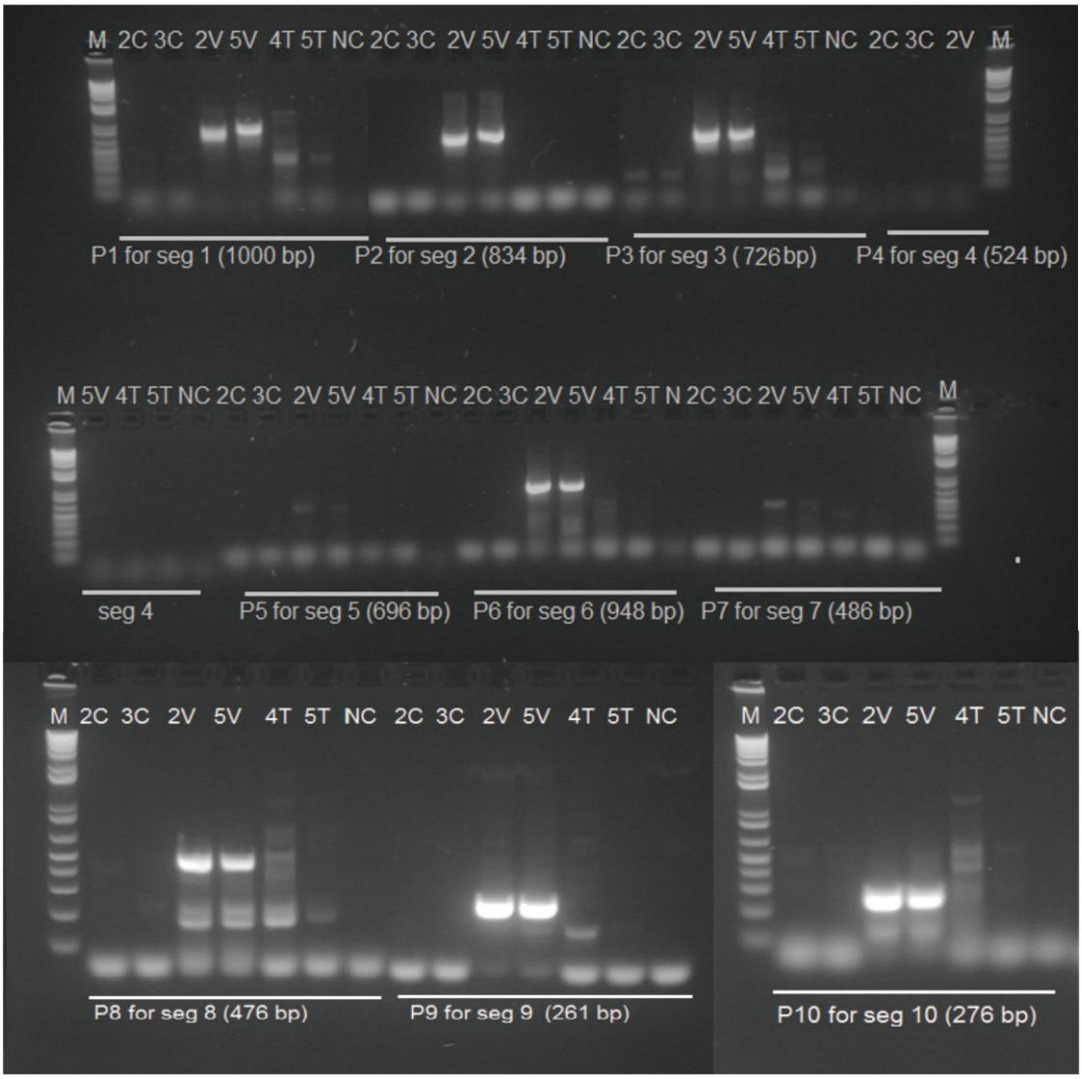

Figure 1: Shows electrophoresis gel analysis of TiLV positive control and the TCF#10 cells and GIFT fish negative control samples tested against TiLV segment 1 -10 for the 10 primers (P1 to P10) enlisted in Table 1. Note that both the positive and negative control samples are tested in duplicates in which the TFC#10 cells cDNA samples are designated as 2C and 3C, GIFT fish cDNA samples are designated as 4T and 5T while the positive virus (TiLV) control samples are designated as 2V and 5V. In addition, a single lane designated as NC for RNase free water was added to each segment tested. The expected amplicon for each primer are shown alongside the name of the segment tested. There were no detectable bands in the TCF#10 cell (lanes 2C and 3C) and RNAse free water (lane NC) negative controls for all the 10 primers tested for segments 1 -10. Note that P1, 2, 3, 6, 8, 9 and 10 showed clear bright bands of the virus positive control (lanes 2V and 5V) while P7 had a faint band in lane 2V and no bands were detected in P4. Finally, the GIFT fish samples showed faint bands in P1, 3, 6, 8, 9 and 10 in lanes 4T and 5T of variable amplicon sizes.

Screening of Nile tilapia samples from Lake Victoria

Table 2 shows a summary of the number of samples examined for the presence of TiLV nucleic acids by PCR using segment-2 primers. Of the 191 fish examined, 28 were found positive by PCR for TiLV nucleic acids with a prevalence of 14.7% (N=191). The prevalence in caged and wild fish was 17.8% (N=45) and 13.7% (N=146), respectively. There was no significance difference in the prevalence ($p=0.136$) among wild fish from the Tanzania side (16.67%, N=108) compared with the Uganda side (5.3% N=38). However, there was a significant difference ($P<0.0028$) in tissue distribution among organs examined. PCR products were detected in 10.99% (N=191) spleen, 7.7% (N=65) head kidney, 3.5% (N=29) heart and liver 0.71% (N=140) samples while no PCR products were detected in brain samples (0.0%, N=17). In summary, Table 2 shows that the lymphoid organs, mainly comprising

of the head kidney and spleen had the highest prevalence followed by heart samples.

Table 2. Sampling sites and number of fish samples

Country	Sampling site	Culture system	Positive fish/ total	Positive samples/ total	Organs (Positive/total)				
					Liver	Heart	Head kidney	Spleen	Brain
Tanzania	Maganga beach-1	Wild	4/19	4/38	0/19	-	-	4/19	-
	Mchongomani-1		7/28	7/56	0/28	-	-	7/28	-
	Maganga beach-2		6/35	6/70	0/35	-	-	6/35	-
	Mchongomani-2		1/26	1/52	0/26	-	-	1/26	
Uganda	Kigungu	Cage farms	5/8	5/32	0/8	0/8	3/8	2/8	
	Lwera		1/8	1/16			1/8	0/8	
	Kasenyi		0/8	0/8				0/8	
	Entebbe		0/5	0/5				0/5	
	Bukanama		0/5	0/5				0/5	
	SON		1/6	1/23	1/6	0/6	0/5	0/6	
	Kome		1/5	1/11			1/6	0/5	
	Lake Victoria-1	Wild	0/5	0/10			0/5	0/5	
	Lake Victoria-2		0/6	0/12			0/6	0/6	
	Lake Victoria-3		0/6	0/12			0/6	0/6	
	Lake Victoria-4		0/3	0/6			0/3	0/3	
	Lake Victoria-5		1/6	1/30	0/6	0/6	0/6	1/6	0/6
	Lake Victoria-6		1/6	1/30	0/6	1/6	0/6	0/6	0/6
	Lake Victoria-7		0/6	0/26	0/6	0/3	0/6	0/6	0/5
Total			28/191	28/442	1/140	1/29	5/65	21/191	0/17
Prevalence			14.7%	6.3%	0.7%	3.5%	7.7%	10.9%	0.0%

Sequence alignment and phylogenetic analysis

Table 3 provides a summary of sequenced samples showing their origin, organ, size of the sequence product and Genebank accession numbers. The average length of the 28 sequences retrieved was 768 bp. A blast analysis against the NCBI sequence database showed that all sequences obtained were homologous to TiLV segment-2 sequences obtained from viruses isolated from Israel and Thailand. The samples clustered into two groups (Figure 2) that were slightly different from each other and corresponded to different isolates that were already reported in the Genebank database. Group-I comprised of a total 25 Lake Victoria sequences of which nine were from Uganda and 16 from Tanzania that were identical with the Israeli KU552132 sequence deposited by Tal *et al.* (2016) and Thailand KX631922.1 sequence deposited by Surachetpong *et al*. (2017). Group-II consisted of only three Lake Victoria sequences of which one was from Uganda and two were from Tanzania that were closely related to the Israeli KU751815.1 and NC029921 sequences deposited by Eygor et al. (2014b) and Bacharach *et al*. (2016b), respectively

Table 3. Description of samples used for TiLV sequencing

Sample ID	Country	Source	Organ	Size (bp)	Genebank Acc #
UG2016-01	Uganda	Wild	Headkidney	830	MF536423
UG2016-02	Uganda	Wild	Liver	782	MF536429
UG2016-03	Uganda	Wild	Headkidney	332	MF536432
UG2016-04	Uganda	Wild	Heart	805	MF536426
UG2016-05	Uganda	Wild	Spleen	818	MF536427
UG2016-06	Uganda	Wild	Headkidney	830	MF536424
UG2016-07	Uganda	Wild	Spleen	819	MF536425
UG2016-08	Uganda	Wild	Spleen	787	MF536428
UG2016-09	Uganda	Wild	Headkidney	540	MF536430
UG2016-10	Uganda	Wild	Headkidney	724	MF536431
TZ2015-01	Tanzania	Maganga beach	Spleen	777	MF526992
TZ2015-02	Tanzania	Mchongomani	Spleen	767	MF526988
TZ2015-03	Tanzania	Mchongomani	Spleen	827	MF526987
TZ2015-04	Tanzania	Maganga beach	Spleen	755	MF526989
TZ2015-05	Tanzania	Mchongomani	Spleen	828	MF526980
TZ2015-06	Tanzania	Maganga beach	Spleen	669	MF526982
TZ2015-07	Tanzania	Maganga beach	Spleen	706	MF526991
TZ2015-08	Tanzania	Maganga beach	Spleen	675	MF526981
TZ2015-09	Tanzania	Mchongomani	Spleen	725	MF526993
TZ2015-10	Tanzania	Mchongomani	Spleen	827	MF526983
TZ2015-11	Tanzania	Mchongomani	Spleen	578	MF526994
TZ2015-12	Tanzania	Mchongomani	Spleen	758	MF526984
TZ2015-13	Tanzania	Mchongomani	Spleen	792	MF526985
TZ2015-14	Tanzania	Maganga beach	Spleen	576	MF526995
TZ2015-15	Tanzania	Maganga beach	Spleen	731	MF526996
TZ2015-16	Tanzania	Maganga beach	Spleen	765	MF526990
TZ2015-17	Tanzania	Maganga beach	Spleen	794	MF526986
TZ2015-18	Tanzania	Maganga beach	Spleen		

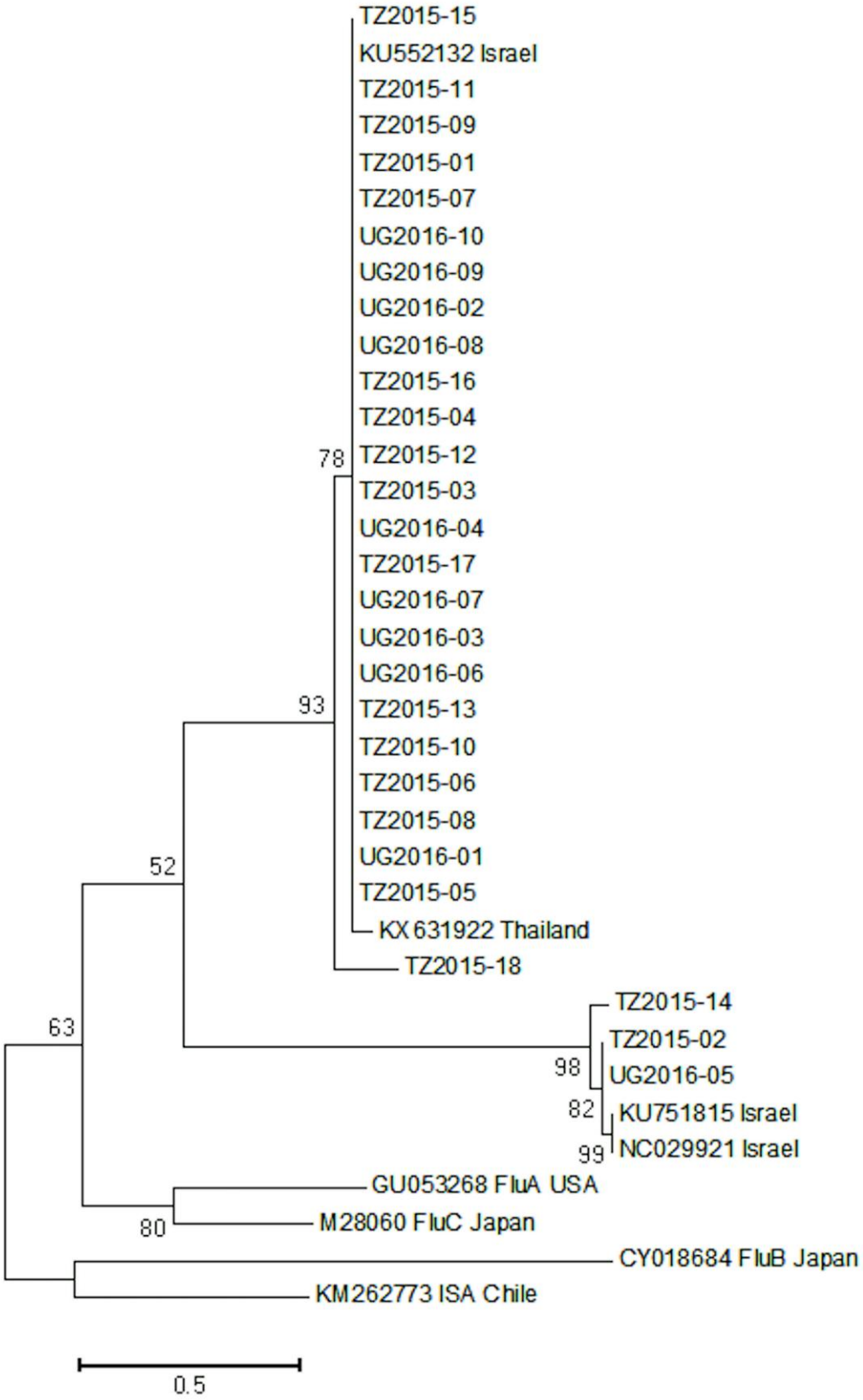

Figure 2: Phylogenetic analysis of the 28 Nile tilapia samples from Lake Victoria (TZ for Tanzanian and UG for Ugandan samples) sequenced using segment -2 primers. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993)**.**

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Infection status determined by post-challenge seroconversion

In unvaccinated cattle seroconversion is an indicator of AlHV-1 infection. This can be used in combination with PCR results to determine the proportion of unvaccinated cattle that became infected during challenge. Of 97 initially seronegative unvaccinated cattle, 40 seroconverted during the challenge phase, while a further 26 remained seronegative but were PCR positive (Table 1). In total 66 out of 97 (68%) unvaccinated cattle showed evidence of post-challenge infection, from Lake Victoria using PCR. Although we did not fulfill the Koch's postulate (Evans, 1976, Gradmann, 2014, Fredericks and Relman, 1996) by establishing the disease-casual factor relationship based on isolation, characterization, culture and re-infection to demonstrate the induction of clinical disease in susceptible fish, our data are strongly suggestive of TiLV infecting Nile tilapia in Lake Victoria. These findings have significant implications on tilapia farming and in countries using parent stocks from Lake Victoria. It is likely that as the demand for high production outputs increases, the use of intensive farming systems based on high stocking densities and artificial feeds aimed at enhancing growth rate is also bound to increase. These factors have the propensity to induce stress in cultured fish (Munang'andu, 2016), which could lead to underlying viruses to replicate culminating in disease outbreaks. Moreover, high stocking densities are proponents of a high transmission index (Munang'andu, 2016), which could increase the risk of inducing TiLV outbreaks in farmed Nile tilapia. Therefore, the detected TiLV nucleic acids in this study serve as an early warning system in which future outbreaks should be thoroughly investigated in order to confirm the possible existence of TiLV in Nile tilapia in Lake Victoria.

Our findings show an overall population prevalence of 14.7% (N=191) suggesting that in subclinical infection, TiLV could be present in a population at low prevalence only increasing to high levels reaching up to 90% mortality during massive die-offs (Fathi *et al*., 2017, Surachetpong *et al*., 2017). Detection of TiLV nucleic acids in the liver, heart, head kidney and spleen in this study is in line with previous studies in which it was shown that TiLV has a tropism for different organs inclusive of the liver, brain, spleen and head kidney when clinical signs of disease are observed (Bacharach *et al*., 2016b, Fathi *et al*., 2017, Ferguson *et al*., 2014, Surachetpong *et al*., 2017, Tsofack *et al*., 2016). Previous studies have pointed to the brain and liver of which fourdied of MCF. The proportion of infected unvaccinated cattle that died of MCF was 6%.

DISCUSSION

In this study, we detected TiLV nucleic acids in an area with no record of previous outbreaks. In line with Louws et al (Louws *et al.*, 1999) who pointed out that the three Ds of PCR analyses are detection, diversity, and diagnosis, we have; (i) detected; (ii) shown phylogenetic diversity; and, (iii) diagnosed the presence of TiLV nucleic acids in Nile tilapia as target organs of which it has been associated with syncytial formation in the liver as a pathognomonic feature of the disease, at least in one study (Del-Pozo *et al*., 2017a). The high prevalence of TiLV nucleic acids in the headkidney and spleen coupled with a low presence in the liver and absence in brain samples shown in this study suggests that lymphoid organs could be ideal for screening the presence of TiLV nucleic acids during surveillance. However, there is a need for more studies to consolidate this observation.

Phylogenetic analysis clustered our sequences in two similar groups. It is interesting to note that based on segment 2 fragments, these groups correspond with Israeli isolates as shown that group-I sequences were clustered together with the Israeli KU552132 sequence while group-II sequences were clustered with Israeli KU751815 and NCO29921 sequences suggesting that TiLV sequences found in Lake Victoria are similar to strains found in the Sea of Galilee in Israel. In addition, group-I sequences that formed the largest cluster were similar to the Thailand isolate KX631992 suggesting that TiLV sequences in Thailand, Israel and Lake Victoria might have a common origin. Given that Nile tilapia is originally a freshwater teleost species native to the Nilo-Sudanian ecoregion of Africa (McAndrew, 2000), which in recent decades has been introduced into more than 85 countries in the world (Casal, 2006, Molnar *et al*., 2008, Dong *et al*., 2017), it is likely that its dispersal could have contributed to the spread of TiLV. The existence of TiLV sequences shown in our findings suggests that the virus could have been in existence for a long time such that as tilapia was being dispersed across the world, they carried the virus unnoticed. Its emergence as a fish pathogen is most likely due to stress related factors induced by current intensified aquaculture systems as well as the increasing environment changes that stress fish in natural waterbodies. However, there is need for detailed studies to determine its distribution and to identify factors linked to its dispersal in aquaculture. Moreover, future studies should seek to

establish whether genomic differences seen between group I and II strains in this study account for differences in virulence and persistence linked to subclinical infection, tissue tropism or other factors. Although the homology between sequences obtained in this study and those from previous outbreaks in Israel and Thailand suggests that Lake Victoria sequences could be originating from a virus having the potential to cause outbreaks in Nile tilapia it is important that these findings are supported with virus isolation, culture and re-infection in future studies.

Conclusions

This is the first documentation of TiLV genomes in a none-outbreak area (Lake Victoria). The findings clearly demonstrate that viral nucleic acids were present at low-level in seemingly healthy fish. Future studies should focus on isolating the virus from Nile tilapia and demonstrate its ability to cause disease in susceptible fish.

ACKNOWLEDGEMENTS

This study was supported in part by the TRAHESA project, which is the Capacity building for training and research in aquatic and environmental health in Eastern and Southern Africa, funded by the Norwegian Development Agency, Project No. TAN/13/0027.

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Tilapia lake virus threatens tilapines farming and food security: socio-economic challenges and preventive measures in Sub-Saharan Africa

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SUMMARY

Tilapiais a traditional and favorite dish in almost all countries of Sub-Saharan Africa (SSA) and the second most produced fish worldwide. A deadly viral disease caused by Tilapia Lake Virus (TiLV) currently threatens tilapia production. This study aimed to describe TiLV disease, discuss its related socio-economic impacts in SSA, and envisage preventive measures applicable in SSA countries. PubMed, Web of Science, Scopus, Google Scholar and Research Gate were searched. Results reveal that TiLV is an RNA virus causing the disease of over 90% mortalities in tilapia. It attacks early developmental stages of tilapia, transmitted horizontally between fish, and is a potential trade-influencing transboundary animal disease. It is currently confirmed in six countries: Ecuador, Israel, Colombia, Egypt, Thailand and Taiwan. 10 SSA countries have likely imported TiLV infected tilapia fingerlings from hatcheries in Thailand and Tanzania, Burundi, Congo, Mozambique, Nigeria, Rwanda, South Africa, Togo, Uganda, and Zambia are suspected infected with TiLV. Approximately 6 Million jobs with subsequent 18 million livelihoods are expected to be affected. Food insecurity is likely to hit over 400 Million lives in the course of TiLV disease. An estimate of US$ 3 billion per year could be lost in SSA countries due to TiLV. In SSA, technologies to achieve effective control of TiLV based on measures suggested by OIE, and FOA do not exist. There is a crucial need for capacity building among farmers and technical personnel on prompt diagnostic procedures and effective remedial action and establishment of outbreak response systems.

Keywords: Tilapia; fish diseases, aquatic health, aquaculture, food security, Tanzania.

INTRODUCTION

Globally, fish currently represents16.6% of animal protein supply and 6.5% of all protein for human consumption (World Bank, 2013). Tilapines, is a generic term for edible fish belonging to the family Cichlidae and commonly known as tilapia. They are fast growers, efficient food convertors and relatively disease-resistant (Nicholson *et al.,* 2017). These assets render them most suitable for farming and their demand is growing as populations grow (Lem *et al.,* 2014). Tilapia are farmed worldwide and serve as an important protein source, because of their affordable price, omnivorous diet, and tolerance to high-density farming methods (Tsofack *et al.*,2016). Tilapia serve as the second most important group of farmed fish worldwide with an annual production of 4.5 million tons and the current global tilapia trade has value of more than USD 7.5 billion (Dinesh *et al*., 2017).

In Africa, Egypt ranks first and second after China worldwide with regard to global tilapia output (Nicholson *et al.,* 2017). Cultureof tilapia in Sub-Saharan Africa (SSA) has grown at an annual average rate of 20% over the last decade with about 150,000 tons produced in 2012 (De Graaf and Garibaldi, 2014).The contribution of sub-Saharan Africa to global aquaculture production remains very small but is increasing significantly; between 2000 and 2008 there was an increase in production from 55 802 to 238 877 tons (Handisyde *et al.,* 2014).Although aquaculture is still recent in Africa and mostly concentrated in a few countries, it already produces an estimated value of almost US$3 billion peryear (De Graaf and Garibaldi, 2014).

Most disease reports in tilapia implicate bacterial pathogens, e.g. *Aeromonas hydrophila* and *Streptococcus* species with fewer reports of viral

diseases (Nicholson *et al.,* 2017). Viral pathogens in tilapia include betanodavirus, iridovirus, and herpes-like virus (Surachetpong *et al*., 2017). At the time this paper is being written, six countries have reported a new viral disease named "Tilapia Lake Virus" (TiLV) disease. The affected countries are Ecuador (Ferguson *et al.,* 2014), Israel (Eyngor *et al.,* 2014), Colombia (Tsofack *et al.,* 2017), Egypt (Fathi *et al*., 2017; Nicholson *et al.,* 2017), Thailand (Dong *et al.,* 2017a; Surachetpong *et al*., 2017) and Taiwan (FIS, 2017). Outbreak reports and experimental studies with TiLV have demonstrated mortalities from 10% to 90% and up to 100% during co-infections with other microorganisms (Dong *et al.,* 2017b; Surachetpong *et al.,* 2017; Tattiyapong *et al.,* 2017).Cohabitation trials by Tattiyapong *et al.* (2017) demonstrated that the disease is contagious and can spread from one fish to another and that under controlled conditions mortalities occur within few days post-infection, leading to the death of 80-100%. Recent updates from Dong *et al.* (2017a) reveal that tilapia egg, fry and fingerling samples collected from previous disease outbreaks in several tilapia hatcheries in Thailand during 2012-2017 have tested positive for TiLV. These records suggest that many countries have been translocating tilapia fry/fingerlings prior to and even after the first reports of TiLV in Thailand (Dong *et al.,* 2017b).

Besides the six already confirmed countries, it seems that 43 countries, including 10 SSA countries, i.e. Burundi, Congo, Mozambique, Nigeria, Rwanda, South Africa, Tanzania, Togo, Uganda, and Zambia, may have imported TiLV-infected tilapia from hatcheries in Thailand (Dong *et al.*,2017a). Although the TiLV disease has not yet officially been detected and reported in any of these SSA countries, the Food and Agriculture Organization (FAO) has raised alertsand called for awareness and active surveillanceof TiLV worldwide (FAO, 2017). Human and animal infectious diseases usually spread fast and often uncontrolled in SSA countries and TiLV has the potential to cause serious socio-economic impact on tilapia farmers and fishers in SSA. This study therefore aimedto review the available literature on TiLV and discuss the potential socio-economic impact and disease control and management challenges faced by SSAcountries where disease control is known to be difficult compared to other countries in the world.

MATERIALS AND METHODS

Data sources and searches

We conducted a systematic literature review on the global situation of TiLV disease. Scientific reports were identified by searching PubMed, Web of Science, Scopus, and Google Scholaras well as ResearchGate. The search was performed in English during 25 June to 25July 2017with no time limitation imposed. The search string used was the following: "Tilapia Lake Virus", "Syncytial hepatitis of tilapia", "update TiLV", "alert Tilapia lake virus" "TiLV Africa", "TiLV developing countries", "TiLV Sub-Saharan Africa". Moreover, specific documentation was selected from FAO and WorldFish websites and other literature sourceson the socio-economic importance of aquaculture and tilapia farming in Sub-Saharan Africa as well as challenges related to fish health management in SSA countries.

Data collection and eligibility criteria

For this review, only articles written in English were considered. We first studied titles and abstract of all the articles and retrieved data. Several criteria were used to select eligible studies which included that studies should be conducted on Tilapia lake virus; reports of TiLV in Africa; importance of tilapia farming in SSA; reports on economic aspects of aquaculture in Africa and studies describing biosecurity and disease control challenges in SSA aquaculture. Information about TiLV disease included country of study, risk factors and detection methods of TiLV, clinical manifestations, geographic distribution, epidemiology and etiologic information. Reference lists of full-text publications and textbooks were also examined to identify studies not retrieved by the original search. Other papers not related to TiLV disease description were selected from the literature purposively to respond to challenges and prevention strategies for aquatic health management in low-income countries.

RESULTS AND DISCUSSION

Current research on TiLV

A total of 42 scientific papers, 13 books, reports, and six webpage were retrieved with the search queries and only 18 were retained and used in the study based on the aforementioned eligibility criteria for TiLV disease description. However, none of these studies was conducted in Sub-Saharan Africa, nor did they address SSA specific matters. They were concentrated on large-scale commercial fish production rather than small-scale fish farming with proposed solutions that can hardly be implemented in low-income countries in SSA.

Description of the causative agent of TiLV disease

Tilapia lake virus (TiLV) disease is caused by a novel RNA virus in the family Orthomyxoviridae (Eyngor *et al.*, 2014; Bacharach *et al*., 2016; del-Pozo *et al.*, 2016). It is a round to oval enveloped and filamentous/tubular virus of 60 to 80 nm size with negative-sense, RNA genomes of 10-segments and10, 323 kb total length (Dong*et al*., 2017b; Tattiyapong *et al*., 2017). Electron micrographs of the TiLV are shown in Figure 1 (Tattiyapong *et al.*, 2017). In its segmented genome, the largest segment (segment 1), contains an open reading frame with weak sequence homology to the influenza C virus PB1 subunit, while the other nine segments show no homology to other viruses but have conserved, complementary sequences at their 5' and 3' termini, consistent with the genome organization found in other Orthomyxo viruses (Bacharach *et al*., 2016). Genetic information of the virus can be found trough the GeneBank accession numbers provided in the NCBI database by the authors. Available sequences from Israel include KJ605629 (clone 7450, ORF) (Eyngor *et al*. 2014) and KU751814 to KU751823 (whole genome, segments 1 to 10) (Bacharach *et al.*, 2016). Thailand strains include KY615742 (segment 1), KY615743 (segment 5) and KY615744 to KY615745 (segment 9) (Dong *et al.*, 2017a), as well as KX631921 to KX631930 (whole genome, segments 1-10) and KX631931 to KX631936 (segment 1, originating from different Thailand provinces) (Surachetpong *et al.*, 2017).

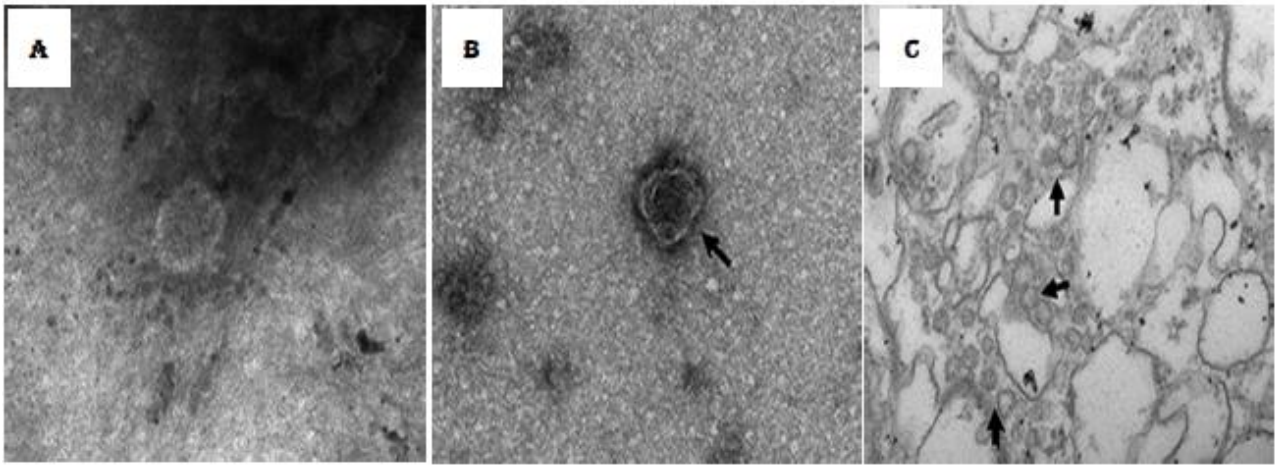

Figure 1. Transmission electron micrograph of infectedE-11 cells and ultrathin sections of infected tilapiabrain tissue. (A and B) High magnification of a freevirion showing a round enveloped viral particle with60 to 80 nm diameter. (C) Ultrathin section of infected tilapia brain showing multiple viral particlesin the cytoplasm of infected cells marked with black arrows (Source: Tattiyapong *et al.*, 2017)

Habitats and hosts of TiLV

The virus is found in fresh and brackish water (OIE, 2017) and have so far been observed only in wild tilapia *Sarotherodon galilaeus*, farmed tilapia (*Oreochromis niloticus*) and commercial hybrid tilapia (*Oreochromis niloticus* X *Oreochromis aureus*) (Bacharach *et al.*, 2016; Ferguson *et al.*, 2014; Eyngor *et al.*, 2014). Thus, TiLV has a narrow host range infecting only tilapines while other species which are reared with tilapia remain unaffected (Dinesh *et al.*, 2017). Although only tilapines have been shown to be susceptible to date, it is possible that other species will be found susceptible since the virus can undergo mutations. Nevertheless, it is still not clear why only tilapines are infected and what are the specific receptors of the virus in tilapia compared to other fish species. The only controversial report in terms of species affected so far is from Egypt, where

mortalitiesaround 9% due to TiLV were observed in medium to large sized Nile perch, whichbelong to the Latidae and not the Cichlidae as reported by the World Organization for Animal Health (OIE, 2017). Additional, studies are needed to confirm that Nile perch is also susceptible to TiLV.

TiLV tropism and susceptible host age

The virus has a multiple tissues tropism including liver, brain, kidney, spleen, gills and connective tissues of muscle (Dong *et al.,* 2017a). However, the central nervous system (brain) and the liver of tilapia remain the main targets of the virus where it probably has its best receptors by inducing viral encephalitis and syncytial hepatitis (Bacharach *et al.,* 2016; Tattiyapong *et al.,* 2017). It is possible that the pathogenic agent can also be found in musculature of infected fish (OIE, 2017).

Outbreaks and experimental studies have indicated that TiLV affects mainly early developmental stages of tilapia, i.e. fertilized eggs, yolk-sac larvae, fry and fingerlings (del-Pozo *et al.,* 2016; Dong *et al.,* 2017b; Tattiyapong *et al.,* 2017). Surachetpong *et al.* (2017) reported that higher mortalities were seen among fish weighing 1 to 50 g, whose immunity is still low, while adults or larger sized tilapia seems to resist the virus. TiLV disease is frequently reported within one month after fry or juvenile tilapias have been moved from hatcheries to the grow-out cages (Tattiyapong *et al*., 2017; OIE, 2017). The affected age group brings suspicions of possible vertical transmission ornonspecific absorption of viral particles on these life stages, an issue that requires further investigation. Additional questions to be resolved also include the replication cycle and pathogenesis of the virus to understand its infection process, specific receptors involved and immunology.

Ferguson *et al.* (2014) noted that a strain of tilapia (genetically male tilapia) incurred a significantly lower level of mortality (10-20%) compared to other strains. This was concluded by the OIE (2017) as evidence those certain genetic strains of tilapia are resistant to TiLV. Moreover, Dinesh *et al.* (2017) noticed no more outbreaks in the same pond once the initial mortality ceased. Such information gives hope for vaccine development and for gene profiling for the promotion of genetically improved tilapia strains that are resistant to TiLV. However, since TiLV infects very early developmental stages of tilapia (fertilized eggs, fry, and fingerlings) when fish immune system is not fully developed, the use of vaccines may not be an effective control approach.

Mode of transmission of TiLV

The main transmission route of TiLV is horizontal through cohabitation giving a high likelihood for disease transmission via water and transport of live fish (Tattiyapong *et al.,* 2017). Nicholson, *et al* (2017) noticed that the Egyptians strains of TiLV have high nucleotide identity with Thai and Israeli strains. Similar observations were made by Dong *et al*. (2017a) who concluded that TiLV strains circulating in Asia are all genetically related, confirming the risk of cross country and cross continents spread of the virus. Nevertheless, there are possibilities of vertical transmission since the virus is present in early developmental stages of fish such as larvae, fries and fingerlings (Dong *et al.,* 2017b). It could be that the immune system of adult tilapia makes them resist the disease's manifestations and they are therefore, in most cases, asymptomatic carriers that pass the virus to their offspring. There is limited information about TiLV biophysical properties and the risks associated with aquatic animal products. Since biophysical characteristics of the virus are not well described, it is unclear to determine the significance of indirect transmission by fomites (OIE, 2017). Infected populations of fish, both farmed and wild, are the only established reservoirs of infection but the original source of TiLV is not known (Ferguson *et al.,* 2014; Dong *et al.,* 2017b). The most important risk factors are associated with stress (Ferguson *et al.,* 2014) but no studies have provided evidence on other physico-chemical risk factors such as temperature, salinity, Ph, etc.

Current geographical distribution of TiLV

Countries that officially reported TiLV disease when this paper is being written are Ecuador (Ferguson *et al.,* 2014), Israel (Eyngor *et al.,* 2014), Colombia (Tsofack *et al.,* 2017), Egypt (Fathi *et al.,* 2017; Nicholson *et al.,* 2017), Thailand (Dong *et al.,* 2017a; Surachetpong *et al.,* 2017) and Taiwan (FIS, 2017). However, 43 other countries are currently at risk of occurrence of the disease and this include 10 SSA countries such as Burundi, Congo, Mozambique, Nigeria, Rwanda, South Africa, Tanzania, Togo, Uganda, and Zambia (Dong *et al.,* 2017a). Investigations of TiLV are lacking in most SSA countries and we do not know the actual distribution of the virus in the SSA region. For instance, the etiology of mortality in tilapia in Ghana and Zambia in 2016 (OIE, 2017) have not yet been confirmed.

Clinical manifestations of TiLV disease

Natural and experimental infections of fish by TiLV display the same clinical signs that reflect the tropisms of the virus. Ferguson *et al.* (2014) and del-Pozo *et al*. (2016) described TiLV disease as a syncytial hepatitis because of its liver related symptoms. However, other authors reported liver and brain affected symptoms in TiLV infected fish (Dong *et al.,* 2017a; Tattiyapong *et al.,* 2017). Symptoms comprising lethargy, endophthalmitis, skin erosions, renal congestion, and encephalitis were reported by Dinesh *et al*.(2017).General clinical manifestations of TiLV disease include black discoloration, skin abrasions, and ocular alterations like opacity of the lens (cataract), ruptured lenses with endophthalmitis accompanied by swelling of the eye ball with occasional perforated cornea and shrinkage and loss of ocular functioning in advanced cases (OIE, 2017; CGIAR, 2017).Other lesions include skin erosions and moderate congestion of the spleen and kidney as well as lesions of the brain such as edema, focal hemorrhages in the leptomeninges, and capillary congestion in both the white and gray matter (Dinesh et al., 2017; Tattiyapong *et al*., 2017). Indicative signs in infected tilapia ponds include mass mortality (20-90%), loss of appetite, pale color, gathering in the bottom, slow movement, and stopped schooling prior to death (Eyngor *et al.,* 2014; NACA, 2017). Figures 2 and 3 illustrate clinical signs of TiLV disease.

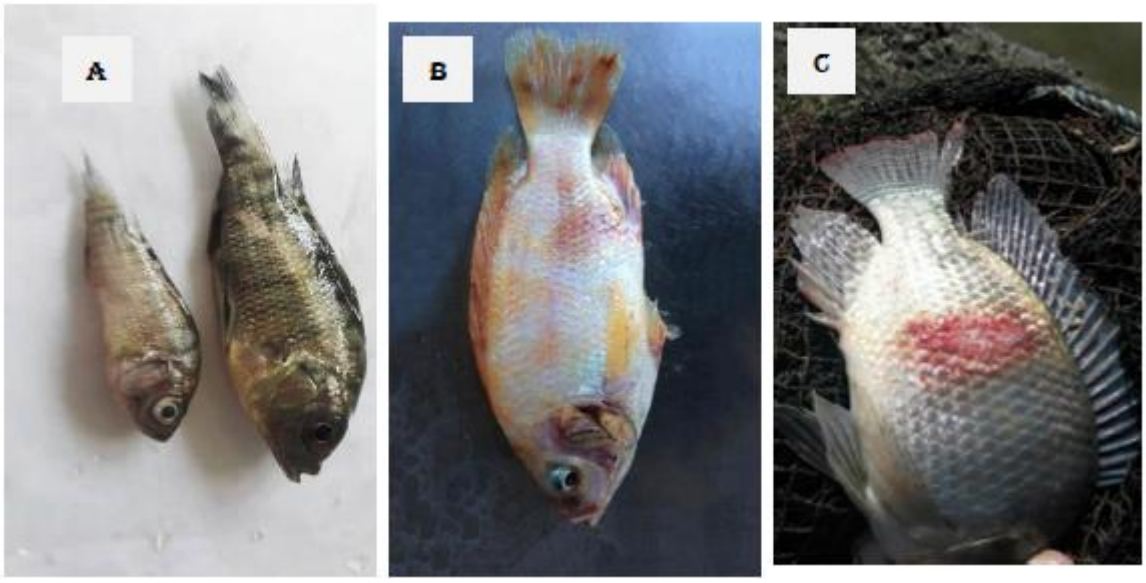

Figure 2. (A) Naturally TiLV-infected Nile tilapia revealed discoloration, and scale protrusion; (B) Naturally TiLV-infected red tilapia juveniles with congestions; (C) Skin lesions in TiLV-infected tilapia. (Source: Jansen and Mohan, 2017).

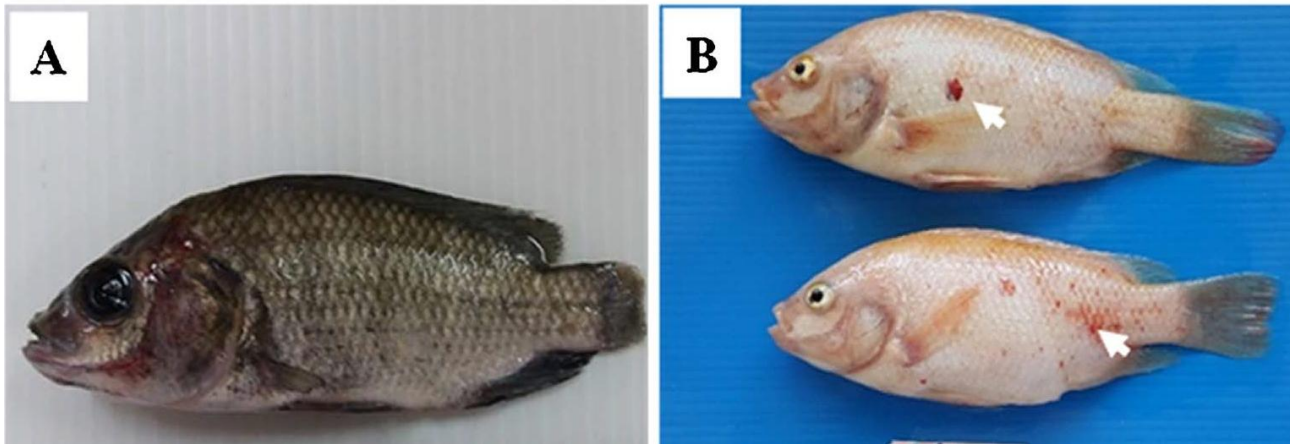

Figure 3. (A) Experimentally TiLV-infected Nile tilapia with skin erosion, hemorrhage and ocular alterations**;** (B) Experimentally infected Red tilapia with skin hemorrhage (arrow), mild exophthalmos and abdominal swelling. (Source: Tattiyapong *et al.,* 2017).

Detection methods for TiLV

Several detection methods are available and range from isolation of the virus to molecular techniques. Reported molecular methods for TiLV detection include nested RT-PCR, semi-nested RT-PCR, SYBR (Synergy Brand) quantitative RT-PCR and *in situ* hybridization (Bacharach *et al*., 2016; Dong *et al*., 2017a; Tsofack *et al.*, 2017). Moreover, electron microscopy (Tattiyapong *et al*., 2017), high nucleotide sequencing, unbiased high-throughput sequencing (UHTS), Northern hybridization and mass spectrometry (Dinesh *et al.*, 2017) were reported efficient in TiLV diagnosis. Isolation of the virus is feasible by propagation in E-11 cells lines, a continuous cell line from snakehead fish *Ophicephalus striatus* (Tsofack *et al.*, 2016; Tattiyapong *et al.*, 2017). Advanced detection methods include viral metagenomics with the potential to identify novel viruses without prior knowledge of their genomic sequence data and may provide a solution for the study of uncultivable viruses (Munang'andu *et al.*, 2017). Currently, the easiest detection method for laboratories that do not have necessary biosafety level for virus isolation is the alternative semi-nested RT-PCR described by Dong *et al*. (2017c). It is an improved semi-nested RT-PCR protocol based on a previous protocol described by Eyngor *et al*. (2014) and Tsofack *et al*. (2017) targeting TiLV genome segment 3 by omitting the primer Nested ext-2. Actually, sequencing results of amplicons from the first procedure of Eyngor *et al*. (2014) demonstrated that the primer Nested ext-2 was similar to a fish gene and lead to false positive results. Since the error was likely to be from that primer Nested ext-2, Dong *et al*. (2017c) then developedan alternative semi-nested RT-PCR in which the primer Nested ext-1 and ME1 are employed in the first round amplification, then 7450/150R/ME2 and ME1 are used in the second round PCR. With this new protocol, heavily infected samples generate two amplicon bands of 415bp and 250 bp while lightly infected samples generate a single 250-bp amplicon band (Dong *et al*., 2017c).

Prospective socio-economic impacts of TiLV diseasein SSA

The fisheries sector in SSA is confronted with serious depletion of most wild captures because they either have reached their production limit or are over-fished (World Fish, 2009). Additional losses due to TiLV will certainly worsen the situation and subsequently cause poverty, malnutrition, unemployment and food insecurity in the region. The majority of small-scale fish farmers in SSA stock their ponds with fingerlings obtained from other local farmers (De Graaf and Garibaldi, 2014). Since TiLV is more virulent in early life stages of tilapia, a crucial fish scarcity is plausible with subsequent impact on fish availability among poor communities whose livelihoods depend on this activity. The impact on local fish trade is expected to be significant in low-income settings although it might not be felt in the global fish market since the level of tilapia production in SSA is very low (World Fish, 2009). In Egypt for instance, the overall impact of mortalities observed in tilapia aquaculture in the summer of 2015, which was suspected to be caused by TiLV, was estimated at 98,000 tons of lost production at a value of USD 100 million affecting 37% of the country´s fish farms (Fathi *et al*., 2017). Egypt is however, a leading aquaculture producer as compared to the entire SSA countries and even if the economy of most SSA countries does not heavily depend on tilapia farming, the economy of populations whose life directly depends on aquaculture and fisheries are likely to be severely affected. The value added by the African fishery sector as a whole in 2011 was estimated at more than US$ 24 billion, 1.26% of the GDP of all African countries (De Graaf and Garibaldi, 2014).

According to FAO, the fisheries sector employs 12.3 million people in Africa as full-time fishers or full-time and part-time processors, representing 2.1% of Africa's population of between 15 and 64 years old (De Graaf and Garibaldi, 2014). Since tilapia is the second most encountered fish in this system, it is therefore predictable that the impact of TiLV that causes up to 90% mortalities could be catastrophic in SSA. Nevertheless, most tilapia in SSA countries are wild caught (*Handisyde et al.*, 2014) and this could reduce the impact because although wild tilapia is indicated susceptible to TiLV, most reports on TiLV disease showed high mortalities in cultured tilapia only (Ferguson *et al.*, 2014; del-POZO *et al*., 2016; Dong *et al.*, 2017a). However, if we stick to the fact that both wild and cultured tilapia are susceptible to TiLV (Eyngor *et al.*, 2014; Bacharach *et al*., 2016) and can develop mortalities up to 90%,we could estimate that the income source of at least 6 million people (half of the 12.3 million involved in the fisheries in SSA is at risk. Based on this estimation, if we suggest for instance that each of these 6 million people supports three dependents, the livelihood of 18 million people including children is at risk of serious food insecurity in SSA. Such situation is susceptible to lead to social crisis such as theft and exodus towards

the already crowded urban areas with all the associated health and environmental corollaries.

About 400 million Africans consume fish, predominantly tilapia, which contributes essential proteins, minerals and micronutrients with annual demands for fish expected to increase by 2.6 million tons by 2030 (World Fish, 2009, World Fish, 2011; Lem *et al.,* 2014). Food availability and accessibility is therefore endangered. A disease that can make vulnerable the food source of over 400 million people (Africa alone) needs serious attention and consideration. So far, few initiatives e.g. from international organizations such OIE (2017) and FAO (CGIAR, 2017) has considered locally adapted preventive measures in SSA countries to limit the emergence of the virus in the region.

The global economic impact of TiLV is estimated at US$7.5 billion per annum, especially among the top tilapia-producing countries (NACA, 2017). According, to Dong *et al*. (2017b), 10 SSA countries are already at high risks of emergence of TiLV and some of them have recorded suspicious mortalities in farmed tilapia (OIE, 2017). As TiLV is mainly transmitted horizontally through infected live fish (Eyngor *et al.,* 2014), the spread from aquaculture systems to natural water bodies is very likely because the outlet water of most aquaculture ponds in SSA are connected to natural water bodies such as lakes and rivers. If TiLV gets into African great lakes and rivers, it will create serious unprecedented food insecurity and economic crisis on the continent especially among populations whose income depend on fisheries and small-scale fish farming. Further model-based risk assessment studies are needed to quantify and appreciate the possible losses that can be due to TiLV when it occurs in natural waters in Africa.

Baylis *et al.* (2017) reported that the Chilean Infectious Salmon Anemia (ISA) outbreak bankrupted the aquaculture industry in 2007 and left debts of US$1.8 billion. Low-income rural communities were particularly badly affected, and an estimated 13,000 jobs were lost (Baylis *et al.,* 2017). This is evidence that SSA countries, because of their poor disease control systems should begin active surveillance to prevent TiLV outbreaks in order to avoid such irreversible losses. Another example is the outbreaks between 2010 and 2014 of acute hepatopancreatic necrosis disease (AHPND) in shrimp culture which was estimated to have caused a US$1 billion annual loss to the shrimp farming industry (Baylis *et al.,* 2017). Therefore, TiLV should not be given such chance to worsen poverty, malnutrition and unemployment in SSA.

Challenges related to the management of TiLV diseasein SSA

Control measures and biosecurity procedures are provided by FAO and OIE on TiLV management (OIE, 2017; CGIAR, 2017; Jansen and Mohan, 2017). However, these measures are yet to be applied in SSA because aquaculture in this region is still at small-scale level whereas the proposed solutions are based at commercial larger scale operations. The fear of global spread of TiLV together with the fact that most imports of fish products from commercial hatcheries are directed towards locations where TiLV has not been reported, have resulted in distorted solutions and policies in respect of disease management and trade in fish products (OIE, 2017). According to OIE (2017), live fish imported for aquaculture should undergo screeningand possibly be quarantined. However, experiences from the veterinary field have proven that such measures hardly work in SSA countries where farmers have inadequate resources and infrastructures to quarantine animals (Lupindu *et al.,* 2012). Poor capacity in biosecurity remains a major obstacle to most agricultural trade in Africa and limits farmers' incomes. In SSA countries aquaculture production is still in the hand of rural farmers. Most farmers have limited resources, little or no knowledge of aquaculture health management and with inadequate opportunities to improve management skills and respond effectively to diseases. Moreover, most of them have little knowledge about symptoms of different diseases or when to apply treatments (Idowu *et al.,* 2017). In a situation of total stamping out for containment when the virus emerges in a particular farm, there is typically no compensation policy for the farmers in SSA, making the solutions proposed by OIE and FAO difficult to apply in African settings. Moreover, 'stamping out' often results in large numbers of fish being destroyed, which is unacceptable in most poor SSA countries (Thomson, 2009).

The transboundary nature of most water bodies in SSA countries is a serious vulnerability factor for the rapid continental spread of the virus when it jumps from aquaculture to natural environment and becomes hard to control. There is a high probability that infected fish from aquaculture systems in Burundi, Tanzania, Congo, Mozambique, Uganda and Zambia can get into the African great lakes of southeastern Africa mainly Lake Victoria, Lake Tanganyika, Lake Malawi, Lake Kivu, Lake Edward, the Congo River, the river Nile and more. Nigeria on the other hand shares a basin with Lake Chad, another African great lake of the continent. In

the west, the Volta and Mono Rivers are at high risks of contamination by TiLV especially because Togo, which is declared at risk, is in this gulf.

Since some fish, especially adults (Ferguson *et al.*, 2014; Fathi *et al.*, 2017) have recovered from TiLV disease; vaccination was regarded as a possible prevention method besides sanitary prophylactic measures (OIE, 2017; Jansen and Mohan, 2017). Nevertheless, there are various challenges that can hamper the effectiveness of TiLV vaccination in SSA. First is the cost-effectiveness. Most aquaculture farms in SSA are small-scale and cannot afford expensive vaccines as reported in other animal diseases (Lupindu *et al.*, 2012). The currently available vaccine (TV1 by KoVax in Israel made of attenuated strain of TiLV) requires some levels of technology and infrastructures as well as financial necessities that may limit its use in SSA countries. The required cold storage and vaccine delivery are constraints for field application of the vaccine. It is unlikely that vaccination will be possible for tilapia in natural water bodies like African great lakes.

Most SSA countries are characterized by poor laboratory services, which are hampering disease control. In many parts of the subcontinent, laboratories lack resources and expertise (Bankolé *et al.*, 2015). As a result, they cannot keep up with diagnostic demands, and proper diagnosis and response is delayed (Mwabukusi *et al.*, 2014). In SSA countries, most laboratories have no vital expertise and skills for fish diseases for several reasons. These include lack of capacity in this particular field, professionals retiring and emigrating. The consequence is a lack of mentorship and proper training for new experts. A further problem is that many veterinary and fisheries technologists have not kept up with current knowledge and new technologies.

TiLV disease can be classified as a trade-influencing transboundary animal disease (TAD) because it is able to spread quickly and affect a large number of animals (fish) over a wide geographic area in a short period of time and can affect trade of tilapia from countries that are declared infected. In SSA, the control of most TADs (including TiLV disease) is challenging for a variety of technical, financial and logistical reasons (Thomson, 2009). Sub-Saharan Africa is consequently confronted with a complex problem that contributes significantly to retarded rural development, which, in turn, impedes poverty alleviation. The fact is that the epidemiology of most TADs, not to mention performance characteristics of vaccines and other control mechanisms, preclude any realistic SSA-based prospect of success for effective control. Therefore, instead of adopting technologies from developed countries whose solutions are based on large-scale production, it makes more sense to conjugate efforts on managing the impact of TiLVusing local realities because the contexts differ.

Preventive and control measures for TiLV disease in SSA and perspectives for further investigations

Effective control of TiLV in SSA countries should aim at preventing introduction of the disease orits propagation in case it occurs. Most diseases affecting fish including TiLV are stress related (Ferguson *et al.*, 2014), thus affordable disease prevention and control practices should center ongood husbandry (management) practices; good water quality management, nutrition and sanitation.

The use of locally produced larvae and fingerlings should be promoted from local and regional farmers or breeders known to have no record of mass mortality with acceptable safety levels controlled by mandated fisheries officers. Healthy fish obtained from such reputable sources must possibly be quarantined using locally available means before being released to culture ponds. Other local measures could include the use of mobile technologies, which are already proven great in timely disease reporting in SSA countries (Mwabukusi *et al.*, 2014) to give alerts on occurrence of TiLV for prompt and adequate response. Innovations such as rapid field diagnostic tools for TiLV must be considered for SSA countries where special laboratory skills are scarce. Since TiLV is still a new virus, continuous capacity building is needed to train and strengthen laboratory technicians and field aquatic health officers in SSA to be able to detect and respond to TiLV outbreaks. At elementary level, a relatively disease-free water supply is vital. Introduction of organic matter to the pond water must be controlled. Proper and appropriate feeding schedule should be ensured. Maintaining a suitable stocking density is also necessary as overcrowding stress fish and eventually predispose them to infections. In addition, proper handling of fish is necessary to reduce the risk of surface injuries capable of predisposing fish to TiLV. Governments in SSA countries may impose and sponsor TiLV screening to all fish feed or fish products imported to the countries to minimize the risk. In a farm with many ponds and despite the limited resources available in the farms, it is advisable to have separate nets for each pond so that

TiLV outbreak in one is prevented from being transferred to the others. General prophylactic measures applicable at resource-limited levels include pond disinfection by fallowing and liming, which can prevent diseases in pond from being carried over to subsequent culture year.

Vaccination against TiLV could be considered in SSA countries if the vaccines are thermostable, cost-effective, applicable at low dosage for small farms and cheap, accessible and affordable to low-income farmers. Sponsored vaccines by Governments or locally produced vaccines against TiLV are encouraged for the control of TiLV in SSA.

Governments of SSA countries and agricultural extension services are expected to take measures adapted to low-income settings such as local sensitization for awareness rising on the virus, elaboration of manuals with locally applicable Biosecurity rules and affordable containment procedures. Collaborations between SSA countries for contextualized solutions are needed for integrated and sustainable control and prevention of TiLV because the virus does not need a visa to cross borders. There is a need to protect fisheries and aquaculture trade by enhancing international collaboration in fighting TiLV via integrated holistic approaches like One-Health approach although human health is not at risk. It urges that SSA countries invest as soon as possible in prevention and control of TiLV by designing SSA-based (i) regional guidelines; (ii) national strategies on TiLVdisease management; (iii) rapid diagnostics and therapy; (iv) Surveillance and reporting; v) research; (vi) institutional strengthening and manpower development (education, capacity building and extension, diagnostic services). Possibilities should also explore natural product research for TiLV prevention and treatment because most animal diseases in Africa can be treated or prevented by means of locally available medicinal plants, which are freely accessible for farmers (Dougnon *et al.*, 2017).

Moreover, OIE recommended strict restrictions on the movement of live tilapines from farms and fisheries where the virus is known to occur (OIE, 2017). To facilitate rapid dissemination of information, participatory approaches at all stakeholder levels should be encouraged. Collaborative programs between theprivate sector and relevant governments should be promoted to limit the impact of TiLV and the associated disease.
In summary Tilapia Lake Virus disease is devastating fish disease causing high mortality in Tilapia with subsequent losses in the fisheries sector mainly in aquaculture. It is a serious threat to tilapia farming and food security worldwide. Every government should support researchers to begin active surveillance of the virus for early detection and control. Based on local realities of SSA countries, awareness creation and capacity building among farmers, veterinarians, and laboratory staff and fisheries officers is highly needed for effective management of TiLV disease in these countries. Pond water and natural water bodies also need to be sampled and analyzed for thorough risk characterization.

ACKNOWLEDGEMENTS

The authors acknowledge The African Centre for Health of Aquatic Resources for providing free access to the internet for data search and sorting.

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Molecular characterization of infectious bursal disease virus detected in Morogoro, Tanzania

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SUMMARY

Infectious bursal disease (IBD) virus (IBDV) is a double-stranded RNA virus that belongs to the genus *Avibirnavirus* of the family *Birnaviridae*. IBDV is a causative agent of IBD, the highly contagious viral infection of young chickens aged 3 to 6 weeks. IBD outbreaks occur frequently in both vaccinated and non-vaccinated chickens in Tanzania causing significant economic loss among poultry keepers. The control of IBD is mainly done through vaccination, which requires the understanding of molecular and biological characteristics of circulating virus strains in particular geographic location. This study was conducted to determine the genotype of IBDV recovered from confirmed IBD outbreak(s) in 2014 in Morogoro, Tanzania. The investigation was performed by reverse-transcription polymerase chain reaction (RT-PCR), sequencing and phylogeny analysis of nucleotide sequences corresponding to the VP2 hypervariable (VP2-HVR) domain of IBDV. The findings indicated 100% detection rate (n = 10) of IBDV genome from infected bursa of Fabricius samples. Phylogenetic analysis revealed that the sequenced virus belonged to the African very virulent IBDV (VV-IBDV) genotype and was genetically closely related to KZC-109 strain detected in Zambia in 2004. Taken together, our findings suggest that the African VV-IBDV detected in this study was responsible for the IBD outbreak(s) in Morogoro. Further studies are required to examine the transmission dynamics, evolutionary characteristics and antigenicity of field IBDV strains order to design the appropriate control method(s) of IBD in Tanzania and neighboring countries.

Key words: IBDV, VV-IBDV, Sequencing, Phylogeny, Tanzania

Infectious bursal disease (IBD) or Gumboro disease is a highly contagious immunosuppressive viral infection of young chickens (3–6 weeks old) causing severe economic and production losses worldwide (Müller *et al*., 2003). The disease is highly contagious affecting young chickens and characterized by destruction of lymphoid organs and in particular the bursal of fabricius where B lymphocytes mature and differentiate. The cloacal bursa is the target organ of IBDV infections; however, IBD viral replication also occurs in other lymphoid structures including the spleen, thymus, Harderian gland, and ceca tonsil. The immunosuppressive effects of IBDV infections not only enhance the chicken's susceptibility to secondary opportunistic infections such as gangrenous dermatitis, chicken anemia agent, inclusion body hepatitis, respiratory diseases, and E. coli infections among others, but frequently interfere with effective immune responses to vaccination (Jacqueline, 2010).

The disease was initially recognized in 1957 as clinical entity responsible for acute morbidity and mortality in broilers on Delmarva peninsula (Hirai *et al*., 1972). Predominant signs of illness included trembling, ruffled feathers, watery diarrhea, anorexia, depression, severe prostration, and death. In addition, hemorrhages in the thigh and leg muscles, increased mucus in the intestine, liver lobe infarction, renal damage, and enlargement of the bursa of Fabricius were lesions commonly observed at necropsy (Cosgrove, 1962). Early studies suggested that the causative agent was a nephropathogenic strain of infectious bronchitis virus due to similar gross changes observed in the kidneys (Winterfield and Hitchner, 1962; Pejkovski *at al*., 1979; Winterfield *et al.*, 1978). However, revealed that IBV immunized birds could still be infected with the infectious bursal agent and develop changes in their cloacal bursas specific for the disease. Following successful isolation of IBA in embryonated chicken eggs (Winterfield *et al.*, 1962; Hitchner, 1970), proposed that the disease be termed infectious bursal disease due to its pathognomonic bursa lesions. The immunosuppressive effects of infectious bursal disease virus (IBDV) infections were first disclosed by Allan *et al.* (1972). In 1980, a second serotype was reported McFerran *et al*. (1980).

The condition spread rapidly and was recognized throughout the US broilers and commercial egg production areas by 1965, and by 1967 the highly infections nature of infectious bursal disease virus was recognized followed by reliable developments of methods to isolates the virus in embryonated eggs and to adapt it to tissue culture. In1976, the agent was characterized as a virus belonging to a new taxonomic group. The immunosuppressive property of IBDV was first recognized in and was confirmed in 1976.The first isolate of IBDV was isolated in

Gumboro, Delaware in USA in1965 (Winterfield, 1962) and from there spread worldwide causing greater economic lossesin poultry industry (Hirai *et al*., 1972). The immune suppression that results from an IBDV infection has a major economic impact on the broiler and layer chicken industries. Often the immune suppression goes unnoticed because the disease is sub-clinical in nature. Thus, the true economic impact of IBDV as the underlying cause of opportunistic respiratory and enteric diseases and vaccination failures is difficult to estimate (OARDC, 2004).

Infectious bursal disease (IBD) is caused by Infectious bursal disease virus (IBDV) a double-stranded dsRNA virus that has a bi-segmented genome and belongs to the genus Avibirnavirus of the family Birnivirida (Swai *et al*., 2011). Although turkeys, ducks, guinea fowl and ostriches may be infected, clinical disease occurs solely in chickens. Only young birds are clinically affected. Severe acute disease of 3–6-week-old birds is associated with high mortality, but a less acute or subclinical disease is common in 0–3-week-old birds. This can cause secondary problems due to the effect of the virus on the bursa of Fabricius. Two distinct serotypes of infectious bursal disease virus (IBDV) are known to exist. Serotype 1 virus causes clinical disease in chickens younger than 10 weeks. Older chickens usually show no clinical signs. Antibodies are sometimes found in other avian species, but no signs of infection are seen. Serotype 2 antibodies are very widespread in turkeys and are sometimes found in chickens and ducks. There are no reports of clinical disease caused by infection with Serotype 2 virus. (OIE, 2008).

IBDV genome consists of two segments of double stranded RNA (dsRNA), segments A andB. The large segment A contains partially overlapping open reading frames (ORFs), ORF1 and ORF2. The small ORF1 encodes a non-structural protein VP5, whereas the large ORF2 encodes a precursor polyprotein (NH2-VP2-VP4-VP3-COOH), which is cleaved by auto proteolysis to produce the viral capsid protein (VP2), the ribonucleoprotein (VP3) and the viral protease (VP4). The smaller segment B encodes VP1 and RNA-dependent RNA polymerase (RdRp) responsible for viral genome replication and RNA synthesis.

Based on serology and in vivo challenges, serotype 1 IBDV strains are classified into very virulent (VV), classical (virulent, mild/intermediate or attenuated), antigenic variant, and Australian classic genotypes. The hypervariable region (HVR), which spans amino acids from position 206 to 350 within VP2 (VP2-HVR), is known to be critical for determination of the conformational epitopes responsible for recognition of virus neutralizing antibodies in VP2. The VP2-HVR has the highest amino acid sequence variation among serotype 1 strains and the nucleotide and deduced amino acid sequences within this region are widely usedfor molecular diagnosis and genotyping of IBDVs **(**Kasanga *et al*., 2007**).**

Since its discovery in the USA in 1961, numerous IBDV isolates have been detected continually in chickens from different parts of the world (Cosgrove 1962). Reassorted viruses have recently been discovered in different parts of the world. In China, two reassortant strains which have a segment A evocative of a cell-culture-adapted vaccine virus but a VV-IBDV-like segment B, have been described (Wei *et al*., 2006). Also, the study by (Kasanga *et al*., 2012) to sequence the full-length genome of an IBDV field strain detected from Zambia and to analyze its genetic characteristics, determined the entire coding sequences of KZC-104 strain which provide the first evidence for the occurrence of reassortment of natural genome segments A and B in IBDV in Africa.

Furthermore, the study revealed that the KZC-104 genome had 96 to 99.5 % nucleotide sequence identity to that of the other very virulent strains, and it was most closely related to the KMRG-48 strain from Tanzania, T09 from Nigeria, D6948 from Netherlands and KS from Israel. Since 1987, pathotypic IBDV variants with enhanced virulence, called very virulent IBDVs (VV-IBDVs), emerged in Europe, and have spread to many places of the world. These strains were called European VV-IBDV. The first IBDV isolates from various locations in Tanzania were characterized as very virulent (vv) type in 2007.

These 'variants' were found to be widely distributed throughout Tanzania and demonstrated great similarities with isolates from western Africa and European/Asian vv IBDV variants (Swai *et al*., 2011). In Tanzania is insufficiently studied but it appears that IBDV is the most important recurring disease every year. Although IBDV represents one of the most severe poultry diseases and is responsible for marked economic losses, few studies of IBDV have been done on chickens in Tanzania, which hinders the implementation of effective disease control measures (Swai *et al*., 2011).

Infectious bursal disease (IBD) is among the most important constraints for commercial and local chicken production in Tanzania (Matovello and Maselle, 1989). Genetic and antigenic characteristics of circulating infectious bursal disease virus (IBDV) strain have not been extensively studied in Tanzania. The vaccine used in controlling IBDV is of classical type discovered in 1960's without considering variationsdue to continuous genetic mutation of IBDV stains. Control of IBDV in Tanzania is mainly done through vaccination. However, the vaccines used do not match with the antigenic features of the prevailing viruses. Therefore, this study will focus on the seasonality of IBD virus infection and strain identification so that preventive and control programmes can be designed. The aim of the study was to establish genetic characteristics involved with specific objectives to identify IBDV genome and determine genotype of field IBD viral strain involved in IBDV infection Morogoro Municipality

MATERIALS AND METHODS

Study area and sample size

The study was conducted in Morogoro municipality-Tanzania in which 10 broiler chickens were collected in Kihonda ward following an outbreak. The area lies within longitude 37.67^{o}E and latitude 6.82^{o}S and receives the total annual rainfall of 935mm. According to 2012 census, the population is 315,866. The area is located in the southern highlands of Tanzania 169 km west of Dares salaam, 223km East of Dodoma and 511meters high from sea level. The temperature varies depending on the season with average annual range of 17°C to 28°C.A total of ten broiler chickens three month were selected and collected from one household keeping broilers at Kihonda ward where outbreak of IBD occurred based on clinical signs of the disease. A cross sectional was adopted and study was conducted within a period of three months from September to December

Sample collection and preparation

The chickens were collected and humane euthanized in the laboratory by air embolus in which air was introduced in blood circulation via wing vein. Then, burse of fabricius were aseptically removed from 10 affected broiler chickens, chopped them using two scalpel blades and a small amount of peptone broth containing penicillin and streptomycin (1000 µg/ml each) was added following homogenizing them in tissue blender. Then the homogenate was centrifuged at 3000 ***g*** for 10 minutes and supernatant fluid was harvested for use in the investigations.

IBDV RNA extraction

The IBDV RNA was extracted from bursal of fibricius using Viral RNA Min Extraction Kit (Qiagen) followed by Reverse Transcription. The process was performed by preparation of RT master mix as shown in Table 1. Then 10µl of the prepared RT master mix was put in each epedorff tube followed by addition of 10µl of RNA sample in each tube. The overall process was conducted under suitable cold chain. The sample mixture was subjected into thermal cycler under the following conditions; cycler for RT under the following conditions; 25ºC for10 min, 37ºC for 120min, 85ºC for 5min.

Table 1. Master mix for Reverse Transcription

No.	Component	Volume (µL)
1	10×RT buffer	10.0
2	25×dNTPs	4.0
3	10×RT Random primers	10.0
4	Reverse Transcriptase	5.0
5	RNase Inhibitor	5.0
6	Nuclease free water	3.2
	Total	37.2

Polymerase chain reaction (PCR)

The PCR was done targeting the VP2 HVRs which have molecular diagnostic features using theprimers V1 forward primer (5-CCA GAG TCT ACA CCATAA-3) and V2 reverse primer (3-TAC GAA AGAGTG GCA ACA GG-5) (Kasanga *et al*., 2007). The process was done by preparing PCR Master Mix using the reagents given in the Table 2. Then 20µl of PCR Master Mix was added in each PCR tube and finally 5µl of template as RT product was added in each sample under ice condition. The sample mixture was put in the PCR machine and the reaction was carried out under the following condition; denaturation at 94^{o}C for 30 sec, annealing at 57^{o}C for 30 sec, elongation at 72°C for 30 sec followed by last extension at 72°C for 5 min.

Table 2. Master Mix for Polymerase chain reaction (PCR)

No.	Component	Volume (µl)
1	2×PCR ready mix	62.5
2	Forward primer (V1)	1
3	Reverse prime (V2)	1
4	Nuclease free water	27.7
	Total	92

Electrophoresis

electrophoresis of the PCR products was performed. That 1.2 g of agarose powder was mixed in 100mls of TBE in a conical flask and boiled using hot plates with magnetic stiller to make clear solution. Then the samples were loaded in wells 10µl electrophoresis and stained in ethidium bromide 0.5 mg/ml was used to stain the DNA. The reaction was carried out at 100V for 45min. UV transilluminator was used for visualization of DNA bands.

Purification of obtained DNA amplicons was performed based on Qiagen quick PCR purification protocol. Cycle sequencing was performed using 5x sequencing buffer, Big Dye Terminator, primer, DNA template and water as reaction reagents. The reaction was performed under the following conditions; 96ºC for 1min in one cycle, (96ºC for 10sec, 50 ºC for 5sec, 60 ºC for 4min and 4 ºC as

Data analysis

The primary sequence data were edited by BioEdit software, then aligned using CLUSTALW and subjected to BLAST searches to determine their identity with other strains. Phylogenetic analysis was performed using MEGA 7 software by using the neighbor-joining method to determine the evolutionary relationship.

Agarose gel electrophoresis of the PCR ampliconsto confirm the PCR reaction, 1.2% agarose gel the storage temperature in 25 cycle). Then, ethanol precipitation was done in the tubes, in which to each reaction 5µl 125Mm EDTA and 60µl 100% EtOH. Then the mixture in the tubes were vortexes and left in the dark for 15min at room temperature. And centrifuged for 30min. All the supernatant was removed following addition of 60µl 70% EtOH and vortexed for few seconds and centrifuged for 30 min following remove all the supernatant. Then Vacu-dry was performed for 15min in the dark following addition of 20 µl Hi-Di Formamide then loading in the sequencing machine. Sequencing of PCR product was performed at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture using AB 3500 Geneticanalyzer.

RESULTS

PCR results

Diagnosis of IBDV was done using PCR employing diagnostic primers V1/V2 (5-CCA GAG TCT ACA CCATAA-3) / (3-TAC GAA AGAGTG GCA ACA GG-5). Following amplification of IBDV DNA, single major amplicons of approximately 472 bp was generated in all ten samples (Figure 1).

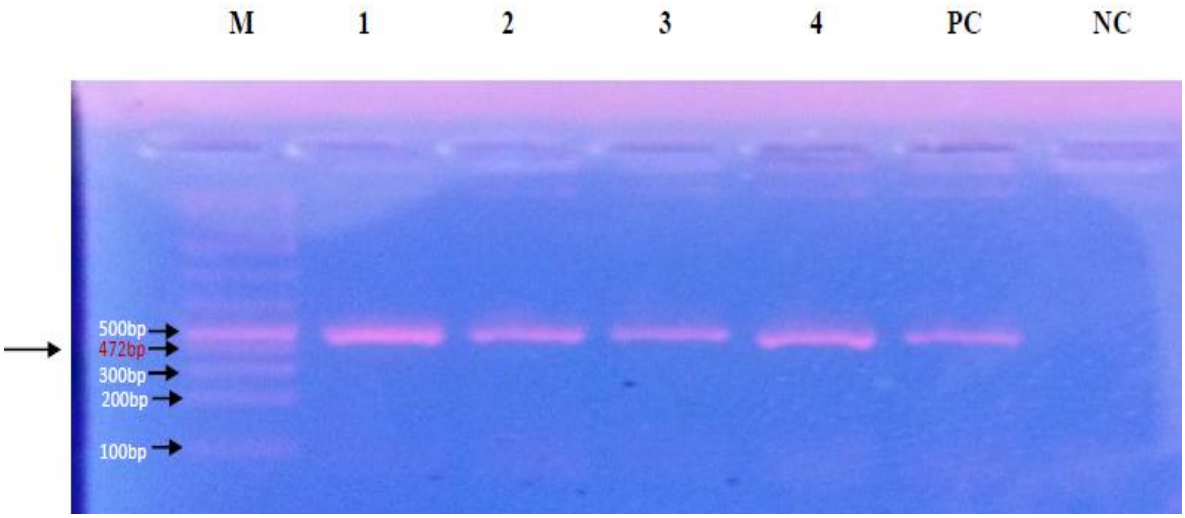

Figure 1. An Agarose gel after performing IBDV PCR using V1/V2 primers. Diagnostic PCR for the diagnosis of IBDV produced a PCR product of approximately 472 bp shown by arrow. All samples were positive for IBDV. Key; M = marker, number 1to4 = samples, PC= positive control, NC= negative control.

Sequencing results

Two PCR products samples 4& 5 out of ten were sequenced and both shows similar results. The strain obtained after sequencing was named M-/MRG/04/2014/C. Obtained sequence was compared to sequences of IBDV available in GenBank for genetic relatedness, in Table 3.

Phylogenic analysis

The data obtained in GenBank above was used to construct phylogenetic tree using neighbor-joining method to determine the evolutionary relationship (Figure 2).

Table 3. Percentage nucleotide identity of T-MRG/0414/C to IBDV in GenBank

% Identity	Strain	Year	Country	Genotype
94	KZC- 109	2004	Zambia	VV- A type
93	KMRG 38	2005	Tanzania	VV- EU type
93	TI/TW	2000	Taiwan	VV- EU type
93	Br/99/BN	1999	Brazil	VV- EU type
92	IBDV/NG2010	2010	Nigeria	VV- EU type
91	HuB-I	2007	China	VV- EU type

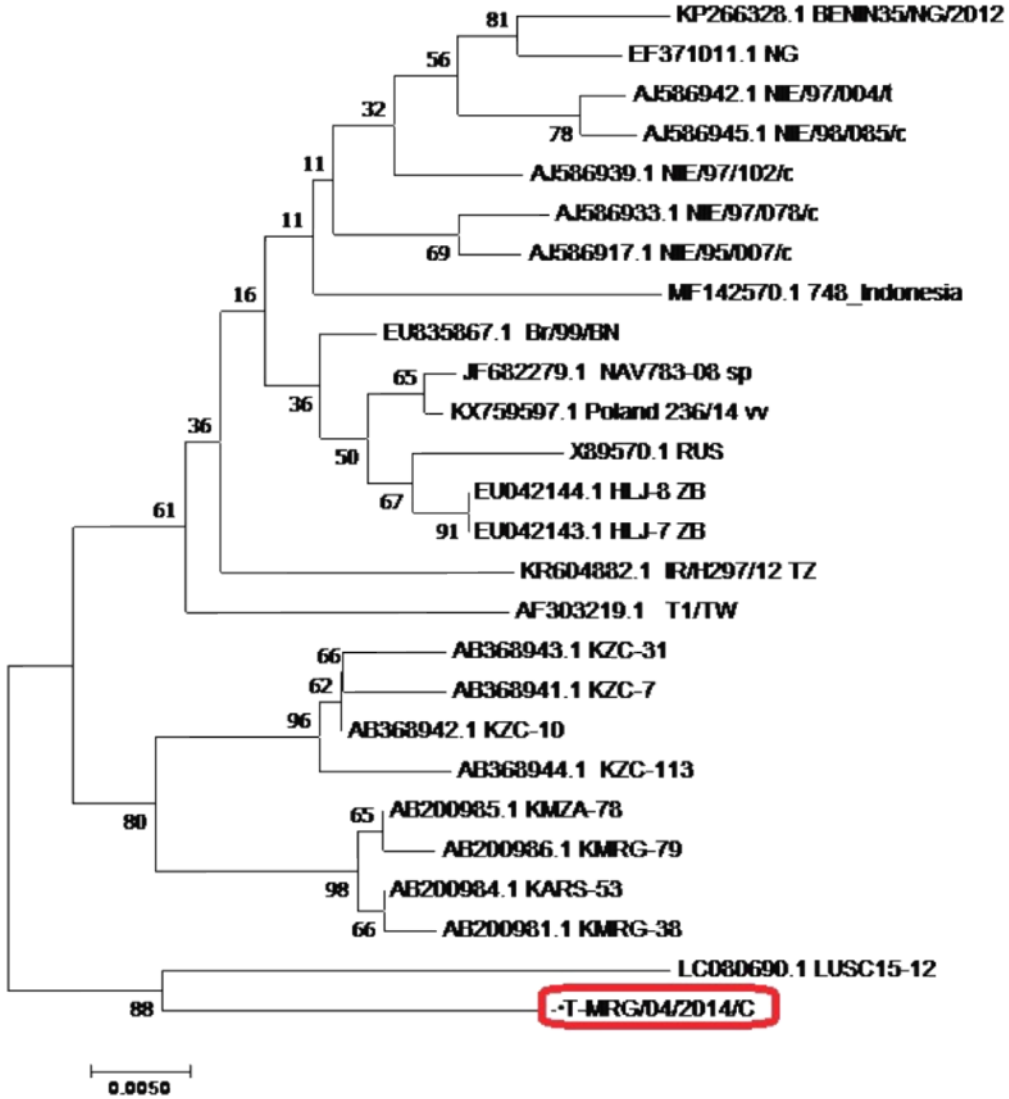

Figure 2. Phylogenetic tree of T-MRG/04/14/C indicating genetic relatedness of IBDV serotype 1

DISCUSSION

The results obtained from the present study confirmed that IBD caused massive death of broiler chickens in Kihonda ward Morogoro. This was confirmed after performing PCR using diagnostic primers V1/V2 that target a conserved region of VP2 HVRs which have molecular diagnostic features. Analysis of M-/MRG/04/2014/C sequences obtained showed that the virus isolate was 94% related to other previously reported outbreaks virulent European and classical type.

Based on the results of this study, it can be concluded that from the results obtained in this study the IBDV strain caused the massive death of young chickens in outbreak occurred in2014 in Morogoro region of Tanzaniais identical to Zambia (KZC-109) which is VV African type. The disease is still a threat in the poultry farming in the region dueto continuous genetic mutation of IBDV stains Zambia(KZC-109) which is VV African type, 93% related to KMRG 38(VV EU type) Tanzania, T1/TW (VV EU type) Taiwan, 92% IBDV 71/NG/2010(VV EU type) Nigeria, 91% HuB-1(VV EU type) China This IBDV isolate belongs to genotype I and also clusters together with the latter outbreaks isolates in the phylogenetic analysis. This shows that M-/MRG/04/2014/C strain is antigenically close related to very virulent African type which are different from very

and the vaccines used do not match with the antigenic features of the prevailing viruses. It is recommended that **f**urther studies are required to investigate**;** Evolutionary characteristics of the A – VV-IBDVFactors responsible for its maintenance and spread as well as development of rational vaccine for control of the African type VV-IBDV.

ACKNOWLEDGEMENTS

We are grateful to the IFPHTM project sponsored by Wellcome Trust for kindly financing this research. The laboratory technologist; Mr. Hashim Matimbwa, Ramadhan Juma, Ms. Herbertha Mpete and Ms. Arafa Rupia for their technical support as well as farmers in Morogoro for their cooperation and providing us the samples. Thanks to everyone who supported this work in one way or the other.

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Retrospective study on laboratory results of African Swine fever virus in Tanzania

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SUMMARY

A 10-years records on diseases was retrieved so as to investigate the occurrences of African Swine Fever (ASF) disease in Tanzania over a period of year 2007/2008 to 2016/2017. A total of 640 samples were tested for ASF virus at the Centre for Infectious Diseases and Biotechnology (CIDB), Dar es Salaam. The samples included whole blood, swabs and internal organs from domestic pigs in different areas of Tanzania. Annual, monthly and origin distribution of suspect cases was analyzed. ASF occurrences was confirmed in each year from 465 (72.6%) samples. High numbers of positive cases were in recorded in February 94 (20.2%), March 198 (42.5%), April 35 (7.5%), May 36 (7.7) and June 56 (12.0%). Regional distribution of positive samples were 45.8% from Iringa, 16.9% from Dar es Salaam, 12.3% from Mbeya, 10.3% from Coast,9.4% from Morogoro and the remaining 0.51% were from Kilimanjaro, Rukwa, Arusha and Tanga, Kagera regions. Largest number of samples were tested in 2012/2013 (255 (39.8%) and 2011/2012 (96 (15%). The study reveals endemicity of ASF disease in Tanzania and its trend in occurrence. Further studies need to be undertaken to establish factors for the endemicity of the disease so as to mitigate its occurrence and spread for the control of the disease.

Keywords: African Swine Fever disease, endemic, Retrospective study, Tanzania.

INTRODUCTION

African swine fever (ASF) is a highly contagious and fatal disease of domestic pigs caused by double-stranded DNA virus that belongs to genus Asfivirus and family Asfarviridae. The transmission of the virus is direct and vector-borne, and the disease has sylvatic and domestic cycles. In a sylvatic cycle, it involves soft ticks of the Ornithodorus species and warthogs as well as in domestic pig populationswith or without involvement of Ornithodorus ticks. The domestic cycle involves domestic pigs spreading the virus to other domestic pigs through direct or indirect contact (Penrith and Vosloo, 2009). The virus is highly resistant in tissues and the environment, contributing to its transmission over long distances (Wilkinson, 1989). Wild pigs act as reservoir hosts; this poses a constant threat to domestic pigs.

In the last two decades, pig production has shown remarkable growth in Tanzania in terms of pig population, pork production and consumption (FAO, 2005; URT, 2012, FAO, 2012a). Similar developments have been observed in other parts of Eastern and Southern Africa (ESA) countries (Waiswa et al., 2009; Mutui *et al.,* 2010, FAO, 2012b) and Asia (Delgado *et al.*, 1999; Psilos, 2008); however, the industry is hampered by ASF disease.

The aim of the study was to investigate the occurrence of ASF disease in Tanzania based on the retrospective data retrieved from CIDB for the years spanning 2007/2008–2016/2017 to give an insight in the epidemiology of the disease in Tanzania.

MATERIALS AND METHODS

Data of all samples tested for ASF virus at the Centre for Infectious Diseases and Biotechnology during 2006/2007-2016/2017 were retrieved, compiled and analysed. The samples were submitted to CIDB from different areas of the country. The types of the samples submitted were whole blood and internal organs. Examination of ASFV was done by polymerase chain reaction (PCR). DNA was extracted using a QIAamp nucleic extraction kits (Qiagen, Hilden, Germany) according to manufacturer's instructions. Examination of ASFV was done by polymerase chain reaction (PCR) targeting a conserved region of the *B646L* (p72) gene as described by Aguero et al., 2003. Data analysis was done by descriptive and inferential statistics.

RESULTS

During a period of ten years, from 2007/2008 to 2016/2017 a total of 640 samples were submitted at CIDB for diagnosis of ASF disease with average of 64 samples being submitted yearly. The total

positive cases were 465 (72.6%) and the disease was diagnosed in each year during the entire period under study. On average the annual occurence of ASF during the 10 years retropsective study was 64 (10%). The occurrence of ASF disease according to the regions is summarized in Figure 1. It was noted that, ASF frequently occurred in Iringa, Dar es Salaam, Mbeya, Coast and Morogoro regions.

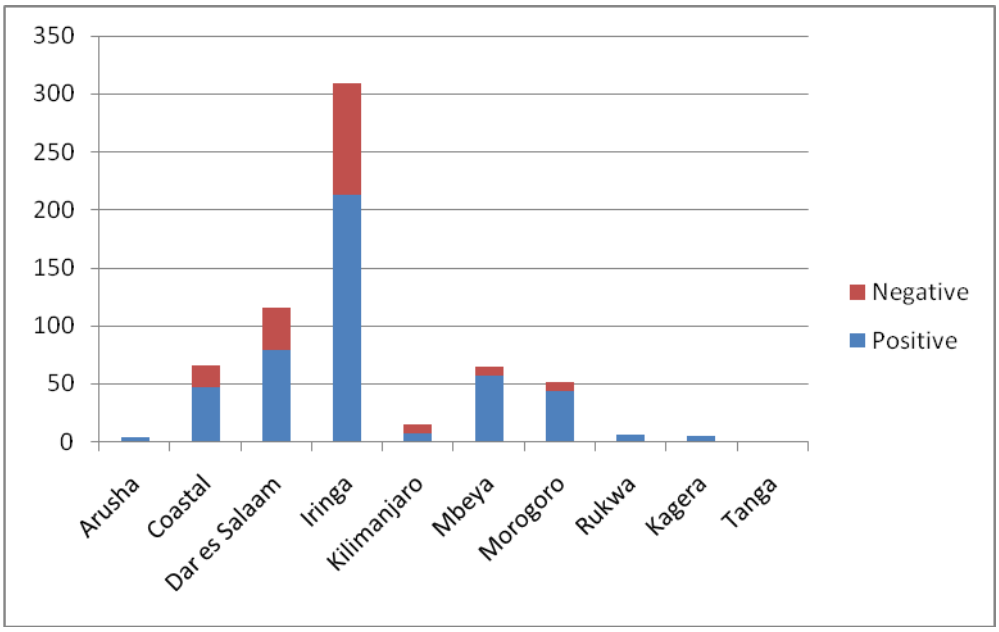

Figure 1. Regional distribution of ASFV tested samples for the period 2007/2008- 2016/2017

The high numbers of positive cases were diagnosed during February, March, April, May and June than during the remaining months of the year (Figure 2). The months of July had the lowest number of disease occurrence. According to this data, it was apparent that the occurrence of ASF was associated with rainy season.

There is a steady sample tested with exception of the year 2012 and 2013 where the number was high 96 (15%) and 255 (39.8%) respectively (Table 1).

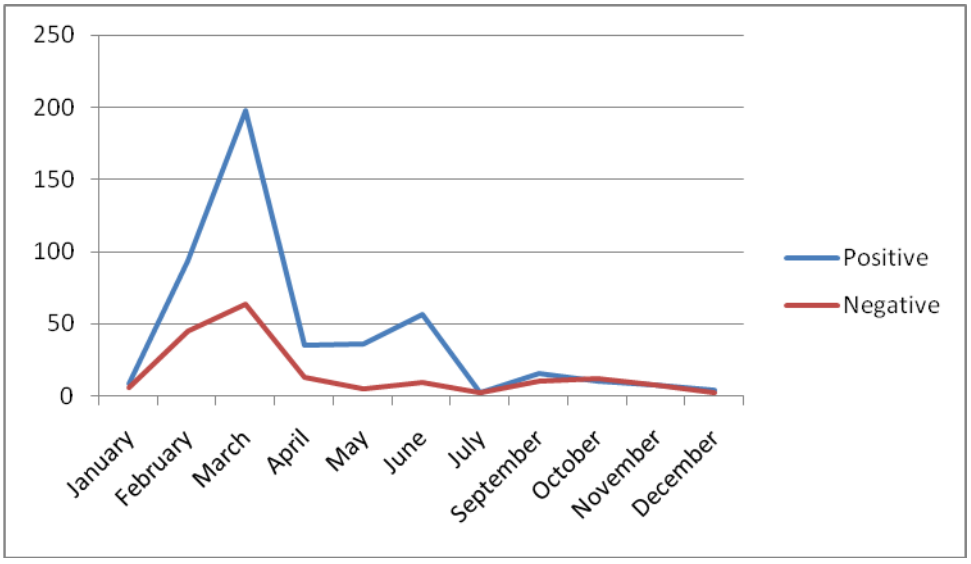

Figure 2: Monthly distribution of ASFV tested samples for the period between 2007/2008 and 2016/2017

Table 1: Annual distribution of ASFV tested samples for the period 2007/2008- 2016/2017

Year	Number of samples tested	Number (%) of positive	Number (%) of positive
2008	24	18 (2.8)	6 (0.9)
2009	56	40 (6.3)	16 (2.5)
2010	19	8 (1.3)	11 (1.7)
2011	34	22 (3.4)	12 (1.9)
2012	96	76 (11.9)	20 (3.1)
2013	255	178 (27.8)	77 (12.03)
2014	16	12 (1.9)	4 (0.6)
2015	33	24 (3.8)	9 (1.4)
2016	51	32 (5.0)	19 (3.0)
2017	56	55 (8.6)	1 (0.2)
Total	640	465 (72.7)	175 (27.3)

DISCUSSION

This study aimed at elucidating the trends of ASF disease occurrence in Tanzania based on the retrospective data retrieved from CIDB sample register books. ASF disease was suspected and confirmed annually during the entire period of the study (2007/2008-2016/2017) indicating endemic state. These findings agree with a report by the OIE which indicated that ASF is an endemic disease in Tanzania (OIE, 2010).

The distribution of ASF showed no specific pattern. However, there were higher occurrence of ASF disease in Iringa, Dar es Salaam, Coast, Mbeya and Morogoro regions and yet they have highest pig population. Most pigs in Tanzania are kept in high altitude areas, where the human population density is high and the land is of high agricultural potential. About 54% of the pigs in the country are thus found in the Southern highlands of Tanzania (SHT) regions (Mbeya, Iringa, Rukwa and Ruvuma) (URT, 2012). Pig production in most of these areas are dominated by traditional production systems and practices thus practices free ranging, characterized by small herds, low level of biosecurity and productivity.

Moreover, the consumption of pork has increased in Tanzania especially in town centresand cities with the Dar es Salaam region leading the demand. This could cause a surge in movement of pigs by traders from most parts of the country to the cities e.g. Dar es Salaam, hence the high prevalence of the disease. Transmission through direct contact can occur up to 30 days after infection. Meat from infected pigs or contaminated pork products is another source of infection due to the virus's long persistence in tissues (Costard *et al.,* 2009) and environment. Additionally, pigs recovering from infections can remain persistently infected for 6 months and act as a source of transmission to susceptible pigs (Costard *et al.,* 2009). It is also found that in the most rural and city centres ofTanzania, pig slaughter slabs are small, poorly equipped and waste is directly accessible to other animals such as dogs or roaming pigs (personal observation); and that many pig owners sell their pigs as soon as they suspect ASF in their animals or in the neighborhoods.

Furthermore, most of these regions are adjacent to the national parks; it could be that sylvatic cycles are involved. The sylvatic cycle involves wild species of swine spreading the virus by soft ticks of the genus ornithodoros (Penrith and Vosloo, 2009). In Africa the major host for the ASF virus is the warthog, but all wild species of swine can be silent carriers. The wild pig and their ticks can come in contact with domestic pigs and be a source of infections especially in traditional free – ranging systems.

There were fewrecords of occurrence of the disease in the western and lake zone regions. This difference could be partly due to underreporting, the different husbandry practices, and vigilance in disease control, animal movements and other virus transmission dynamics. In north western regions the majority of the population are pastoralists, this could be the reason that no ASF diagnose in those regions. More so the north western regions have low number of pigs (URT, 2012).

In the present study, there was a tendency to a seasonal pattern with higher frequency of ASF occurrence reported during and immediately after the months with moderate to high rainfall compared to the parts of the year with dry season. This is in contrast with the study by Atuhaire *et al.,* 2013 who were revealed that the ASF outbreaks were common during the dry season. The rain season favors the tick activities; hence increase in the transmission of the virus. Very dry season reduce survival and

increases mortality of ticks as a result of desiccation by water loss (Perret *et al*., 2003). Apart from affecting the development and mortality, temperature and relative humidity affects tick questing, a mechanism by which ticks find a host, climb on it, and feed (Busby *et al*., 2012).

General trend on the samples submitted showed that large numbers of samples were submitted at CIDB in 2011/2012 & 2012/2013 compared to the rest of the years. This is because there was a project on ASF diagnosis capacity building and through the project field ASF surveillance was enhanced hence many samples were collected from the field, particularly Iringa, Mbeya and Njombe regions. However, there is an increase of sampled submitted for the last three consecutive years compare to the period of year 2007/2008-2010/2011. This indicates the significant mounting of the disease.

Based on the findings of this study it is concluded that there is a trend of ASF disease occurrence in the country at an average of 64 samples per year. The disease is widely distributed in the country and mostly occurs during the rainy season. It is strongly recommended that ASF control strategies should encompass a holistic chain analysis. More detailed and systematic studies should be undertaken to investigate further specific risk factors and patterns of occurrence of ASF in Tanzania.

ACKNOWLEDGEMENTS

The authors would like to acknowledge TVLA and CIDB for making records to be available for the study to be conducted. The authors also wish to appreciate funding of the Government of Tanzania through the Ministry of Science and Technology, that funded a project titled "Enhancement of infectious diseases surveillance and risk management for increased livestock production in Southern zones of Tanzania" whose activities made it possible to build steady laboratory diagnostic capacity and assisted some field surveillance of active foci of ASF.

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Seroimmune responses to strategic vaccination in chickens against Newcastle disease using commercially available vaccines

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SUMMARY

Evaluation of the Newcastle Disease (ND) antibody level after different vaccination strategies using I-2 and La Sota Vaccines was experimentally conducted on broiler chicken using standard HI test. Three vaccination strategies employed were 12.5%, 25% and 50% of the chickens were vaccinated; positive and negative control groups were used. At 12.5% vaccination strategy, for I-2 vaccine, 12.5% of the chickens were sero-converted to protective level (HI titre results≥log3base 2) and was not significantly different ($p>0.05$) to the negative control group. For La sota vaccine 62.5% of the chickens seroconverted to protective level and was not significantly different ($p>0.05$) compared to control positive group.At this strategy, La sota vaccine has proved to do better than I-2 vaccine in activating humoral immune response.At 25%vaccination strategy, I-2 vaccine has shown that, 75% of the chickens were seroconverted to protective level, the results was significantly different ($p<0.05$) to the negative control group. La sota likewise, 75% of the chickens were seroconverted to protective level which was significantly different ($p<0.05$) to the negative control. At this strategy, both vaccines have the same activation. At 50% vaccination strategy, for I-2 vaccine, 81% of the chickens were seroconverted to protective level and for La sota Vaccine, 94% of the chickens were seroconverted to protective level. For both vaccines their results were significantly different ($p<0.05$) to negative control. At this strategy, both vaccines have similar effect in humoral immune response activation. Therefore vaccinating 25% and above of the chickens will results in a flock immunity in intensive farming.

Key words: Chicken, Newcastle disease, vaccination, La sota

It is estimated that Tanzania had about 36.2 million chickens by 2008, out of which almost 95% are Free Range Village Chickens (FRVC) and the rest are exotic breeds (RLDC, 2011) and most of these village chickens are kept mainly in the rural areas by women and children. Village poultry production plays an important contribution to household food security and income generation (Goromela, 2009; Goromela 2009). The proportion of traditionally raised birds kept in Tanzania, majorities are chicken (94.1%) followed by ducks and geese (5.3%), guinea fowl (0.4%) and turkeys (0.2%) (Melewas, 1989). The growth rate of the chicken industry has been increasing at the rate of 2.6% per year since 2003 (Msami,2008; Msami 2008). The rapid increase of the chicken industry has been influenced by the increased demand of chicken meat and eggs as source of animal protein due to increasing fast food vendors in urban settings (Gyles, 1989) and taken as an opportunity by people both in urban and rural settings. In Tanzania chicken industry is divided into the traditional and the commercial sectors. Indigenous free range local chickens dominate in the traditional sector. Chickens are poorly managed, poorly housed and some roost on trees, no feed supplementation and hardly given veterinary attention and if happens traditional medicines are practices. Chicken management in a family is the responsibility of women and children and men show little interest in chickens except in some areas where chicken sale fetch higher selling price especially in areas where traders from big cities and town come to buy chickens for urban consumption. Village chicken supplies 100% of all the chicken meat and egg demands for rural people and about 20% of urban demand (Ministry of Livestock Development, 2006).

The effort in developing the chicken industry is directed to both the commercial and traditional sectors (Melewas, 1989). Importation of hatching eggs and day old chicks for commercial purposes and production and increased used of Thermo stable Newcastle disease vaccine for village chickens are all effort to develop the chicken industry in the country (Ministry of Livestock Development, 2008). This is because the industry provides employment to people both in urban and rural settings and provides income to government through levy. Therefore, Tanzanian government through Ministry of Livestock Development (MLFD) has been encouraging chicken keeping for income generation and therefore poverty alleviation (Ministry of Livestock Development, 2006).

The major hindrance to rural chicken prosperity is Newcastle Disease (ND). The disease may cause 90% of mortality rates and sometimes clears the whole flock during an outbreak (Buza and Mwamuhehe, 2000). Vaccination against the disease has remains the most effective means of controlling ND (Orajaka, 1999). In Tanzania mostly available commercial ND vaccines are La Sota and I-2 vaccine. La Sota, a lentogenic live vaccine is used mainly in commercial poultry sector and having setbacks in application in rural areas due to the problem of heat intolerance of the vaccine strain, large dose presentation, affordability, reliability, transport and cold chain for effective administration of the vaccine (Dias, 2001) The avirulent, thermostable ND vaccine strains I-2 provide rural poultry farmers with an effective, affordable and reliable means of controlling ND in their flocks (Dias, 2001) and have been used widely and effectively in village chickens population in many Asian and African countries (Dias, 2001). The vaccine is currently widely used in Tanzania (Wust, 2010), the vaccine has been accepted as suitable for use in Vietnamese villages (Tu, 1997) and has been used in Nigeria, Kaduna state (Nwanta, 2006). However, apart from the vaccine availability, ND is still a bottleneck to local village production.

In spite of the availability of vaccines against the ND, there is inadequacy of controlling the disease; this could probably be due to failure of following manufacturer's indications to vaccinate individual chicken in a flock, the free range nature of chickens and short time available due to farmers' engagement to other economic activities. Therefore, this study looked for the best vaccination strategy which will ensure highest level of flock protection and at the same time lessen the work of vaccinators or farmers because vaccination so far remains the most effective strategy for controlling Newcastle Disease (Orajaka,1999). The study investigated the extent of horizontal transmission of vaccine virus and protective antibody response to non-vaccinated in contact chickens following strategic vaccination of chickens against

MATERIALS AND METHODS

Study area

The study was conducted in Mpwapwa District at Kikombo basin. Mpwapwa District is one of the six districts in Dodoma Region. Mpwapwa is located 120 kms from Dodoma Regional headquarters and 54 km from Morogoro-Dodoma main road at a village called Mbande. Mpwapwa lies between latitudes 6°00" and 7°30" South of the Equator and between longitude 35°45" and 37°00" East of Greenwich (Council 2009). It borders Kilosa District on the Eastern part, Kongwa District on the Northern part, Chamwino District on the Western area and Kilolo District on the Southern part. The district covers a total area of 7 379 square kilometers about 18.1% of total area of Dodoma Region (Mpwapwa DC, 2009). Kikombo is an area where institutions under the ministry of Livestock and Fisheried Development including National Livestock Research Institute (NLRI), Veterinary Investigation Centre (VIC) and Livestock Training Institute (LITI) are found.

This study area was chosen because of availability of poultry units which needed little modification to suit the present experiment at LITI poultry units, to disseminate the knowledge about chickens rearing, sample collection and processing to LITI students using VIC laboratory facilities, the area is accessible to Dodoma where chickens inputs can be fetched.
The experiment site was located at latitude 6^{o} 20'66''South and longitude 36^{0} 30' 60'' E. with an altitude of 948m above sea level (GPS. Geko 101, Hampshire, UK). During the study period, November 2011 to March 2012, Kikombo experienced the following maximum average dry temperatures of 30.7 ^{o}C, 28.5 ^{o}C, 28.5 ^{o}C, 29.7 ^{o}C, 28 ^{o}C from November to March respectively and average rainfall of 35.6 ml, 244 ml, 214 ml, 136 ml and 123 ml during the same period but with uneven daily rainfall distribution in a month(Centre 2012).

Experimental Study Design and Methodology

House preparation and chicks managements

Cleaning and disinfection of the housing using broad spectrum disinfectants was done using Rhino White disinfectant containing tar acids at 7-8 % (v/v), a product of SAPA Chemical Industries, Dar Es Salaam, Tanzania. Disinfection was done twice, a month before and two weeks before stocking the chicks. Chicken house was divided into non communicating compartments (Figure 2 below) and spacious enough (90 cm wide x 240 cm long x 210 cm high) to accommodate 16 chickens until the end of study period. The non-communicating compartments where enough to prevent cross contact amongst chickens of different experimental groups. Foot bath carrying tar acids 8% (v/v) was placed at the entry point to prevent contaminants brought in by the attendant. Attendant had to wear overcoat and gumboot when attending chickens and not allowed to work for chickens/animals anywhere else to avoid introduction of contaminants.

Figure 2. A section of chickens in their none-communicating pans

Feed and water were given ad libitum using commercially prepared feed sourced in the market (Igo products, Dar Es Salaam, Tanzania), commercial vitamins supplements (A, B, C, D, E and K) were provided to the chicken through drinking water. Light source was made available to provide heat and light twenty four hours for the first three weeks during brooding (Figure 3 below) and only during night hours thereafter until the end of the study period. Anticoccidial products (Ancoban, Ipswich, UK) were provided to chickens as prophylaxis and treatment during the study period. The deep litter (saw dust) 3 inch was set at the beginning of study and was not changed till the end of the study period.

Figure 3. A Section of chicks during brooding stage

Chickens source and vaccination

About two hundred day old broiler chicks (Figure 3 above) were purchased from a local supplier who sourced them from Interchick®, these chicks of similar age sex and breed came from the parent stock with a history of being vaccinated against ND using live vaccine (Nobilis Clone 30, a product of Intervet, South Africa). The chicks were brooded in the isolation unit for three weeks before being transferred to the experimental site. All chicks were vaccinated against Infectious Bursa Disease (IBD) at the age of two weeks and against Pox at the age of four weeks.

Experimental design

At the age of three weeks, 160 chickens were randomly selected and divided into ten groups of 16 chickens each to be vaccinated using commercially available vaccines either I-2 or La Sota. The first five groups were for I-2 vaccine and the remained five groups were for La Sota vaccine. Each chicken was wing tagged and identified by type of vaccine, group number and specific number of chicken example GL. 1. 1-16 where GL stood for Group La Sota vaccine, 1 stood for 12.5% vaccinated and 1-16 refer specific number of chick within the group.

Yellow colored tags were for none vaccinated and red colored tag for vaccinated ones.

In this experimental trial, the first five groups were vaccinated using I-2 vaccine (CVL product, Dar Es Salaam, Tanzania) batch ND 1107VD111). From group 1 to 5 strategic vaccinations were 12.5% (2 chicks vaccinated), 25% (4 chicks vaccinated), 50% (8 chicks vaccinated), 100% (16 chicks vaccinated) and 0% (None is vaccinated) respectively (Figure 4). I-2 vaccine was given as an eye drop(Young 2002).

The same strategic vaccination was applied for the La Sota vaccine (BIOVAC product, Israel, batch 101414) and was given through drinking water. For both vaccines groups, 100% and 0% vaccination was used as positive and negative controls respectively.

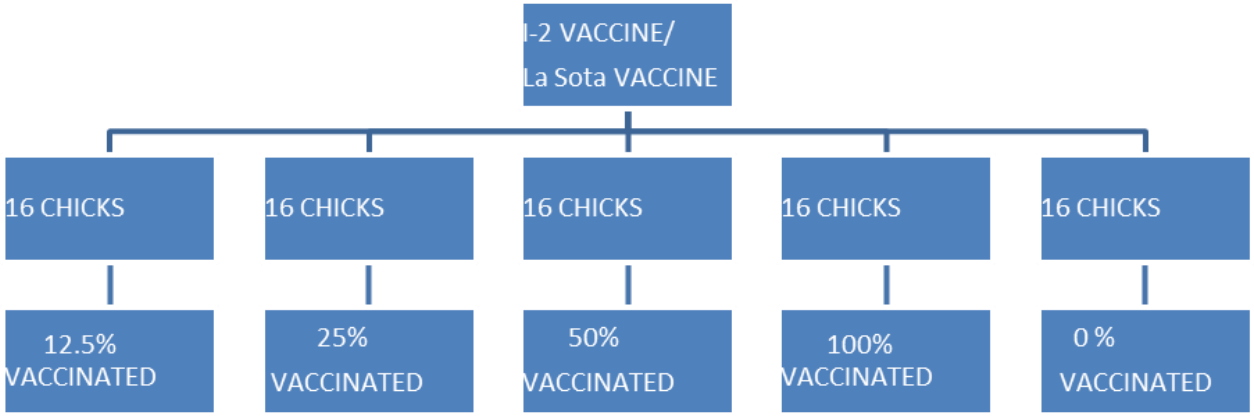

Figure 4. The design of the experimental groups per vaccine used, number of chickens in each group and strategic vaccination in percentage of chickens in a group.

Blood Sample collection

From all the chickens, the first blood sample were collected at the age of three weeks before vaccination and thereafter after every two weeks five times post vaccination (Appendix 1). Using 2mls sterile syringe and needle, 1-2 mls of blood was collected from each chicken through wing vein (Brachial vein), before blood collection, feathers were removed and the site was disinfected using cotton wool soaked in 70% alcohol (Yongolo, 1996) and put in the plain vacutainer tubes and the tubes were labeled.

Blood was left to coagulate in a refrigerator at 4^{o}C overnight (Allan and Gough, 1974a) and centrifuged the following day at 1500 rpm for 2 minutes for clear serum collection. Each serum was kept in a labeled cryovial and stored in the deep freezer at -20^{o}C until HI testing (Allan and Gough, 1974b)
Wing tag number (ID number of chicken) and date of blood sample collection were marked on the vacutainer tube and corresponding cryovial.

Serum Testing Procedure

The Haemaglutination Inhibition (HI) titre of sera from experimental chickens was measured by using standard procedure of microplate HI test (Allan and Gough, 1974a). HI test was performed using four HA units (4HA) of ND virus and a 1% suspension of chicken red blood cells (Allan and Gough, 1974a) in V shaped well microtitre plates. All titres were recorded as log2 of the reciprocal of the end point dilution. In this study the HI titre ≥3 (Log2) was considered positive based on the findings of Allan and Gough (1974) and Bell *et al*. (1991a) who reported that birds with HI titre ≥3 (log2) were protective against challenge with a virulent strain of ND virus. The end point dilution forming a stream following tilting the plate was recorded as a true positive.

Preparation of Newcastle disease virus antigen for use in HI tests

Antigen was prepared by inoculating embryonated chicken eggs free from NDV (SPF) from SUA farm. The driller was used to open the allantoic cavity and 100µL of sample of NDV from I-2 vaccine was inoculated, the opening was sealed by wax and then eggs were incubated for four days before harvesting allantoic fluid. A volume of 60ml of allantoic fluid was harvested and centrifuged at 1,200 g to clarify and remove contaminating red blood cells. The working antigen was stored in a refrigerator at 4°C

and the stock antigen kept at -20°C (Allan and Gough, 1974a).

Preparation of Washed Red Blood Cell Suspension

About 6 mls of blood was collected from 3 chickens found in SUA poultry farm by using 2mls syringe and needles and then transferred to a vacutainer tubes impregnated with an anticoagulant (EDTA), then blood was gently mixed. The blood was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and the tube was refilled with PBS and centrifuged again at 1500 rpm for 5 minutes, again the supernatant was discarded. This step was repeated three times. The last round of centrifugation was done without adding PBS and afterwards the supernatant was discarded. The small part of RBC was diluted to a 1% solution, by adding PBS to prepare a working RBC solution (1ml RBCs: 99 ml PBS).

The control sera

For control purposes, positive secondary laboratory standard serum from SUA VET Virology Laboratory (SUA) was used. This secondary laboratory standard serum was developed following comparative testing with the standard serum from Veterinary Laboratory Agency (Ministry of Agriculture. Weybridge, Surrey, UK). This secondary standard serum was used to confirm our prepared antigen by HI test and had HI titre of log25 and therefore used as control positives, the consistency of results when tested with 4HA units of antigen was observed (Allan and Gough, 1974a). For negative control PBS was used. The positives and negative controls were run simultaneously in the plates and acted as a golden standard for the results.

Preparation of 4HA units of Newcastle disease virus antigen

The standard amount of Newcastle disease virus used in the haemagglutination inhibition (HI) test is 4HA units (OIE, 2012). It was necessary to prepare and test a suspension of Newcastle disease virus containing 4HA units in order to carry out the HI test. This involved a series of following steps.
The antigen prepared was tested by Haemagglutination test (HA)
Procedure (OIE, 2012).

i) 25 µL of PBS was dispensed into each well of a plastic V-bottomed microtitre plate.
ii) 25 µL of the virus suspension was placed in the first 4 wells of the first column of the microtitre plate.
iii) Two fold dilutions of 25µL a volume of the virus suspension was made across the plate iv) 25 µL of PBS was dispensed to each well.
v) 25 µL of 1% (v/v) chicken RBCs was dispensed to each well.
vi) The solution was then mixed by tapping the plate gently. The RBCs were allowed to settle for 40 minutes at room temperature.
vii) HA was determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming) (Figure 5); this represents 1 HA unit (HAU). The found to have end point titre at 210 (1:1024) (Figure 5).
Therefore, to get 4HA Units = Titre/4
=1024/4= 256
Therefore, 1ml of Antigen was mixed with 255ml of PBS to make an antigen working solution.

The results of the back titration of the diluted antigen and the HI titre of the laboratory standard positive (SUA VET Virology Laboratory) were both used to confirm the antigen if has been diluted to a concentration equivalent to the standard 4 HA units.

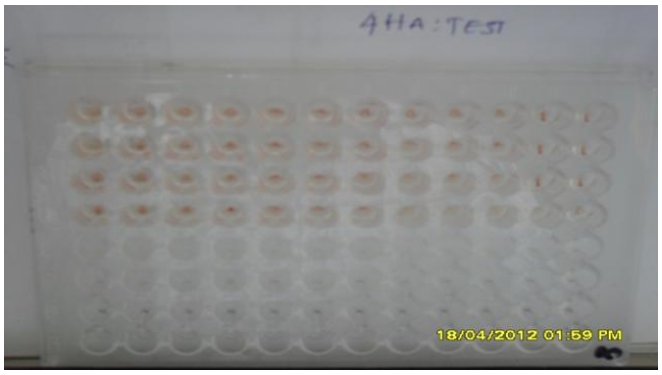

Figure 5. 4HA testing (HA titre of 210 = 1:1024) was recorded

Haemaglutination Inhibition Test Procedure according to Allan and Gough, 1974

Materials required
-Thawed serum samples in racks
-V-bottom microwell plates and covers
-Phosphate Buffered Saline (PBS)
-1 percent washed red blood cells
-V-bottom reagent trough
-25 µL single and multichannel pipettes and tips
-Microwell plate recording sheet.
-Newcastle disease virus antigen diluted to 4 HA units per 25 µL
-Standard positive and negative control (PBS)

Procedure

i) Each test serum was recorded correspondingly to the well on a microtitre plate
ii) 25µL of PBS was dispensed into each well of V botton micro well plate.
iii) After shaking the cryovial of test serum, 25 µL of each test serum was added into the first well on a column and the last well of a row.
iv) By using a multichannel pipette two fold serial dilutions was done along the row until the second last well from the end and discarding the last 25 µL from the second last well.
v) Then, 25 µL of the 4HA dilution of antigen was added into each well except the control wells in the last column.
vi) Then, the loaded microwell plate was gently shaken to allow the reagents to mix. Followed by covering the plate with a lid and allowed to stand for 30 minutes at room temperature.
vii) Then, 25 µL of 1 percent washed red blood cells was added into each well including the control wells in the last column.
viii) The plate was gently held and shaken to allow mixing of the reagents. The plate was then covered by a lid and allowed to stand at room temperature for 45 minutes before start reading.
Reading Results and interpretation

In the well where antibodies are present there will be haemagglutination inhibition, free red blood cells will settle down, tears of RBCs will be made in a well when the plate is tilted at an cute angle, it was done so because sometimes it is not easy to determine degree of haemagglutination inhibition by looking the size of the button and control wells (Allan and Gough, 1974a/b).

The end point of titration is the well that shows complete haemagglutination inhibition by forming tears when a plate is tilted at an acute angle (Figure 6).-In the well where antibodies are not present, there is agglutination and therefore no free RBCs and tears formation when the plate is tilted at an acute angle (Figure 2).

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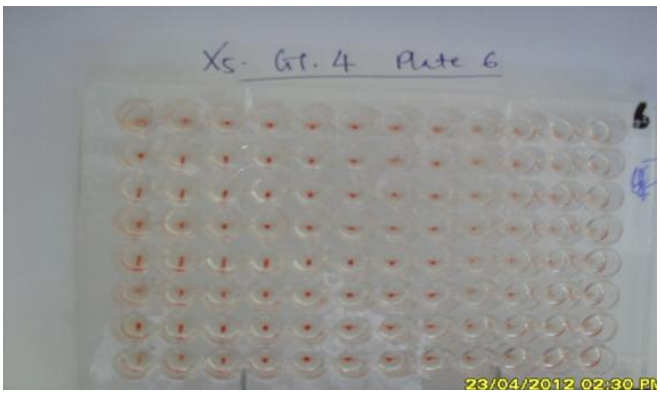

Figure 6. The HI test, Row 1 reads HI negative throughout, Row 5 read HI positive up to well 3 (Log23).

Data Analysis of the test results

The microsoft office excel 2007 spread sheet was used to enter data of end point of dilution showing haemagglutination inhibition of each chicken (vaccinated and non- vaccinated) in each vaccination group (HI titre) before and post vaccination. The excel spread sheet was used to store, summarize, analyse, design and present data. Geometric Mean titre to determine the flock immunity in each vaccination group was calculated by calculating an average of the individual chicken HI titre in a group (Appendix 2). The level of significance before and after vaccination in the same group was calculated by using Chi square test. The number of seropositive and seronegative chickens before and after vaccination was used to find out the effect of vaccination (Appendix 3).

The level of significance between the two vaccines in a similar vaccination strategy was calculated using paired student's T test by comparing their respective GMTs (Table 3). The level of significance to determine the difference in antibodies level in control positive between the two types of vaccines was calculated by using paired student's T test by comparing their respective GMTs before and after vaccination (Table 3).

The level of significance when comparing the GMT of the vaccination strategy and the respective control was calculated by using paired student's T test as well.

RESULTS

12.5% vaccination strategy

For the group vaccinated using I-2. The HI before vaccination (X0) indicated that 9 chickens (44%) were seropositive (HI positive) and 56% of the

chickens were seronegative (HI negative) before vaccination (Table 1). At the 8th (X4) and 10th week (X5) post vaccination no in contact chicken had protective antibodies level except for the two vaccinated ones. The highest Geometric Mean titre (GMT) of 2.00 was recorded at two weeks post vaccination (X1) and 1.13 at the 8thweek (X4) and 10th week (X5) at the end of the experiment (Table 1).

For the effect of vaccination, there was no significant difference in numbers of seropositve chickens before and post vaccination ($p>0.05$). Likewise, there was no significant difference ($p>0.05$) in GMT when compared to negative control group. But when compared to the positive control group (100% vaccinated), there was significant different between the two groups ($p<0.04$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 50% of the chickens were seropositive and 50% of the chickens were seronegative (Table 1). The number of seropositive and seronegative chickens keep changing, at the 8th week (X4) 63% of the chickens were seropositive and 37% of the chickens were seronegative which was maintained until the end of the experiment at the 10th week (X5) (Table 1).

The GMT of 2.75, 3.00, 2.13 and 3.19 were recorded at the 2nd week (X1), 4th week (X2), 6th week (X3) and 8th week (X4) post vaccination respectively (Table 1). The highest GMT of 3.31 was recorded 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of the vaccine, there was no significant difference in number seropositive and seronegative before and after vaccination ($p>0.05$). When the GMTs of this vaccination strategy were compared to the GMTs of the positive control (100%), the two groups were not significant different ($p> 0.05$) in protecting chickens. However, at this vaccination strategy (12.5%), when the effect of La Sota and I-2 vaccines were compared using their GMT, they were significant different ($p< 0.05$) in protecting chickens.

Vaccination strategy 25 %

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 38% of the chickens were seropositive and 62% of the chickens were seronegative (Table1). At the 8th week (X4) post vaccination 56% of the chickens were seropositve and 44 % of the chickens were seronegative (Table 1). At 10th week (X5) 75% of the chickens were seropositive and 25% of the chickens were seronegative (Table 1) at the end of the experiment. The highest GMT of 3.25 was recorded at 6th weeks (X3) post vaccination followed by a subsequent falling in the GMT to 2.50 in the 10th weeks (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccination, the number of seropositive and seronegative chickens before and after vaccination was found to be significant different ($p<0.05$). When the GMTs are compared to the GMTs of the positive control group (100% vaccinated) the two groups were not significant different ($p>0.05$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 44% of the chickens were seropositive and 56% chickens were seronegative (Table3). At the 4th week (X2) post vaccination, 69% of the chickens were seropositive and at the 10th week (X5) post vaccination, 75% were seropositive and 25% were seronegative (Table 1). The highest Geometric Mean titre of 3.25 was recorded at two weeks (X1) post vaccination and 3.06 in the 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccine, the number of seropositive and seronegative chickens before and after vaccination were statistically significant different ($p<0.05$). When the GMTs of this strategy are compared to the GMTs of the positive control the two were not significantly different ($p> 0.05$).
However, at this vaccination strategy, when the effect of La Sota and I-2 vaccines in chickens were compared themselves using their GMTs, statistics shows that they are not significant different in protecting chickens ($p> 0.05$).

Vaccination strategy 50%

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 63% of the chickens were seropositive and 37% of the chickens were seronegative (Table 1). At the 2nd week (X1) post vaccination 31% of the chickens were seropositive and 69% of the chickens were seronegative. The number of seropositive was increased to 37% of the chickens at 4th and at 6th week (X3) post vaccination 87% of the chickens were seropositive followed by subsequent fall in seropositive chickens to 81% in the 8th week (X4) which was maintained to the 10th week (X5) which was the end of the experiment (Table 1). The highest GMT of 3.00 was recorded at 6th weeks (X3)

post vaccination followed by a subsequent falling in the GMT to 2.75 in the 10th week (X5) post vaccination which was the end of study period (Table 1). For the effect of the vaccine, number of seropositive and seronegative chickens before and post vaccination were not significantly different ($p>0.05$). When the GMTs of this vaccination strategy were compared to those of positive control the two groups were not significantly different ($p>0.05$) in protecting chickens. For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 31% of the chickens were seropositive and 69% of the chickens were seronegative (Table 1). At the 2nd week (X1) and 4th week (X2) post vaccination 63% of the chickens were seropositive and 37% were seronegative. The number of seropositive chickens at 6th week post vaccination (X3) was increased to 81%. The number of seropositive chickens was increased to 94% at the 10th week (X5) post vaccination at the end of the experiment (Table 1). The high Geometric Mean titre of 4.38 was recorded at 6thweeks (X3) post vaccination and followed by subsequent rising to the highest 5.56 in the 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of vaccine, the numbers of seropositive and seronegative chickens before and after vaccination were compared and found to be significant different ($p < 0.05$). When the GMTs of this vaccination strategy were compared to those of positive control, the two groups were not significantly different ($p>0.05$) while significant different to the control negative ($p<0.05$). However, at this vaccination strategy, when La Sota and I-2 vaccines were compared using their GMTs in this vaccination strategy (50%), they were not significant different in protecting chickens ($p>0.05$).

Table 1. HI titre and GMT results for each vaccination strategy and controls

Vaccination Strategy	Vaccine Type	HI TITRE RESULTS												Comparison of GMTs (Means) (p=0.05)
		X0		2nd (X1)	Week	4th (X2)	week	6th (X3)	week	8th week (X4)		10th week (X5)		
		+	-	+	-	+	-	+	-	+	-	+	-	
12.5%	I-2	44%	56%	44%	56%	6%	94%	13%	87%	13%	87%	13%	87%	
	GMT±1.01	2.6		2.00		0.94		0.93		1.13		1.13		
	La Sota	50%	50%	56%	44%	50%	50%	31%	69%	63%	37%	63%	37%	
	GMT±1.16	2.19		2.75		3.00		2.13		3.19		3.31		
														P<0.05
25%	I-2	38%	62%	38%	62%	38%	62%	50%	50%	56%	44%	75%	25%	
	GMT±2.03	1.81		2.25		2.56		3.25		2.31		2.50		
	La Sota	44%	56%	62%	38%	69%	31%	25%	75%	31%	69%	75%	25%	
	GMT±1.9	2.25		3.25		3.00		1.44		1.75		3.06		p>0.05
50%	I-2	63%	37%	31%	69%	63%	37%	88%	12%	81%	19%	81%	19%	
	GMT±1.44	2.63		2.00		2.75		3.00		2.73		2.79		
	La Sota	31%	69%	63%	37%	63%	37%	81%	19%	69%	31%	94%	6%	
	GMT±2.29	2.00		3.38		2.94		4.38		3.50		5.56		p>0.05
100%	I-2	56%	44%	12%	88%	88%	12%	81%	19%	81%	19%	81%	19%	
	GMT±1.46	2.63		1.53		3.00		3.25		3.06		3.00		
	La Sota	25%	75%	100%	0%	69%	31%	88%	12%	94%	6%	100%	0%	
	GMT±1.78	1.69		3.94		2.50		5.00		4.56		4.13		p>0.05
0%	I-2	69%	31%	19%	81%	12%	88%	6%	94%	0%	100%	0%	100%	
	GMT±1.11	2.50		1.69		0.69		0.88		1.38		1.69		
	La Sota	44%	56%	44%	56%	19%	81%	6%	94%	0%	100%	0%	100%	
	GMT±1.30	2.06		2.31		1.88		1.19		1.50		1.58		p>0.05

+ = ≥3Log base 2, - = <3Log base 2 GMT= Geometric Mean Titre, GMT± SD

The control positive (100% vaccinated)

For the group vaccinated using I-2, the HI results before vaccination (X0) indicates that 56% of the chickens were seropositive and 44% were seronegative (Table 1). At the 2nd week (X1) post vaccination 13% of the chickens were seropositive and 87% were seronegative. At the 4th week post vaccination (X2) 88% of the chickens were seropositive and 12% were seronegative. Seropositive chickens were reduced to 81% at 8th week (X4) and maintained until the end of the experiment at 10th week (X5) (Table 1). The highest Geometric Mean titre of 3.25 was recorded at 6th weeks (X3) post vaccination and followed by fall in the Geometric Mean titre to 3.00 in the 10th week (X5) post vaccination at the end of the experiment (Table 1).

For the effect of the vaccine, when the number of seropositive and seronegative chickens before vaccination were compared to the number of seropositive and seronegative chickens post vaccination and found to be significant different ($p < 0.05$).

For the group vaccinated using La Sota, the HI results before vaccination (X0) indicates that 25% of the chickens were seropositive and 69% were seronegative (Table 1). At the 2nd week (X1) post vaccination all 100% of the chickens were seropositive. The percentage of chickens who were seropositive at the 2nd week gradually reduced in the 4th week (X2), 6th week (X3) and 8th week (X4) to 69%, 88% and 94% respectively before peaking up again to (100%) in the 10th week (X5) at the end of the experiment (Table 1). The Geometric Mean Titre recorded before vaccination (X0) was 1.69 and 3.94 two weeks (X1) post vaccination and peaked up in the 6th week (X3) post vaccination to 5.00 before drop down to 4.56 and 4.13 in the 8th week (X4) and 10th week (X5) respectively. For the effect of vaccine, the number of seropositive and seronegative chickens before vaccination when compared to the number of seropositive and seronegative chickens post vaccination was

significant different ($p < 0.05$). When the two vaccines I-2 and La Sota were compared at this positive control vaccination by using their GMTs, the two vaccines were not significantly different ($p>0.05$) in protecting chickens.

The control negative (0% vaccination)

For the control negative group stayed in the I-2 vaccinated chickens, 69% of the chickens were tested seropositive and 31% tested seronegative before vaccination (X0) (Table 1). The seronegative chickens were sharply increased to 81% two weeks later (X1) and there was slight increase to 88% at 4th week later (X2). Seronegative chickens were then increased to 94% and 100% at the 6th week (X3) and 8th week (X4) respectively and maintained until the end of the experiment at 10th week (X5) (Table 1). The highest GMT of 2.5 was recorded during the first sampling (X0) and decreased to 1.69 and 0.69 at the 2nd week (X1) and 4th week (X2) respectively before starts to increase to 0.88, 1.38 and 1.69 at the 6th week (X3), 8th week (X4) and 10th week (X5) respectively at the end of the experiment (Table 1). Statistically there was significant difference ($p<0.05$) in the number of seropositive and seronegative chickens at the beginning and at the end of the experiment.

For the negative control group in the La Sota vaccinated group, 44% of the chickens were seropositive and 56% were seronegative at first sampling (X0) and second sampling two weeks later (X1) (Table 1). Then there was a steady increase in seronegative chickens to 81% and 94% at the 4th week (X2) and 6th week(X3) respectively. Seronegative chickens were then increased to 100% at the 8th week (X4) and maintained at the 10th week (X5) the end of the experiment (Table 1). The GMT of 2.06 was observed during the first sampling (X0) which was then increased to 2.31 at the 2nd week sampling (X1). During the third sampling at 4th week (X2) the GMT was decreased to 1.88 then decreased to 1.19 at the 6th week (X3). Then there was gradual increase in GMT to 1.50 and 1.58 at the 8th week (X4) and 10th week (X5) at the end of the experiment (Table 1). There was significant difference in number of seropositive and seronegative chickens at the beginning when compared to the number of seropositive and seronegative chickens at the end of the experiment ($p < 0.05$). When the negative control groups stayed in the I-2 and La Sota vaccination were compared by using their GMTs, they were not significant different ($p> 0.05$).

DISCUSSION

This experiment shows that chickens primed vaccinated using I-2 and Lasota were protected against Newcastle disease just like many research findings (Mazija, 1990, Tu, 1997, Dias, 2001). For each type of vaccine results shows that, vaccine viruses have the potential of horizontal spread ability from one vaccinated chicken to other in contact chicken(Dias 2001, Nazeri 2011). The usefulness of vaccine in protecting chickens has been described by Darrel *et al.* (Darrel *et al* 2013) when they challenged vaccinated chickens against ND and found no immune depressive elements. This study used day old broiler chicks from parents with a history of being vaccinated against ND because it was not easy to acquire large number of chicks from non-vaccinated parents. This led to the use of chickens with different immune status at the beginning of the experiment due to maternal immunity. OIE recommends that to avoid interference of maternal antibody in chicks, vaccination should be done until the chickens are at the age of 2-4 weeks when most of them would have been susceptible. In this experiment vaccination was done when chickens were 3 weeks of age (OIE, 2012). Al Zubeedy (Al Zubeedy, 2009) recommended early vaccination to enhance not only maternal derived immunity but also cell mediated immunity. The major factors affecting seroconversion and seroreversion in this study were vaccines and individual chicken's response to vaccines. Different strains of NDV used to prepare these vaccines can have effect in the immune response in vaccinated chickens and so was the objective of this study. The individual chicken's response to vaccines was taken care by using the geometric mean titre to find out effect of vaccine in immune response of the vaccinated chickens and non- vaccinated in contact chickens. For the evaluation of the effect of I-2 and La Sota vaccines, GMT obtained from each vaccination strategy were compared amongst the vaccines themselves and to the controls.

In this research, at 12.5% vaccination strategy for I-2 vaccine, none of the in contact unvaccinated chickens was seropositive except for the two vaccinated ones until the end of the study. This finding is similar to the findings of Rahman*et al.* (Rahman*et al*, 2004) when they found only vaccinated chickens where protective. The GMTs of this vaccination strategy was not significantly different ($p>0.05$) to the GMTs of the negative control ($p>0.05$) and significantly different to the positive control ($p<0.05$). The results of having seropositive chickens following vaccination agrees to the findings of Aini*et al*. (1990) and contrast the findings of Bell *et al*. (1991) about significant increase of positive reactors after vaccination. For La Sota vaccine 62.5% of the chickens were HI tested positive at the end of the study and the GMT of 3.31 was recorded which was not significantly different ($p<0.05$) to the control positive group (100% vaccinated) and significant different ($p>0.05$) to the negative control (0% vaccinated) group. When I-2 and Lasota were compared using their GMTs at this vaccination strategy, the two vaccines were significant different ($p<0.05$) in ability for vaccine virus spread and therefore induce antibody production and confer protection to in contact chickens and La Sota has done better than I-2. This finding agrees to the findings of Feizi and Nazeri in 2011 (2011) when they compared the HI titres of Avinew and La Sota vaccines.

In the vaccination strategy 25%, I-2 vaccine showed that, 75% of the chicken were seropositive (protected) until the end of the study and the GMT of 2.5 was recorded and found to be statistically significant different ($p<0.05$) to the negative control group and not significantly different ($p>0.05$) with the positive control group. This suggests that the vaccination has done better similar to as when all chickens were vaccinated. La Sota on the other hand, 75% of the chicken were tested positive and their GMT recorded was 3.06 which was significantly different ($p<0.05$) to the GMT of the negative control group and not significantly different ($p>0.05$) with the GMT of the positive control group. When the GMTs of both vaccines were compared, they were not significant difference ($p>0.05$) in the spread of vaccine and induction of antibody production to protective levels in the chickens.

For the vaccination strategy 50%, for I-2 vaccine 81% of the chickens were tested positive and the GMT of 2.75 was recorded at the end of the study period. When this GMT is compared to the GMT of the control negative the two are significantly different ($p<0.05$) and not significantly different with the GMT of the positive control group ($p>0.05$). This means that this strategy is as good as vaccinating all chickens. The same results was obtained in Mozambique by Dias *et al*. (2001), they found that 6 chickens were protected against ND when stayed in contact with 10 vaccinated chickens (62%). La Sota vaccines on the other hand, 94% of chickens were tested positive and the GMT of 5.56 was recorded at the end of the study period. When this GMT is compared to the GMT of negative control group, the two are significantly different ($p<0.05$) but when compared to the positive control group, the two are not significantly different ($p>0.05$).When both vaccines at this strategy are compared using their GMT, statistics shows that they were not significantly different in stimulating antibody production in vaccinated and in contact none vaccinated chickens at this vaccination strategy.

This experimental study found that, for both vaccines, vaccine viruses have the potential of spreading from one chicken to another, as found in unvaccinated in contact chickens. For the vaccination strategies employed in this study, 50% vaccination strategy for both vaccines has shown to provide better protection when compared to the rest strategies as it gives higher proportion of immunized individual 81% and 94% for I-2 and La Sota vaccines respectively. Furthermore, this strategy induced highest level of GMT of 2.75 and 5.56 for I-2 and La Sota vaccine respectively the levels not attained by other vaccination strategies. This level of GMT attained were not significantly different to their respective positive controls. Therefore, instead of vaccinating the whole flock 50% vaccination is sufficient to provide flock/herd immunity similar to as vaccinating all, but field trials need to be done to comprehend this finding.

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Thermo stability study of Temevac® I-2 Newcastle vaccine

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SUMMARY

Newcastle is a highly contagious disease of domestic poultry and wild birds and is widely regarded as the most important avian disease. Effective Newcastle vaccines are crucial for control of the disease. The objective of this study was to establish the stability of TEMEVAC® (I-2) vaccine at three different temperatures for 204 days. The study was conducted at Tanzania Vaccine Institute laboratories for the period of April-December 2017. Two vaccine batches (Batch 0068 and 0069) were subjected at 37°C for 10 days, 22-25°C for 60 days and 2-8°C for 204 days. The vaccines were 10-fold serial diluted and then inoculated into 9-days old embryonated chicken eggs and incubated for 96 hours. A virus infectivity assay and Standard mathematical technique were applied to confirm the presence and measure the amount of live Newcastle virus in the suspension. The study revealed that TEMEVAC® can maintain potency up to more than10 days, 45 days and 197 days at 37°C, 22-45°C and 2-8°C, respectively. This study underscores the importance of using TEMEVAC® vaccine which can tolerate relatively wider range of temperatures for control of the disease in rural areas.

Keywords: local chickens, Thermotolerant vaccine, Newcastle disease

Newcastle is a highly contagious disease of domestic poultry and wild birds and is widely regarded as the most important avian disease (Young *et al.,* 2012). The best and cost effective means of controlling the disease is through vaccination (Janine *et al.,* 2013; Young *et al.,* 2012; Chakraborty *et al.,* 2014). Available heat labile vaccines are suitable in intensive system (Commercial farming) with limited usefulness in rural areas where most local poultry are kept in extensive system (Chakraborty *et al.,* 2014). The reasons that contribute to the limited use of labile heat include large dose presentation, affordability, limited stable cold chain facilities, and ignorance of the farmers (Young *et al.,* 2012; Chakraborty *et al.,* 2014). Live thermotolerant avirulent I-2 vaccine against Newcastle disease was developed to help famers with small flock sizes and extensive system with poorly established cold chain infrastructures (Young *et al.,* 2012).

Thermostability tests provide evidence of how the quality of a vaccine varies with time and different environmental conditions. The test is important for generating data that allow the establishment of the storage condition and shelf life of the vaccine (WHO 2006). The test data of I-2 vaccine produced at other laboratories are available and can serve as a guide and reference for comparison. It has been suggested that each manufacturer should perform stability test on the vaccine produced in their own laboratories for compliance with Good Manufacturing Practices (GMP) (Young *et al.,* 2012). Temperature is one of the important factors that affect vaccine stability and quality in most developing countries (WHO 2006). Thus the current study aimed to established data for the thermostability of TEMEVAC® vaccines in different environmental conditions.

MATERIAL AND METHODS

Two batches of TEMEVAC® vaccines (batch 0068 and 0069) manufactured by TVI and 9-days old chicken embryonated eggs (Certified MKUZA hatchery) were used for the current study. A total of 158 vaccine vials (droppers) from the two batches were tested by placing in the stability chamber (MEMMERT ICH 110) at temperatures 2-8˚C, 22-25˚C and 37˚C for 204, 60 and 10 days, respectively. The vaccines at 2-8˚C were collected and tested at day 60, 90, 120, 135,150, 169,180, 194,197 and 204. Vaccine samples at 22-25˚C were collected and tested at day 7, 14, 21, 30, 45 and 60 and those at 37˚C were tested at day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10. Each test vaccine were10 fold serial diluted and then inoculated into 9 days old embryonated chicken eggs under sterile condition in the biosafety cabinet level two. The inoculated eggs were the incubated at 37°C for 96 hours for viral replication. Haemaglutination test was conducted to measure the ability of a virus suspension to agglutinate red blood cells according to Thayer and Beard 1998. A virus Infectivity assay and Standard mathematical formula were applied for confirming the presence and measurement of live viruses and determination of Embryo Infectivity Dose fifty (EID_{50}) as described previously (Reed and Muench 1938).

Data analysis

The obtained data were fed in the Microsoft version 2010 Spread-sheet and analyzed by Epi Info™ Version 7 (Centre for Disease Control, Atlanta, USA). The vaccine titres were compared using Chi-square test at a critical probability of $P < 0.05$.

RESULTS AND DISCUSSION

At 37˚C, the titre of the vaccine dropped from $10^{9.5}$ EID_{50}/DOSE to $10^{7.8}$ EID_{50}/DOSE and $10^{7.9}$ EID_{50}/DOSE for batch 0068 and 0069 respectively by day 10, this is above the minimum recommended field titre for I-2 vaccine ($10^{6.0}$ EID_{50}/DOSE) which correlate to other studies (Charkraborty *et al.*, 2014) where the vaccine maintained the titre above the minimum recommended filed titre up to 29 days at this temperature. These findings indicate that I-2vaccine can maintain its potency up to more than 10 days when kept at this temperature.

At 22-25˚C the titre decreased from $10^{9.5}$ EID_{50}/DOSE to $10^{6.1}$ EID_{50}/DOSE for batch 0069 and decreased from $10^{9.5}$ EID50/DOSE to $10^{6.2}$$EID_{50}$/DOSE for batch 0068 at day 45 which is above$10^{6.0}$ EID_{50}/DOSE, the minimum recommended field titre. The current vaccine batches maintained recommended field titre longer than vaccines observed elsewhere (Chakraborty *et al.*, 2014). On the other hand the current vaccines had shorter potency period compared with another study (Spradbow *at al.*, 2012). This difference in time between the previous study and the current study may be attributed to the nature of stabilizer (1% gelatin) that was added to the vaccine under the current study. However, by day 60 the vaccine titre for the two batches decreased to the level that is below the recommended dose. Which were $10^{5.5}$ EID_{50}/DOSE and $10^{5.0}$ EID_{50}/DOSE for batch 0068 and 0069, respectively. Therefore, the findings from this study suggested that, TEMEVAC® can retain its potency up to 45 days when kept at room temperature, hence should not be used beyond 45 days at this temperature.

At 2-8˚C the vaccine retained its infectivity titre up to 197 days where the titre dropped from $10^{9.5}$ EID_{50}/DOSE to $10^{6.3}$ EID_{50}/DOSE and $10^{7.3}$ EID_{50}/DOSE for batch 0068 and 0069, respectively. This suggest the vaccine can be viable up to 197days at 2-8˚C. The infectivity titre plunged to $10^{5.2}$ EID_{50}/DOSE and $10^{5.0}$ EID_{50}/DOSE for batch 0069 and batch 0068, respectively. This indicate that the vaccine should not be used beyond 197 days even if is stored at refrigeration.

It is concluded that the current study has found that the potency of TEMEVAC ®Vaccine can be maintained up to 10 days at 37°C and 45 days at 22-25°C and up 197 days at 2-8°C. This study underscores the importance of using TEMEVAC® vaccine which can tolerate a relatively wider range of temperatures for control of the disease in rural areas. The tolerance to environmental temperature at room temperature for 45 days is advantageous to poultry farmers in villages.

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Trends in diagnosis of Marek´S disease (MD) in poultry at Central Veterinary Laboratory in Dar es Salaam, Tanzania

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SUMMARY

Marek's Disease virus (MDV), is a DNA virus a highly contagious herpes virus is considered endemic in the global poultry environment. Retrospective data from January 2012 to December 2016 was analysed to determine the trend of occcurence of Mareks Disease in Dar es Salaam Region. During the period of 5 years 580 chickens were examined and their age category was 10 – 16 weeks of age were 250 birds whereas above 16 weeks of age were 330. Pooled organs namely liver, proventriculus, bursa of Fabricious, thigh muscles and sciatic nerves were sampled and stored in 10% formalin and histopathological sections were prepared and stained with Haematoxyclin and Eosin. Microscopic observation was done at 10x and 40x magnification where by the pleomorphic infiltration of lymphocytes were observed in tissue sections which suggested Marek disease positive. Marek disease was detected in 376 out of 386 (97.4%) chickens of 10-16 weeks old and 100 (51.5%) out of 194 chickens above 16 weeks old showed MD positive. From the above results, it shows that Marek's disease in Dar es Salaam is endemic with high prevalence. If not controlled, Marek's disease will keep on causing a great economic losses and unhealthy condition to chickens. Hence education on vaccination against the disease should be encouraged to reduce the risk of disease occurance in da es Salaam and other places in Tanzania.

Key words: Control, diagnosis, Marek´s disease,vaccination

INTRODUCTION

Consumption of poultry and poultry products in most developing countries has grown by 5.8%, faster than that of human population growth; as a result, the poultry industry has over the years recorded dramatic changes (State *et al.*, 2010). There is no doubt that high rate of intensification may suffer management defects by deteriorating standards, interplay between environment, host and organism genes, slow responses to prevention and treatment that may ultimately subject a large number of birds to further suffering with a consequent high rate of disease spread (State *et al.*, 2010). Almost all industrialized nations had at one time experienced losses due to Marek's disease (MD) in their poultry industry and a crude estimate of the cost of Marek's disease is said to be in the range of several billion US dollars (Okwor and Eze, 2011). Marek's disease is common in intensively managed commercial layer type chickens (Marek ' s disease, 2008), though few reports have been documented in broilers and geese in some countries. It is a transmissible lymphoproliferative disease of primarily chicken that is caused by an alpha herpes virus and characterized by malignant tumour formations in internal organs, (Marek ' s disease, 2008) in, eye and peripheral nerves. The Marek's disease virus (MDV) is cell associated in body organs and tumors, it replicates and exists as enveloped free form in the feather follicles making feathers particularly dander, dust and litter materials loaded with MD virus, thus facilitating virus transmission by air borne route (Cuxhaven, 2001). Susceptible chickens infected with the pathogenic MDV suffer cytolysis of the lymphoid organs and a concomitant immunosuppression (Cuxhaven, 2001).

Such birds mainly die as a result of tumors development in the visceral organs and peripheral nerves. Though morbidity and mortality vary with virus strain, it is a major economic risk for poultry flocks, not only because of its worldwide distribution, but because it majorly affects young adults about to be utilized for meat or egg production, thereby reducing the profitability of an affected flock (Cuxhaven, 2001).

Single vaccination against MD either *in ovo* or at day of hatch in genetically susceptible stock has been effectively used to control the occurrence of clinical disease in the recent pass (Cuxhaven, 2001). But more recently, double vaccination against MD has also been reported to be more effective, however, the evolution of very virulent MD virus strains, inevitable exposure of flocks to other immunosuppressive diseases or conditions, exposure of birds to Marek's disease virus before vaccine immunity develops and inappropriate vaccine handling and vaccination procedures have resulted to "vaccine break and failure" leading to

increasing number of MD outbreaks despite consistent vaccination in many countries .Another problem associated with MD control through vaccination is evaluating vaccine induced protection following vaccine administration (Cuxhaven, 2001). According to the 2002/2003 National Sample Census of Agriculture, out of 4.9 millions smallholder households in Tanzania, 3 million (62%) kept chickens and of these, 99% kept indigenous chickens and the remaining 1% kept commercial birds (Msami *et al.,* 2007). The livestock sector contributes 18% of Agricultural GDP and 4.7% of national GDP Chickens contribute 16% of livestock GDP, 3% of agricultural GDP and 1% of national GDP. Thus the contribution of chicken to the national economy is significant.

Dar es salaam region has so many smallholder poultry farmers and they experince a number of diseases which has been causing high morbidity and mortality rates. The purpose of this retrospective work was to determine the trend in diagnosis of Marek´s disease (MD) in poultry at Central Veterinary Laboratory in Dar-es-salaam, Tanzania for the period of five years between 2012 and 2016.

MATERIALS AND METHODS

Study area

This study was conducted in Dar es salaam region which is located in Eastern zone of Tanzania. Dar es Salaam experiences get annual rainfall that ranges between 500 and 1800 mm. The region has bimodal rain pattern with about 83% of the rain falling between late February and end of May, and short rains between October and January. The average relative humidity ranges between 60 to 80%. The day temperatures are almost constant ranging from 27^{o}C to 35^{o}C with minimum night temperature of 16^{o}C in the coolest month.

Sources of data for this study

A five-year diagnosis record (2012-2016) at the Central Veterinary Laboratory was retrived to get the data for this study. Data were arranged based on year, month and age. A case was considered as the farmers reported outbreak of a disease and diagnosed based on the history, clinical signs, postmortem findings and laboratory results. The age of birds in this study were categorized as follows: (i) 10-16 weeks (ii) >16 weeks (Mussa *et al.,* 2013).

Samples collections and storage

Carcasses or live birds submitted to Central Veterinary Laboratory that originated from Dar-es-salaam mainly were used for diagnosis. Each chicken carcass was subjected to routine post-mortem examination paying attention to changes in size, color and the transactional appearances in different organs that may be suggestive of neoplastic growth. Samples of liver, proventriculus, muscle, sciatic nerve and bursas of fabricius either grossly affected by tumors' or not were collected for histopathology examination. The samples were taken to Histopathology Laboratory of the CentralVeterinary Laboratory for processing. Flock history, clinical and pathological lesions observed in reported outbreaks served as bases for tentatively diagnosis of MD, while histopathology findings complemented the diagnoses.

The tissue samples collected were fixed in 10% neutral buffered formalin for 3 days, embedded in paraffin and sectioned with microtome (3-5 μm). These were stained with haematoxylin &eosin (H&E) and examined under light microscope at 10 and 40x magnifications for evidence of histological changes.

RESULTS

Table 1 shows a total of 476 MD cases out of 580 cases recorded over a period of 5 years (2012-2016). The rainy (95.2%) seasons in this study appear to have more cases of MD (Table 3). Two high monthly incidences occurred in September-June. The favourable age for MD occurrence was at 10 to 16 weeks (97.4%) Table 2.

Table 1. Distribution of Marek's disease per year in Dar es Salaam

Year	Marek's disease positive	Percent infection rate
2012	130	86.6
2013	94	75.8
2014	84	84.8
2015	82	80.3
2016	86	81.9
Total	476	82.1

Table 2. Spatial distribution of Marek's diseases based on age and season of the year

Parameter	Category	Number of positive birds	Percent
Age	10-16 (n=250)	376	97.4
	Above 16 (n=330)	100	51.5
Season	Rainy	400	95.2
	Dry	76	47.5

History of the chicken, clinical signs and observed lesions

From history, vaccination against MD was almost always questionable because farmers believed that MD vaccination was the responsibility of the hatchery, while few farmers revaccinated irrespective of whether MD vaccine was given at the hatchery or not. The consistent clinical signs observed were whitish-yellow diarrhea and ruffled feathers; nervous signs (Figure 1A) which are characteristic of MD were rarely seen. The gross pathological lesions observed in most cases were severe emaciation, thickened proventriculus (Figure 2A). The organs like liver had numerous greyish-white coalescing tumour nodules (Figure 2B) Tumor growths in affected organs were also rarely seen. Microscopically, focal to diffuse infiltration or proliferation of immature and mature lymphocytes in the affected organs (Figure 1B) were consistent lesions (Calnek and Calnek, 2017)**.**

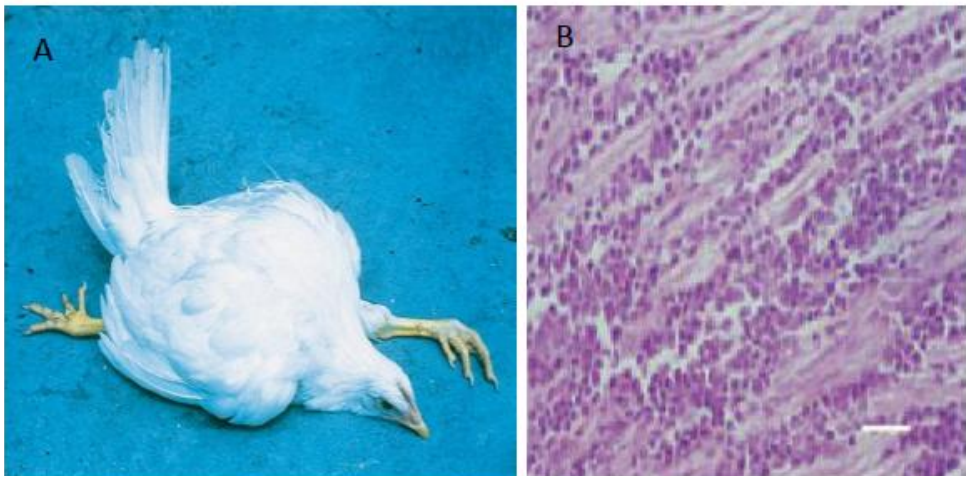

Figure 1. Chicken with legs paralysis (A) and diffuse infiltration of lymphocytes of liver (B) infected with MD

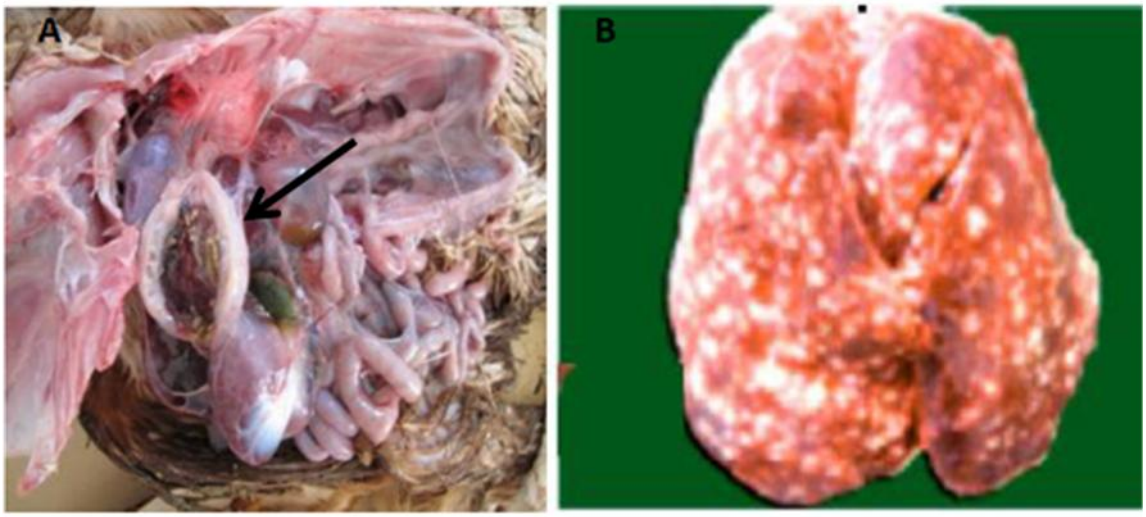

Figure 2. Swollen proventriculus (A) and liver with numerous greyish-white coalescing tumour nodules (B) in chicken infected with MD.

DISCUSSION

Since the 1960s, increasingly virulent strains of MDV have caused continued poultry industry production losses worldwide. In developing countries in addition, improper handling and administration of MD vaccines contribute to continuous outbreaks (Okwor and Eze, 2011). This is because Marek's disease vaccine especially the cell associated type is the most sensitive vaccine requiring storage at -196°C until use, and administration of reconstituted vaccine must be within the first two hours (Cuxhaven, 2001). One other major issue of continuous MD outbreaks in the developing countries is that of vaccine handling.

Results of the current study show that MD is endemic in Dar es Salaam and has been causing high morbidity and mortalities in chicken. The high (95.2%) occurrence of MD in Dar es Salaam was during rainy season and low prevalence of 47.5% in dry season. During the heavy rain season in the study area is characterized by high ambient temperatures which is a serious stressful factor to birds and may have devastating effects on the birds' immune system. Poultry are kept on low nutritive but high energy grower feed within this age range (9 weeks to point of lay) in most farms worldwide. Therefore, bird's immune response is not at its best to protect against this avian cancer and most other immunosuppressive conditions.

Flock history (age of birds), organ/and or tissue neoplastic gross lesions, peripheral nerve lesions and histopathology findings have been our strong diagnostic evidences of MD in the clinic. Nervous signs have not been consistent in assisting the MD diagnosis in our environment, thereby posing further difficulty in differentiating it from avian leucosis (Okwor and Eze, 2011). It has earlier been documented that only a small proportion of the MD affected flocks show nervous signs and that morbidity, mortality rates and other gross manifestations of MD may vary with virus strain, immune status, age and sex of the host (Cuxhaven, 2001).

From this report, it is clear that MD is on the increase despite farmer's efforts to prevent and control it through vaccination. Vaccination alone is therefore not completely reliable in preventing MD outbreaks. More research should be carried out to develop a more potent user friendly MD vaccine, while for the time being, high hygienic standards should be maintained at the hatcheries and farms. Farmers are hereby being advised to keep chicken that are resistant to MD should.

ACKNOWLEDGEMENTS

The authors thank the technical staffs of Central Veterinary Laboratory and histopathology laboratory for making available the required records and processing the tissue slides.

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Current situation for antimicrobial use, antimicrobial resistance and antimicrobial residues in the food and agriculture sectors in Tanzania: A review

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Abstract

Antimicrobials are used worldwide at sub-therapeutic levels as growth promoters in the agriculture sector. The global consumption of antimicrobials in food animal production was estimated at 63 151±1 560 tons in 2010 and is projected to rise by 67%, to 105 596 ±3,605 tons by 2030. Although limited reports describe the situation in Tanzania, the trend of antimicrobials use in the livestock sector is alarming in the country. In Tanzania, studies have indicated that there are rampant and indiscriminate uses of antibiotics in the Livestock sector fueled by trade liberalization. Antibiotics are openly sold on the streets in Tanzania and are distributed over the counters without prescriptions. Farmers in Tanzania do not observe withdrawal periods as recommended by government policies. Most Tanzanian poultry farmers (up to 90%) treat their chicken by themselves with antibiotics. Inappropriate use of antimicrobials for growth promotion and treatment in the animal sector in Tanzania has contributed significantly to the emergence and spread of antibiotic resistance in the livestock sector in this country. Methicillin- resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase (ESBL) were also reported in the livestock sector in Tanzania. Antibiotic residues are present at high levels in foods of animal origin in Tanzania. Antibiotic residues have also been detected in egg, chicken meat, ready-to-eat beef and milk samples from different regions in Tanzania. The use of antimicrobials as growth promoters should be terminated and One Health approach should be used to guide policy and effective use of antibiotics.

Keywords: Antimicrobial resistance; growth promotion; Livestock; food safety; Tanzania

INTRODUCTION

Increasingly, antimicrobial resistance (AMR) and drug resistant infections are being recognized as a crosscutting threat to global health. Despite the increasing global focus, considerable gaps remain in our understanding of AMR. It is now accepted that increased antimicrobial resistance (AMR) in bacteria affecting humans and animals in recent decades is primarily influenced by an increase in usage of antimicrobials for a variety of purposes, including therapeutic and non-therapeutic uses in animal production (Caudell *et al.,* 2017). Antimicrobial resistance is an ancient and naturally occurring phenomenon in bacteria. However, the use of antimicrobial drugs – in health care, agriculture or industrial settings – exerts a selection pressure, which can favour the survival of resistant strains (or genes) over susceptible ones, leading to a relative increase in resistant bacteria within microbial communities (Kashoma *et al.,* 2015). In intensive livestock production systems, resistant bacteria can spread easily between animals and this can be exacerbated if biosecurity is inadequate. Food of animal origin is likely to be quantitatively the most important potential transmission pathway from livestock to humans (Kimera *et al.,* 2015; Kashoma *et al.,* 2015). The current paper presents a short review on the contribution of the food and agriculture sector in the general antimicrobial resistance in Tanzania.

Antimicrobial use in the agriculture sector in Tanzania

Apart from their utilization for livestock treatment, antimicrobials are used worldwide at sub-therapeutic levels as growth promoters in the agriculture sector. The global consumption of antimicrobials in food animal production was estimated at 63 151 ±1 560 tons in 2010 and is projected to rise by 67%, to 105 596±3,605 tons, by 2030 (Laxminarayan *et al.,* 2015). Although limited reports describe the situation in Tanzania, the trend of antimicrobials use in the livestock sector is alarming. In Tanzania, studies have indicated that there are rampant and indiscriminate uses of antibiotics in the Livestock sector fueled by trade liberalization. In the National action plan on antibiotic resistance, 2017-2022 it is indicated that there are high levels of inappropriate use of antimicrobials in the human and animal sectors in Tanzania (MoHCDGEC, 2017). A recent study conducted by Mdegela and colleagues in Morogoro revealed that Antibiotics are widely used as additives in poultry feeds and in treating animals when they are sick. There was also a high routine use of antibiotics in animals and in particular in

commercial chickens and dairy cattle. Respondents in that study affirmed that from day one, chicks are given tetracycline. They also found that Antibiotics are openly sold on the streets in Tanzania and arealso distributed over the counters without prescriptions (Mdegela, 2015). A number of studies reported that farmers in Tanzania do not observe withdrawal periods as recommended by government policiesand therefore keep injecting animals with antibiotics due to ignorance and perceived economic losses (Nonga *et al.,* 2009; Katakweba *et al.,* 2012; Kimera *et al.,* 2015; Mdegela, 2015; Caudil *et al.,* 2017).Caudil *et al.,* (2017) reported ahigh use and self-administration of antimicrobials in cattle herds in Northern Tanzania. The study also indicated fewer adherences to withdrawal of meat and milk from consumption during and following antimicrobial treatment especially among the Maasai, Arushaand Chagga herders. Oxytetracycline, Penicillin dihydrostreptomycin, Tylosin and Sulfonimides were by far the most commonly used antibiotics in the study area (Caudil *et al.,* 2017). In a study conducted in Dar es Salaam poultry farmers, all interviewed participants reported to use antibiotics in their flocks for both prevention and treatment and the most frequently used antibiotic drugs belong to the group of tetracycline and sulfonamides (Mubito *et al.,* 2014). Furthermore, some prohibited antimicrobial agents like furazolidone were found in some veterinary drug stores and poultry farms. All interviewed poultry farmers were aware of drug withdrawal period but none of them declared to observe this requirement because they fear investment losses (Mubito *et al.,* 2014). A significant proportion of Tanzanian poultry farmers treat their chicken by themselves (Nonga *et al.,* 2010; Mubito *et al.,* 2014). In 2009, 90% of broiler chicken farmers interviewed in Morogoro frequently used tetracycline, amprolium, sulphonamides, thrimethoprim, neomycine and flumequine in their chicken flocks and 95% of them slaughtered their chicken before withdrawal period (Nonga *et al.,* 2009). A study by Katakweba *et al.* (2012) reported that 40% of Tanzanian small-scale livestock keepers did not know if antibiotics they used in livestock might pose risk to human health. Similar observations were reported among smallholder poultry farmers in Morogoro where 85% of them were unaware of possible effects of antimicrobial residues in humans (Nonga *et al.,* 2010). In Zanzibar, all investigated broiler farmers use antibiotics to treat their animals but only 45.5% reported to comply in fear of losses and limited awareness of health effect associated with antibiotic residues (Nonga *et al.,* 2013). Most chicken farmers investigated in Morogoro use antimicrobial drugs as prophylaxis and treatment of common chicken diseases namely fowl typhoid, infectious bursa disease (Gumboro), infectious coryza, collibacilosis, coccidiosis, Newcastle disease, helminthosis and fowl pox and Antimicrobials accounted for 85% of the drugs used in the farms (Nonga *et al.,* 2010). Tons of different antimicrobial agents are imported officially in Tanzania in the veterinary sector every year (Figure1). However, official figures do not exist for antimicrobials that illegally enter the country and probably outweigh the official numbers.

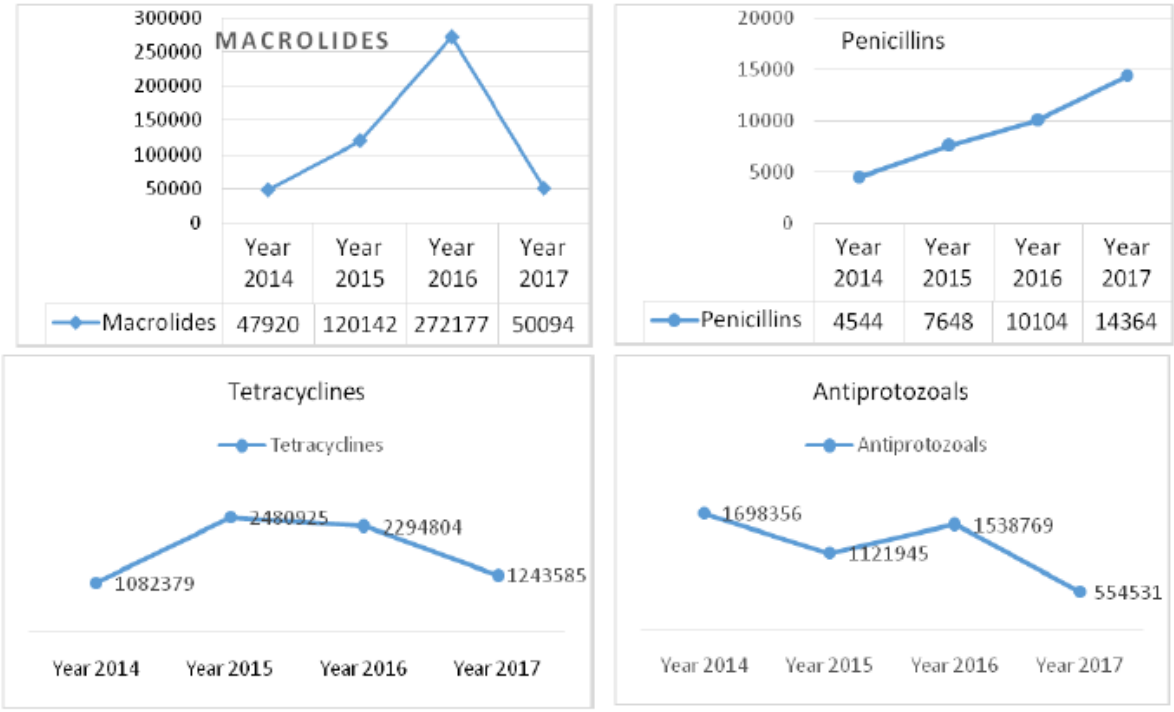

Figure1. TFDA data on trend of Antimicrobials of Veterinary use that officially entered TZ the last 4 years (Kg)

Antimicrobial resistance in the livestock sector in Tanzania

Antimicrobial resistance is a major animal and human health problem in Tanzania. High levels of inappropriate use of antimicrobials for prophylaxis, growth promotion and treatment in the animal sector in Tanzania has contributed significantly to the emergence and spread of antibiotic resistance in the livestock sector in this country. The available studies show a number of multidrug resistant bacteria known to cause mastitis in lactating animals (MoHCDGEC, 2017). High levels of resistance were reported to penicillin G, chloramphenicol, streptomycin and oxytetracycline among *Actinobacter pyogenes, Staphylococcus hyicus, Staphylococcus intermedius* and *Staphylococcus aureus* from cattle with mastitis. Similar results were found in poultry contaminated with *E. coli* strains that were resistant to amoxicillin + clavulanate, sulphamethoxazole and neomycin (MoHCDGEC, 2017). Moreover, there is an increasing trend in the incidence of antibiotic resistance with significant increase in multidrug-resistant *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*, and Salmonella in food animals in Tanzania as reported by Mshana *et al.* (2013). The same authors indicated an increase in methicillin- resistant Staphylococcus aureus (MRSA) and extended-spectrum beta-lactamase (ESBL) in the livestock sector in Tanzania. Similarly, high Antimicrobial resistance rates of *Escherichia coli* and *Campylobacter* spp. from animals to Ampicillin, Augmentin, Gentamicin Co-trimoxazole, Tetracycline, Amoxicillin, Tetracycline, Erythromycin, Cefuroxime, Norfloxacin and Ciprofloxacin were reported in Tanzania, Mozambique and Zambia (Mshana *et al.*, 2013). Antimicrobial resistant *Campylobacter* in important food animals in Tanzania was reported in isolates from Pigs, Dairy, and Beef Cattle in Tanzania with specific resistance to ampicillin, gentamicin, streptomycin, erythromycin, tetracycline, ciprofloxacin, nalidixicacid, azithromycin, and chloramphenicol, tylosin (Kashoma et al., 2015). Resistant Campylobacter were also isolated from Dressed Beef Carcasses and Raw Milk to Ampicillin, ciprofloxacin, erythromycin, gentamicin, streptomycin, tetracycline, azithromycin, nalidixic acid, chloramphenicol, and tylosin was reported in Tanzania (Kashoma *et al.*, 2016). Another study indicated that *Campylobacter* isolates from duck in Morogoro were resistant to cefuroxime sodium, tetracycline, ampicillin, erythromycin, gentamicin, cloxacillin and amoxicillin (Nonga and Muhairwa, 2009). In 2014, a study revealed that wildlife and cattle in Tanzania harbored high number of resistant *Escherichia coli* and *Enterococci.* (Katakweba *et al.*, 2014). Antimicrobial resistance also occurs in the aquaculture sector in Tanzania. Prominent genes encoding resistance to tetracycline, trimethoprim, amoxicillin, streptomycin, chloramphenicol, and erythromycinIn were identified in integrated fish farming systems in the country (Shah *et al.*, 2012).

Antimicrobial Residues in food of animal origin in Tanzania

Various studies conducted in Tanzania have stated that the majority of animal keepers use antimicrobials but do not adhere to withdrawal periods. The direct consequence of this is the occurrence of antibiotic residues in the foods (milk, eggs, meat etc.) derived from the animals. A study conducted at ILRI on the status of regulations for safety of animal source foods in selected Sub-Saharan African countries revealed that Antibiotic residues are present at high levels in foods of animal origin in Tanzania (Mohammad and Delia, 2012). Nonga *et al.* (2013) detected antimicrobial residues in 76.4% of 72 broiler meat samples from Zanzibar. In a similar study oncommercial egg samples from Morogoro municipality it was found that all 70-egg samples analyzed were positive for antimicrobial residues (Nonga *et al.,* 2010). In a previous study on broiler chickens, the same researchers revealed that 70% of sampled farms had antimicrobial residues in Organization (Mgonja *et al.,* 2016). Similar observations were made in the studies of Kimera *et al.* (2015) who reported Oxytetracycline residues in 71.1% of muscle, liver and kidney of slaughtered cattle, of which 68.3% were above acceptable regulatory levels.

Conclusion and Recommendations to mitigate and reduce threat of AMR

Taking into account that the risk of AMR grows in proportion to the amount andfrequency of antimicrobial use, multidisciplinary and internationalcollaborationsare required to address AMR threat. The use of antimicrobials as growth promoters should be terminated, as they are known to select for cross-resistance to antimicrobials used in human and animal medicine. A One Health approach should be used to guide policy and effective use of antibiotics.

Tanzania has taken a good step by writing down a national action plan for the control of antimicrobial resistance. However, this needs to be scrupulously respected and should involve as much monitoring in the food and agriculture sector as in human medicine.

Further recommendations to address this problem are:

(i) The creation of awareness among policy makers, who are mostly unaware ofthe food their chicken meat (Nonga *et al.,* 2009).In 2006, Kivaria and colleagues reported that raw milk marketed by smallholder dairy producers in Dar es Salaam were positive for antimicrobial residues (Kivaria *et al.,* 2006). Moreover, Kurwijila *et al.* (2006) indicated that Antimicrobial residues were detected in about 36% of milk that are marketed across Tanzania, suggesting an average risk of about 11 exposures per month for a daily consumer of milk. In addition, Mdegela *et al.* (2009) reported contamination of milk with antimicrobial residues in smallholder dairy farms in Mvomero and Njombe districts. Lately, using liquid chromatography-mass spectrometry (LC-MS), a high proportion of beef sampled in Dodoma had oxytetracycline residues (Mgonja et al., 2017). This same research group reported as high as 78% of ready-to-eat beef samples to contain oxytetracycline residues while 25.7% had violative residue levels above the maximum residue limits recommended by the Food and Agriculture Organization and the World Health safety problem, and development of potential interventions to address the problem;

(ii) Promotion of organic farming

(iii) routine testing for antibiotic residues in food during food inspections; and

(iv) further investigation of animal production practices that involve the use of antimicrobials by predominantly smallholderproducers and market agents.

Additionally, thorough risks assessment is needed at national and regional levels to provide a basis for designing appropriate extension messages and effective management strategies for protecting the health of consumers without unnecessarily jeopardizing the livelihood benefits derived from animal keeping by producers and market agents. Furthermore, official guidelines on prudent use of antibiotics should be supplied together with antibiotics when sold over the counter in veterinary shops. Quality assurance of antibiotics sold on the market should be ensured through an official approval system for such drugs in Tanzania and East African Nations as a whole. Regular surveillance of antibiotic resistance in bacteria from food animals and humans should be established and well funded.

ACKNOWLEDGEMENTS

The authors would like to thank all who contributed in one way or the other during gathering of literature and writing of this review work.

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Antibiotic resistance of *Salmonella* isolated from commercial chicken feeds in Dar es Salaam, Tanzania

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SUMMARY

Salmonella resistance to antimicrobials is rapidly growing worldwide. Antibiotics are commonly used in prevention of bacterial infections as well as treatment of infected chickens in Tanzania. A study on Salmonella was conducted in commercially produced chicken feeds from feed mills in Dar es Salaam, Tanzania, between May 2015 and June 2016 with the objective of estimating the prevalence of Salmonella contamination and their antibiotic susceptibility pattern. Feed samples were collected from a total of 384 randomly selected feed bags of different types from six feed mills. Cultural and biochemical tests were performed for identification of Salmonella in the samples. All isolates were subjected to eight commonly used antibiotics for sensitivity test using disc diffusion method. The overall prevalence of Salmonella in the study was 22.1%. Prevalence of Salmonella contamination was 22.2%, 39.1%, 14.7%, 0.0%, 25% and 42.9% of the samples from feed mills named A, B, C, D, E and F respectively. Significantly higher ($p = 0.001$) prevalence of Salmonella contamination was seen in feed mill B. Although Salmonella isolates were sensitive to Ciprofloxacin (89.0%), the sensitivity decreased towards Amikacin (36.6%), Sulphamethoxazole/Trimethoprim (14.6%), Gentamicin (13.4%), Kanamycin (12.2%), Streptomycin (3.7%), Amoxycillin (1.2%) and Tetracycline (0.0%). Isolation of Salmonella from commercial chicken feeds in Dar es Salaam connotes the importance of hygienic processing and handling of feeds for effective control of Salmonella contamination in both humans and farms. The antibiogram pattern shows the presence of antibiotic resistant Salmonella species hence; suitable measures should be implemented to avoid indiscriminate use of antibiotics in chicken feed chain.

Keywords: *Salmonella*. Poultry mash. Feed mills. Antibiogram. Antibiotic sensitivity.

INTRODUCTION

Antimicrobial resistance in *Salmonella* is rapidly growing worldwide from about 20% in 1990s (Kruger *et al.*, 2004) to the currently up to 100% reported cases (Akbar and Anal, 2013). Development of resistance is linked with indiscriminate use of antibiotics in animals for therapeutics, prophylaxis and growth promotion (McEwen and Fedorka-Cray, 2002; Kumari *et al.*, 2013). Resistance in bacteria from animals is of great importance as it can spread to humans (Mølbak *et al.*, 1999; Fey *et al.*, 2000) and thus necessitating the use of more expensive drugs, making treatment less effective, and even leaving infections untreatable both in animals and humans (Mølbak, 2005). A study by Lee *et al.* (2003) showed a rapid increase of the prevalence of resistance of *Salmonella* to one or more drugs from 0% in 1995 to 93.5% in 2001, but later on Taddele *et al*. (2012) indicated that all (100%) *Salmonella* tested were resistant to erythromycin with 86.7% of them showing resistance to nalidixic acid and more than 53% of the strains being up to 100% resistant to kanamycin and tetracycline. A study by Borah *et al*. (2013) revealed that all *Salmonella* strains isolated from human, cattle and poultry were resistant to furazolidone whereas Whichard *et al.* (2007) reported a concurrent resistance to Quinolones and Cephalosporins which are main drugs used in treatment of *Salmonella* in both humans and animals. Antibiotics are commonly used in prevention of bacterial infections as well as treatment of infected chickens in Tanzania (Mubito *et al.*, 2014). Because of the increase in antimicrobial resistance, regular testing of *Salmonella* sensitivity to antibiotics is important in providing and reviewing treatment guidelines.

MATERIALS AND METHODS

A cross-sectional study was carried out in Dar es Salaam to analyse the aspects of *Salmonella* contamination and its antibiogram in commercial chicken feeds. The region has a land area of 1,590.5 km^2 located at 6°48' South, 39°17' East (in the Eastern part of Tanzania) with a tropical hot and humid weather. Dar es Salaam is Tanzania's most important city for business with the highest number

of chicken per household in the country (FAO, 2008; Tanzania National Bureau of Statistics, 2012).

A total of 384 feed samples were collected from six feed mills during the period of May 2015 and June 2016. Selection of feed bags was done using systematic sampling method and the interval of selection was determined according to the expected number of bags from the finished product bins ready for bagging. A sample of about 50g was collected using a sterile zip-lock bag and transported to the laboratory for testing.

Salmonella isolation and identification

Isolation of *Salmonella* in feed samples was done at Central Veterinary Laboratory, Tanzania Veterinary Laboratory Agency (TVLA) according to the standard culture methods (ISO 6579:2002(E), 2002; OIE, 2012). A 25 g portion of feed sample was pre-enriched in 225 ml of buffered peptone water (Himedia, Mumbai, India) and incubated at 37°C for 24 hr. Then 0.1 ml of the pre-enrichment culture was added to 10ml of Rappaport-Vassiliadis broth (Himedia, Mumbai, India) and incubated at 41.5°C for 24 hr. Loopful inoculums were subsequently streaked into Xylose Lysine Deoxychocolate Agar (XLD-Agar, Scharlau Chemie S.A., Barcelona Spain) and McConkey's agar (Himedia, Mumbai, India) and incubated at 37°C for 24 - 48 hr to obtain only single type of colonies. The isolates were identified as *Salmonella* species based on the colony appearance, Gram stain, triple-sugar-iron (TSI) reaction, indole reaction, methyl-red (MR) reaction, Voges-Proskauer (VP), and citrate utilisation according to Ewing (1973).

Antibiotic sensitivity test

Antibiotic sensitivity was tested in vitro using the disc diffusion method as described by Bauer *et al.*, (1966). The antimicrobial agents tested were gentamicin (10µg), tetracycline (30µg), amoxycillin (10µg), kanamycin (30 µg), ciprofloxacin (5 µg), Amikacin (30µg), streptomycin (10 µg) and Sulphamethoxazole/Trimethoprim SXT (23.75/1.25 µg) (Oxoid Limited, Basingstoke, Uk). All (n=85) isolates obtained from the feed sample was tested for antibiotic sensitivity. A pure bacterial culture was inoculated into nutrient broth and incubated at 37ºC for 24 h. The broth was homogenously streaked using sterile cotton swabs on Mueller-Hinton agar plates. Antibiotic discs were placed aseptically using sterile forceps on the surface of the inoculated plates. The plates were then inverted and incubated at 37°C for 24 h. After incubation, the plates were examined and the diameters of the zone of complete inhibition were measured to the nearest millimeter using a millimeter ruler and compared with a zone interpretation chart as described by the Clinical and Laboratory Standards Institute (2014).

Data Analysis

Data were analyzed using IBM SPSS Statistics version 20 computer program. The Pearson's chi-square (χ^2) test at a significance level of 5% was used to determine the prevalence of *Salmonella* contamination among different feeds, between batches and among feed mills (McHugh, 2013). The difference was considered statistically significant if the p-value was less than or equal to 0.05.

RESULTS

A total of 384 commercial chicken feed samples consisting of Broiler starter mash (n=121), Broiler grower mash (n=47), Broiler finisher mash (n=102) and Layers mash (n=114) samples (Table 1) from the selected feed mills in Dar es Salaam were tested for *Salmonella*. Out of the 384 samples, 85 tested positive (22.1%) for *Salmonella.* The prevalence of *Salmonella* was highest in samples from broiler starter (29.8%) followed by broiler finisher (19.6%), layers (18.4%) and grower (17.0%).

Table 1: *Salmonella* isolated from different feed types

Feed type	No. of samples tested	No. of *Salmonella* positive (%)	χ^2-value	P-value
Broiler Starter Mash	121	36 (29.8)	6.077	0.108
Broiler Grower Mash	47	8 (17.0)		
Broiler Finisher Mash	102	20 (19.6)		
Layers Mash	114	21 (18.4)		
Total	384	85 (22.1)		

The prevalence of *Salmonella* in commercial chicken feed was investigated in six feed mills in Dar es Salaam (Table 2). The results showed that 22.2% (n=45), 39.1% (n = 92), 14.7% (n = 75), 0.0% (n = 85), 25% (n = 13) and 42.9 (n = 35) of the

samples from feed mills A, B, C, D, E and F respectively were positive for *Salmonella*.

Table 2: Distribution of *Salmonella* among feed mills

Feed mill	No. of samples tested	No. of *Salmonella* positive (%)	χ^2-value	p-value
A	45	10 (22.2)	50.976	0.001
B	92	36 (39.1)		
C	75	11(14.7)		
D	85	0 (0.0)		
E	52	13(25.0)		
F	35	15 (42.9)		
Total	384	85 (22.1)		

The isolation of *Salmonella* in feed also varied between the two batches sampled from each feed mill (Table 3). A total of 44 out of 186 samples (23.7%) in batch number 1 were positive for *Salmonella* while 41 out of 198 (20.7%) in batch number 2 were positive for *Salmonella*.

Table 3: Distribution of *Salmonella* between batches in each feed mill

Feed mill	Batch no.	No. of samples tested	No. of *Salmonella* positive (%)	χ^2-value	p-value
A	1	23	5 (21.7)	0.407	0.524
	2	22	5 (22.7)		
B	1	24	9 (37.5)	0.036	0.849
	2	68	27 (39.7)		
C	1	32	6(18.8)	0.744	0.389
	2	43	5 (11.6)		
D	1	42	0 (0.0)		
	2	43	0 (0.0)		
E	1	32	12(37.5)	6.933	0.008
	2	20	1 (5.0)		
F	1	26	12 (46.2)	0.449	0.503
	2	9	3 (33.3)		
Total		384	85 (22.1)		

Antibiogram of the isolates are presented in Table 4. The highest sensitivity was recorded in ciprofloxacin (89.0%) followed by amikacin (36.6%), sulphamethoxazole/trimethoprim (14.6%), gentamicin (13.4%), kanamycin (12.2%), streptomycin (3.7%), amoxycillin (1.2%) and tetracycline (0.0%).

Table 4: Antibiotic sensitivity pattern of *Salmonella* isolates from feed mills

Feed mill	Total No. of isolates	No. of *sensitive isolates* (%)							
		S	Ac	Am	Cf	Tc	K	G	SXT
A	10	0 (0.0)	0 (0.0)	2 (22.2)	8 (88.9)	0 (0)	2 (22.2)	2 (22.2)	2 (22.2)
B	36	1 (2.9)	0 (0.0)	13 (37.1)	30 (85.7)	0 (0)	4 (11.1)	5 (14.3)	4 (11.4)
C	11	0 (0.0)	0 (0.0)	5 (45.5)	11 (100)	0 (0)	0 (0.0)	0 (0.0)	1 (9.1)
E	13	1 (8.3)	0 (0.0)	4 (33.3)	12 (100)	0 (0)	1 (8.3)	2 (16.7)	2 (16.7)
F	15	1 (6.7)	1 (6.7)	6 (40.0)	12 (80.0)	0 (0)	3 (20.0)	2 (13.3)	3 (20.0)
Total	85	3 (3.7)	1 (1.2)	30 (36.6)	73 (89.0)	0 (0)	10 (12.2)	11 (13.4)	15 (14.6)

S = Streptomycin (10 µg); Ac = Amoxycillin (10 µg); Am = Amikacin (30 µg); Cf = Ciprofloxacin (5 µg); Tc = Tetracycline (30 µg); K = Kanamycin (30 µg); G = Gentamicin (10 µg); SXT = Sulphamethoxazole/Trimethoprim SXT (23.75/1.25 µg)

DISCUSSION

The present study demonstrated that the prevalence of *Salmonella* in feeds in Dar es Salaam (22.1%) agrees with, but is slightly less than, the previously reported prevalence of 29.4% *Salmonella* contamination in feed mills in Ilala District, Tanzania (Mdemu *et al*., 2016). Although some studies have reported a higher prevalence (Chowdhury, *et al.*, 2011), the present data shows that the levels of contamination in this study are higher compared to some of the reported data (Tavechio *et al.*, 2002; Molla *et al.*, 2010; Nourmohamadi and Shokrollahi, 2014). Plavšić *et al.*, (2009) reported that no *Salmonella* was detected in a study on feeds in Serbia whereas the prevalence of 0% to 5.3% *Salmonella* contamination in poultry feeds has been reported from different states in the European Union (Ekesbo, 2013). The prevalence of *Salmonella* in commercial chicken feeds reported here connotes the importance of hygienic processing and handling of feeds for effective control of *Salmonella* contamination in both humans and farms.

Although broiler starter mash in this study showed a slightly higher prevalence than other feed types, statistical analysis of the data revealed that there was no significant difference (χ^2 = 6.077, P = 0.108) on the prevalence of *Salmonella* contamination among broiler starter mash, broiler grower mash, broiler finisher mash and layers mash. A study by Younus *et al.* (2009) showed a similar trend but with lower prevalence of *Salmonella enteritidis* and *Salmonella typhimurium* in chicken feeds in Pakistan. The results of this study therefore confirms that *Salmonella* contamination do not vary depending on the type of feed.

Statistical analysis of the data indicated that there was significant difference (χ^2 = 50.976, P = 0.001) on the prevalence of *Salmonella* contamination among feed mills A, B, C, D, E and F (Table 2). The differences recorded in this study are in agreement with the previous data (Mdemu, Mathara and Makondo, 2016) indicating that some of the feed mills in the region are more likely to formulate *Salmonella* contaminated feeds than others therefore releasing feeds with different levels of *Salmonella* contamination in the market. Davies and Wray (1997) reported different levels of *Salmonella* contamination in ten feed mills in Great Britain suggesting that feeds can be contaminated at the feed mill. The reasons of these differences are beyond the scope of this study and therefore it is recommended that further studies be undertaken.

The distribution of *Salmonella* in contaminated feeds can be sporadic and uneven (Jones, 2011) such that the levels of contamination might differ between batches. The data presented in this study shows the distribution of *Salmonella* contamination between feeds produced in two manufacturing orders (batches) of each feed mill (Table 3). Statistical analysis of the data indicated that there was a significant difference between batches in feed mill E (χ^2 = 6.933, P = 0.008) where *Salmonella* was isolated from only 5% of the samples in batch number 2 as compared to 37.5% in batch number 2. The difference in the levels of contamination between batches may also indicate inconsistency in the operation of the feed mills hence suggesting that periodic testing of *Salmonella* in feed mills should be conducted frequently (by batch) to provide for a higher degree of assurance.

All *Salmonella* isolates were tested for antibiotic susceptibility by disc diffusion method against the commonly used commercial antibiotics (gentamicin, tetracycline, amoxycillin, kanamycin, ciprofloxacin, amikacin, streptomycin and sulphamethoxazole/trimethoprim).

Of all the tested antibiotics, Ciprofloxacin showed the highest sensitivity (89.0%). Although several authors have reported that *Salmonella* is 100% sensitive to ciprofloxacin (Ranju *et al.*, 1998; Mijovic, 2012), but studies show that sensitivity is rapidly decreasing in *Salmonella* isolates from humans, animals and environment worldwide. The level of sensitivity to Ciprofloxacin recorded here is higher than the sensitivity of 73.68% and 80.8% recorded by Borah *et al*., (2013) and Mushtaq, (2006) respectively, but is slightly less than the sensitivity of 95.79% (or 4.21% resistance) and 86.44% (or 13.66% resistance) reported by Nagshetty *et al.*, (2010) and Choudhary *et al.*, (2013) respectively. In the review of antimicrobial resistance in four countries (Zambia, Democratic Republic of Congo, Mozambique and Tanzania), Mshana *et al.* (2013) reported a rapid increase in Ciprofloxacin resistance in The Democratic Republic of Congo from 0.0% in 2009 to 15.4% in 2012, and recently Wasihun *et al*. (2015) have reported 50% and 12.5% resistance of *S.* Paratyphi and S. Typhi respectively in Ethiopia. The evidence of rapid decrease on sensitivity of *Salmonella* to Ciprofloxacin poses a threat to the public health and prompts a careful and appropriate use of antibiotics. The results of the present study, however, differs with the recent report by Katani *et al.* (2015) which showed that *Salmonella* isolates from House crows in Tanzania were 100% sensitive to ciprofloxacin

and the findings by Mwambete and Stephen (2015) on chicken droppings in Tanzania which indicates that only 41% *Salmonella* were sensitive to Ciprofloxacin , but is similar to the report by Meremo *et al*. (2012) which showed 8% resistance of Ciprofloxacin on Non-Typhoidal *Salmonella* from humans in Tanzania. More surveillance of ciprofloxacin sensitivity is therefore required in the fight against resistance.

Salmonella isolates are shown to be less sensitive to both amikacin (36.6%) and gentamicin (13.4%). The results partially agrees with Över *et al*. (2001) who reported 37.5% and 0% S*almonella* sensitivity to amikacin and gentamicin respectively in isolates from hospitals in Turkey, and closely related to the recent study in slaughtered swine in Philippines which showed all *Salmonella* isolates (100%) were resistant to both Amikacin and Gentamicin (Ng and Rivera, 2014). A study by Wasfy *et al*. (2000) show different scenario in Egypt as *Salmonella* isolated from hospital specimens were about 99% and 80% sensitive to Amikacin and Gentamicin respectively. A similar trend was observed by Obi *et al*. (2004) who reported respectively 100% and 96.6% *Salmonella* sensitivity to Amikacin and Gentamicin on isolates from water in South Africa.

In a study on isolates from hospital samples in Tanzania, Blomberg *et al*. (2007) reported an average of 70% *Salmonella* sensitivity to Gentamicin but Meremo *et al.* (2012) reported a lesser sensitivity of 38.5% in *Salmonella* from hospital samples in Tanzania. The study by Mwambete and Stephen, (2015) shows a similar trend with the present study but with higher percentages of sensitivity (68.3% and 53.3%) for amikacin and gentamicin respectively. It appears that the sensitivity of *Salmonella* on both Amikacin and Gentamicin has rapidly decreased with time to the current levels rendering the antibiotics less effective against *Salmonella*.

Salmonella isolates shows to be less sensitive to Amoxycillin (1.2%) and is comparable to the 0% sensitivity reported by Katani *et al*. (2015). The low sensitivity of *Salmonella* to sulphamethoxazole/trimethoprim (14.6%), streptomycin (3.7%), and tetracycline (0.0%) agrees with Dione *et al.*, (2011) showing that *Salmonella* isolates are resistant to these antibiotics and therefore cannot be effective in the treatment of infections by these bacteria. It is therefore recommended that measures should be implemented to avoid indiscriminate use of antibiotics in the feed chain. Furthermore, sensitivity testing of commonly used antibiotics should be done frequently to monitor the development of microbial resistance.

ACKNOWLEDGEMENTS

The researchers are grateful to The Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) and The Central Veterinary Laboratory, Tanzania Veterinary Laboratory Agency (TVLA) for supporting this work. We wish to extend our profound gratitude to Ms. G. Banda of the Department of Bacteriology, Central Veterinary Laboratory, TVLA for her technical support.

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Prophylactic antibiotics in augmenting surgical wound healing

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SUMMARY

Antibiotics are widely used in treatment of bacterial and protozoan diseases in both human and animals. In production animal systems, the overuse and abuse of antibiotics have contributed to the widespread of antimicrobial resistant bacterial strains and drug residues in food chain. Oxytetracycline for example is a common antibiotic employed for treating various bacterial diseases and wound infection. This study aimed at investigating the use of prophylactic antibiotics in augmenting surgical wound healing in animals. Sixty-four adult guinea pigs were employed in this study and randomly divided into four equal groups (n=16). Animals were fully anaesthetized using Xylazine 5.0 mg/kg and Ketamine 44.0 mg/kg. Laparotomy incisions (3 cm long) were made on aseptically prepared sites on the ventral abdomen. The abdominal muscles and peritoneum were sutured by Catgut® while Silk® was used to suture the skin. Animals in group one received Oxytetracycline intramuscular injection two hours before the operation; group two were injected with OTC immediately after the operation; group three received OTC spray on the wound immediately after the procedure; and group four animals were left untreated to serve as the control group. Animals were monitored for two weeks where general appearance and wound contraction was recorded. The wounds in pre-operative group healed faster (7 days), followed by topical group (10 days). Wounds in post-operative and control groups had delayed healing and showed evidence of infection. The study showed that prophylactic antibiotics administration pre-operatively minimize post-operative wound infection and promotes healing.

Keywords: Antibiotics, Prophylaxis, Surgical wound, Pre-operative, Post-operative

INTRODUCTION

A primary function of a normal, intact skin is to control microbial populations that live on the skin surface and to prevent underlying tissues from becoming colonized and invaded by potential pathogens. Exposure of subcutaneous tissue following loss of skin integrity provides a favorable environment for microbial colonization and proliferation (Bogaard *et al.,* 2002). A wound is a bodily injury causing disruption of normal continuity of structures either accidentally or planned (Bowler *et al.,* 2001). Typical planned wounds are surgical wounds and these have been classified as clean, clean–contaminated, contaminated or infected. The classification however has not proved useful in predicting occurrence of wound infection, which is the key to its effective prevention (Haley, *et al.,* 1985). The number and diversity of microorganisms in any wound is influenced by the wound type, depth, location, quality, and level of tissue perfusion and antimicrobial efficacy of the host immune response (Bogaard *et al.,* 2002).

Wound healing is a complex physiological process that may be divided into three main overlapping stages; inflammation, tissue formation and tissue remodeling (Singer and Clark, 1999). Once the tissue is injured, inflammation occurs leading to hemostasis and clot formation; after which the fibroplasia and neovascularization follows with formation of granulation tissue, re-epithelization and finally the formation of new extracellular matrix and tissue remodeling (Bowler *et al.,* 2001). In planned wounds, proper surgical technique including asepsis is important to prevent wound infection, promote wound healing and ensure likelihood of a satisfactory outcome to the surgical procedure (Baines, 1996; Mangram *et al.,* 1999; Ichikawa, 2010).

Wound infection occurs when microorganisms have the opportunity to proliferate in the tissue and the body's defense cannot combat the organism or its proliferation (Hotston, 1995). Certain patient characteristics can be associated with a higher incidence of wound infections including old age, chronic immunosuppressant, diabetes mellitus, corticosteroid use, obesity, malnutrition, and chronic renal failure (Beauchamp *et al.,* 2001). Infecting organisms are often part of the resident flora of the skin or nearby mucous membranes. *Staphylococcus aureus* is most commonly isolated in cutaneous wound infections although *Escherichia coli*, *Streptococcus, Pseudomonas,* and *Proteus species* may also be responsible (Bowler *et al.,* 2001; Kobayashi *et al.,* 2015) Inadequate

postoperative wound care and poor hygiene can introduce bacteria into a wound, which can lead to infection (Haley, *et al.*, 1985).

Antibiotics have been extensively used in treating various bacterial diseases and in wound management. Broad-spectrum antibiotics with activity against bacteria and protozoa are preferred for prophylactic purposes (Hawkey, 1998; Davies and Davies, 2010). Some formulations of OTC when administered intramuscularly take 30 minutes to several hours to reach peak levels in tissues depending on the volume and site of injection. The long acting products have significant slower absorption after intramuscular injection. OTC is eliminated unchanged primarily via glomerular filtration (Donald and Pharm, 1995). Timing of antibiotics administration is critical to efficacy, since the efficacy depends on the presence of peak antibiotic levels in the tissues at a time when the local concentration of microorganisms would otherwise be high (Jenney *et al.*, 2001). Appropriately administered antibiotics as prophylaxis, reduces the incidence of surgical wound infections (Dellinger *et al.*, 1994). Classen *et al.* (1992) suggested the first antibiotic dose to be given preferably within 30 minutes before surgical incision and re-administered at one to two half-lives of the antibiotics for the duration of the procedure. Since antibiotics are also being administered to cases attended aseptically, and in view of the possibility of occurrence of antibiotic resistance following abuse and overuse of these, there is a need to put on record proven recommendations regarding the use of antibiotics in surgery. The present study was aimed at assessing the appropriate time of antibiotics administration for effective prevention of wound infections in surgical interventions.

MATERIALS AND METHODS

Experimental animals

Sixty-four clinically healthy adult male (n=20) and female (n=44) guinea pigs (*Cavia porcellus*) were used for the study that was carried out at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania. All animals were fasted for 12 hours before operation in order to get actual weight and prevent regurgitation during the procedures.

Antibiotic choice

Oxytetracycline hydrochloride (OTC) was used as it is a broad-spectrum antibiotic as well as the most common used antibiotic in treatment of different conditions (bacterial and protozoan infection) in animals.

Experimental design and procedure

The 64 experimental animals were randomly allocated into four equal groups (n=16) i.e. preoperative, postoperative, topical and control groups. Animals in group one, (preoperative) received intramuscular injection of OTC two hours before the operation; group two (postoperative) received OTC intramuscularly immediately after the operation whereas animals in group three (topical) received topical OTC spray immediately after the operation. Animals in the control group were treated with intramuscular water for injection two hours before the operation.

Wound models

Animals were sedated by intramuscular injection of 5.0 mg/kg Xylazine and anesthetized with intramuscular injection of 44.0 mg/kg Ketamine Hydrochloride (Hall and Clarke, 1991). The surgical sites were aseptically prepared by shaving the hairs around the ventral abdomen, cleaning with water and application of Chlorhexidine, alcohol 70%, and finally iodine. A 3cm ventro-midline laparotomy incision was performed cutting through the skin, subcutaneous tissues, *linea alba* and the peritoneum. The incision was sutured using Surgical gut 3/0 for the peritoneum, *linea alba* and subcutaneous tissues while the skin was sutured with Silk 3/0. Animals were treated as shown on the experimental design. After recovery from anesthesia, the animals were taken back to their respective cages.

Observation and Data collection

Wounds were assessed daily until complete healing by re-epithelialization. Body temperature and signs of infection were monitored and recorded daily for 2 weeks. Presence or absence and nature of exudates and smell of the wound were assessed. Wound size measurement was performed at intervals by using veneer calipers and recorded accordingly.

Data analysis

Data were handled using Microsoft excel and analyzed using STATISTICA program version 8. Means and standard deviation for body temperature, infection cases, wound contraction and wound healing rates were calculated. The contingency 2x2 table was employed to check if there were statistically significant differences between and

within the treatment groups. Values of $p \leq 0.05$ were considered significant.

RESULTS

Significant wound contraction was noted in pre-operative and the topical treated groups (Figure 1). Wound healing in the pre-operative group was observed to be between 7 to 8 postoperative days while in topical, post-operative and control groups healing was between 10 to 12, 13 to 15 and 13 to15 days, respectively (Figure 2). Significant difference ($p \leq 0.05$) was observed between the post-operative, preoperative and topical groups but there was no statistical significant difference in healing durations between the post-operative and control groups.

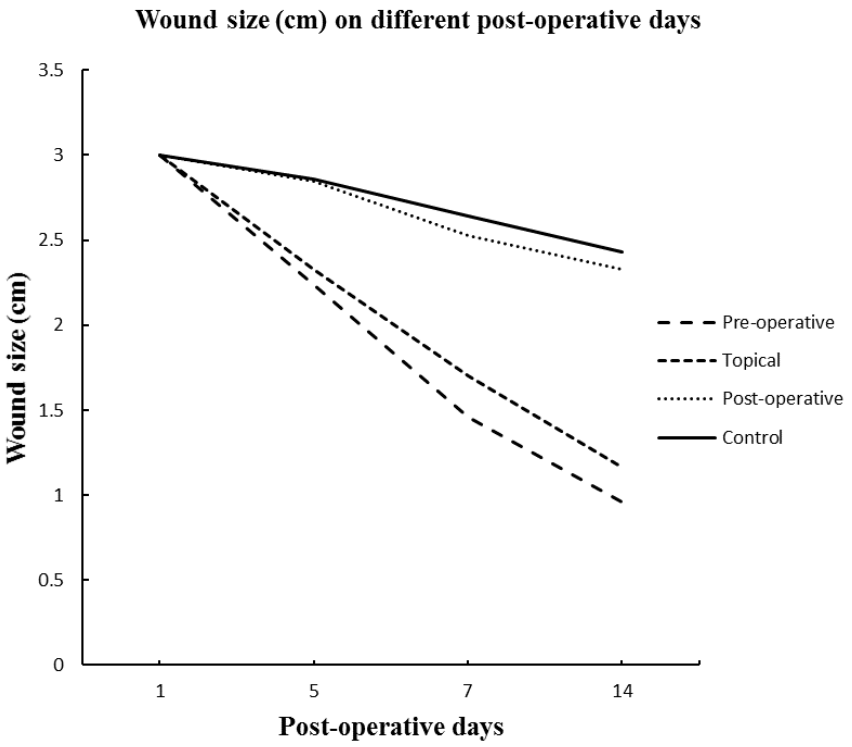

Figure 1. Wound sizes measured at different post-operative days.

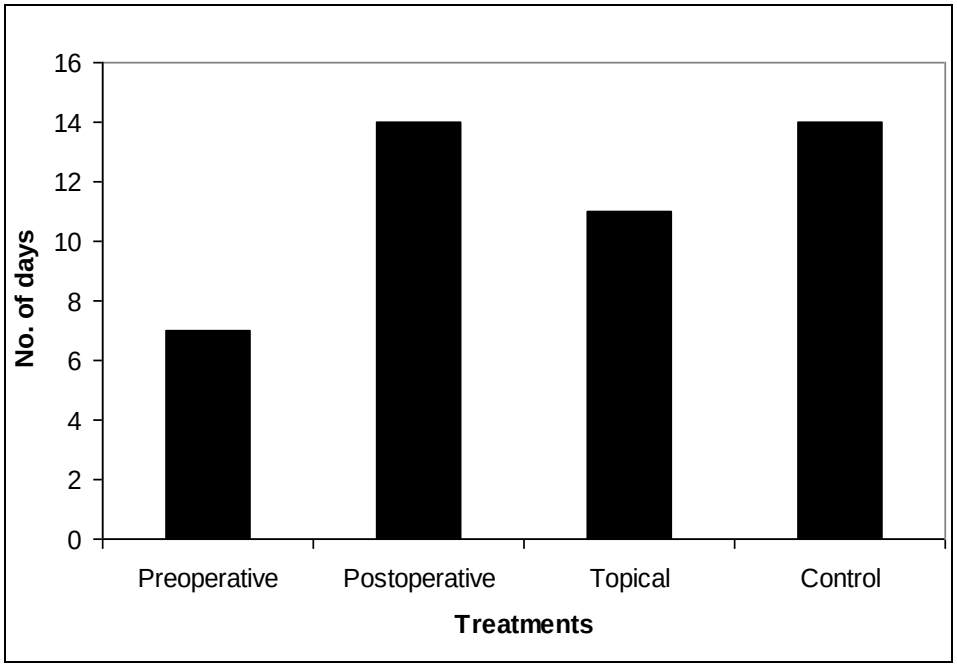

Figure 2. Wound healing durations (days)

Signs of infection such as pyrexia, edema, discharges and pus formation were observed in twelve animals (75%) and ten animals (62.5%) in control and postoperative groups' respectively (Table 1).

Table1. Animals with signs of wound infections

Treatment	Number of animals (%)
Preoperative (n=16)	0 (0.0)
Postoperative (n=16)	10 (62.5)
Topical (n=16)	0 (0.0)
Control (n=16)	12 (75.0)
Total (n=64)	22 (34.4)

DISCUSSION

All surgical wounds are contaminated with bacteria, but not all become infected. The microflora associated with clean, surgical wounds is expected to be minimal, but the presence of foreign materials and devitalized tissue in a traumatic wound is likely to facilitate microbial proliferation unless early prophylactic antibiotic treatment and surgical debridement is implemented (Bowler *et al.,* 2001). Antibiotics have been widely used in veterinary practice to prevent infection in wounds in the perioperative period as it is not easy to define with precision which surgical procedures do and don't warrant prophylactic antibacterial use.

In the present study wounds treated with antibiotic two hours before the procedure healed faster than in the other groups (7-8 days post-operative). Incised wounds begin to heal by 5–8 days with re-establishment of epidermal continuity and a proliferation of fibroblasts from the subcutaneous tissues (Slatter, 1985). The faster uncompromised wound healing in the preoperatively treated animals was attributed to the effect of the antibiotic. During the time of operation, the antibiotic level in tissues was at a peak concentration enabling these animals to fight against the microorganisms thus preventing post-operative wound infection and promote healing.

There was a delay in wound healing in animals under the postoperative group and the control group where most of them (62.5% and 75% respectively) showed typical signs of infection i.e. high body temperature, edema, discharges, and pus formation on the wound. The delayed wound healing in these groups might be explained by the fact that there was no antibiotic in the tissues at the time of intentional tissue damage caused by surgical operation since antibiotic in this group was administered after the operation. The results of this study correlate with the findings of Burke (2001) who reported that administration of antibiotic either shortly before or after inoculation of microorganisms resulted in lesions that were histologically identical to lesions induced by intra-dermal inoculation with killed organisms. Also, our findings are in line with the reports of Page *et al.* (1993) and McDonald (1998) that prophylactic antibiotics administration before operation minimizes or prevents the postoperative infection in surgical patients. Classen *et al.* (1992) noted that delaying administration of antibiotic resulted in lesions identical to those in animals not receiving antibiotic. Since surgical site infection is mostly likely due to colonization of the wound by animal's own endemic flora, inappropriate use of antibiotics peri-operatively may increase the risk of surgical site infection with opportunistic organisms (Hotston, 1995). In the postoperative group and the untreated animals, the body's immune defenses were overwhelmed by the microorganisms and failed to fight and prevent infections. In these animals, the delayed wound healing is thus partly attributed to wound infection.

Wounds that were topically treated with antibiotics had a faster healing rate than the control animals. In this group, no animal showed signs of infection. This is explained by the fact that antibiotic was applied directly to the site of action thus enabled to reduce bacterial proliferation and prevent infection on the surgical incision. When antibiotics are administered locally the effective drug concentration goes straight to the site for action while when administered through parenteral routes, it takes time to be absorbed and distributed to tissues for effective action (Bergamini *et al.,* 1989; Classen *et al.* 1992; Page *et al.,* 1993).

The results of this study justify the use of antibiotics especially in animals because the operation might be conducted aseptically but animals may acquire infection from the surrounding environment after the operation. Normal flora also from the animal itself might take the opportunity after the surgical stress to cause the secondary bacterial infection. Prophylactic antibiotics administration using broad spectrum antibiotics or antibiotics which are effective against the suspected contaminants is therefore useful if administered before the operation in order to prevent or minimize postoperative infections. It should however be noted that where the risk of infection is low, inappropriate use of antibiotics is likely to result in unnecessary cost for the client and can increase the risk of development of antibacterial resistance.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Veterinary Surgery and Theriogenology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania for allowing using their facilities during this study.

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Effect of freezing on stability of oxytetracycline residues in beef from Dodoma region, Tanzania

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SUMMARY

The aim of this study was to determine the effect of freezing on the concentration of oxytetracycline (OTC) residues in beef samples stored at -20 °C (core beef temperature -12 °C and below) for 60 and 120 days. A total of 60 fresh beef samples were randomly obtained from cattle slaughterhouses and butcher shops in districts in Dodoma region in Tanzania. The OTC residues were determined using high performance liquid chromatography (HPLC) with a diode array detector (DAD). Results showed that the mean concentration of OTC residues in 16 positive samples before freezing was 191.71 ± 90.21 ng/g. The mean concentration of OTC after freezing at -20 ºC for 60 and 120 days were 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g respectively. These results revealed a significant ($p<0.05$) reduction of OTC residues of 30% after 60 days and 65% after 120 days of freezing at -20 °C. The percentage reduction of OTC residues was not dependent on the initial concentration or the freezing process but was rather due to unknown time dependent individual beef sample factors. It is concluded that, despite OTC levels in beef decreasing due to non-freezing factors, any residues significantly above Maximum Residues Level (MRL) may not be expected to reduce to acceptable levels as a result of freezing

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Key words: Oxytetracycline, cold storage, HPLC, beef.

INTRODUCTION

The presence of antimicrobial residues (AMRs), in food is a public health concern. The availability of antimicrobial drugs in some countries without effective regulations and with inadequate awareness on appropriate drug use among livestock keepers results in the occurrence of high levels of antimicrobial residues in meat (Nisha, 2008). Some of the effects caused by antimicrobial residues in food include autoimmunity, carcinogenicity, mutagenicity and bone marrow toxicity (Pavlov *et al.*, 2008; Nisha, 2008). Furthermore, AMRs present in meat, milk and other foodstuff can initiate the development of resistant strains of bacteria due to the consumption of sub-therapeutic doses of antimicrobial (Mateu and Martin, 2001; Teale, 2002; Wilson *et al.*, 2003; Hardman and Limbird, 2007).

Several studies have been conducted to determine the levels of AMRs in food products of animal–origin in Tanzania. The prevalence of antimicrobial residues ranges from 2.8% to 100% in beef, chicken meat, milk and eggs in various areas in Tanzania (Mmbando, 2004; Karimuribo *et al.*, 2005; Mdegela *et al.*, 200; Kurwijila *et al.*, 2006). Nonga *et al.* (2010 & 2013); Mgonja *et al.* (2017) reported the prevelence of antimicrobial to be 100%, 76.4%, and 35% in cattle, chicken meat, milk and eggs, respectively.

The destiny of drug residues during heat-treating is still uncertain. Many scientists have been concerned whether antibiotic residues can be destroyed by cooking procedures, pasteurization, or canning processes (Ibrahim and Moats, 1994; Rose *et al.*, 1995; Isidori *et al.*, 2005; Hassani *et al.*, 2008; Hsieh *et al.*, 2011; Mgonja *et al.*, 2016). A study described by EI Atabani *et al.* (2014) reported that out of one hundred local liver samples examined by microbial inhibition test for oxytetracycline (OTC) residues, 5 samples (5%) reacted positive while all the 20 imported frozen liver samples examined were free from OTC residues.

Although freezing is a form of preservation of meat by hindering the development of microorganisms, various researchers have reported many variations in the reduction of antimicrobial residue concentration with time in frozen meat making the reason for the reported reductions to be unclear. The stability of antimicrobials is generally expected to be higher during storage at -20 °C in comparison to storage at 4 °C (Honikel *et al.*, 1978; O Brien *et al.*, 1981; Pavlor *et al.*, 2005). However, O'Brien *et al.* (1981) reported that the concentration of oxytetracycline decreased by 7.4% and sulphadimidine by 20.1% in meat stored at 4 °C for 6 weeks. Gehad, (2002) reported that there was no antibiotic residue detected in cattle muscle and organs after freezing for three months at -20 °C. It is therefore, hypothesized that cold storage could reduce OTC residues in beef from unacceptable levels to

acceptable values. The aim of this study was to investigate the effect of freezing on OTC residues in beef from selected slaughter facilities and butcher shops in Dodoma region, Tanzania.

Materials and methods

Study site

This study was carried out in Dodoma region in Tanzania. Dodoma Region is centrally positioned in Tanzania, lying at latitudes 4º to 7º South and longitudes 35º to 37º East, and is bordered by four regions namely, Manyara in the North, Morogoro in the East, Iringa in the South and Singida in the West.

Sample collection

Beef samples were obtained from cattle slaughter facilities and butcheries in Bahi, Kongwa, and Dodoma Urban and Rural districts. The cattle slaughter facilities and butcheries were selected using a simple random sampling technique. Each sample was transferred in separate sterile and labeled plastic bags in an icebox and transported to Zambia Agricultural Research Institute (ZARI) laboratory for analysis. All samples were analyzed for determination of oxytetracycline residues. The control and test samples were stored in a freezer at −20 °C for approximately 1 week. Both control and test samples were thawed at room temperature for four hours before extraction and analysis of OTC residues. Antibiotic-free meat control samples (blank matrix) were collected from the Central Veterinary Research Institute of Zambia. The sixty samples were analyzed by HPLC and found that only 16 samples were positive for OTC. Sixteen beef samples, which were positive for OTC residues were subjected to cold storage at −20 °C for 60 and 120 days.

Analytical method validation

The procedures for validation parameter were taken from the guidelines for the Germany Society of Toxicology and Forensic Chemistry GTFCh, (2009).

Samples extraction

The extraction procedures were similar for spiked blank samples and test samples. Samples were removed from the -20 ºC freezer and thawed for about 1 hour. Approximately 10 g of muscle sample was weighed and mixed with 25 mg (EDTA) per gram sample. The sample and the EDTA were homogenized using a blender for one minute. The blended sample was then further ground using a mortar and pestle. One gram of the homogenized sample was accurately weighed into a 15 mL polypropylene centrifuge tube. To the sample, 50 µL of 50 µg/mL caffeine solution, equivalent to 2500 ng caffeine, were added. Five millilitres acetonitrile was added using a 5 mL volumetric pipette and the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube by decantation. Five millilitres acetonitrile was then added to the residue, the mixture was vortexed for 1 minute. The sample was centrifuged for 10 minutes at 7000 rpm. Both supernatants were combined into a 15 mL centrifuge tube, briefly mixed using a vortex and gently dried under a stream of nitrogen to 2 mL. Thereafter, 0.5 mL of HPLC grade water and 30 µL of formic acid were added, making the mixture 1.2% acidic. Fifteen milligrams of Supelclean ENVI-carb active coal were added; the sample was mixed for 30 seconds using a vortex and centrifuged for 10 minutes at 7000 rpm. The supernatant was collected into a separate 15 mL centrifuge tube and dried to 0.5 mL.

Sample analysis

The reference standard for OTC and Ethylenediaminetetraacetic acid (EDTA) was supplied by Sigma-Aldrich (St Louis, MO, USA). Acetonitrile and methanol were of high performance liquid chromatography (HPLC) grade (Merck Company, Germany). The determination of OTC residues was carried out using HPLC with a diode array detector (DAD) as describe by Mgonja *et al*. (2016). The HPLC apparatus was equipped with a constant flow quad pump at a flow rate of 0.5 mL/min. Elution of OTC from the analyte was done on an Eclipse XDB C-18 column 4.6 x 150 mm, 5µm I.D with HPLC grade water-acetonitrile containing 0.1% formic acid. A 100 µl of the analyte from each sample was injected to obtain average peak areas of positive samples corresponding to retention times of 5.0 minutes of the reference standard for OTC. The concentrations of OTC residues in the samples were calculated from the linear equation, $Y = 614.8x + 428699$ (where, Y = AUC for sample OTC chromatogram peak, x = concentration of OTC in sample) obtained from the standard curve (Figure 5.1). The Limit of Detection (LOD) was 18.2 ng/g and the Limit of Quantification (LOQ) value was 54.6 ng/g.

Data Analysis

The data was analyzed by using t-test. A probability of $p<0.05$ was considered statistically significant.

RESULTS

The results revealed that there was reduction in concentration of OTC residues in beef after storage at -20 ºC for 60 and 120 days by 2%-30% and 11%-65% (Table 1). The mean concentration of OTC after the cold storage days was significantly lower than the mean concentration of OTC before the cold storage (166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g versus 191.71 ± 90.21. ng/g; $p<0.05$). Only two samples with OTC levels marginally above Codex Alimantarious MRL of 200 ng/g before freezing had their concentration reduced to levels below the MRL during the freezing period. The higher concentration of OTC was associated with a higher peak. The correlation coefficient associated with the linear regression for the OTC standard concentration with AUC is represented by = R^2 0.99 (Figure 1).

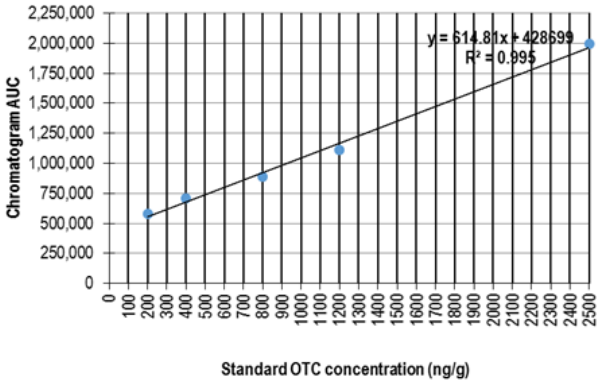

Figure 1. Calibration curve of Oxytetracycline standard

Table 1. Concentration of OTC and percentage reduction after freezing at -20 ºC for 60 and 120 days

Sample no.	OTC concentration in beef (ng/g)			OTC concentration reduction (%)	
	Before storage	After 60 days at -20°C	After 120 days at -20°C	After 60 days at -20°C	After 120 days at -20°C
1	188.20	131.06	66.20	30	65
2	92.09	82.46	74.35	10	19
3	70.68	57.61	42.02	18	41
4	370.42	357.42	315.66	3	15
5	167.32	145.34	114.00	13	32
6	262.16	233.45	212.07	11	19
7	134.07	128.75	114.73	4	18
8	**221.37***	197.27	170.67	11	23
9	318.22	229.7	146.15	28	54
10	228.62	224.01	201.97	2	12
11	143.57	120.71	90.25	28	54
12	322.28	314.33	284.63	2	12
13	132.11	126.6	96.51	30	65
14	108.14	75.25	38.13	4	26
15	207.39*	154.81	108.95	25	47
16	100.77	82.66	59.79	18	41
Range	70.68 -370.42	57.61 – 357.42	42.02 – 315.66	2 - 30	11 - 65
Mean	191.71 ± 90.21	166.40 ± 86.49	133.50 ± 83.24	13 ± 10	33 ± 22

*Samples with OTC levels above Codex Alimantarious MRL of 200 ng/g in muscle before freezing. Shaded rows show samples with concentrations that reduced to levels below the MRL during the freezing period.

Figure 2 shows that the percentage reduction in OTC residues in frozen beef samples was not dependent on the levels of OTC in the sample before storage but rather on individual field sample factors that were not investigated in this study. Levels in all samples continued decreasing despite all of them being subjected to the same freezing conditions.

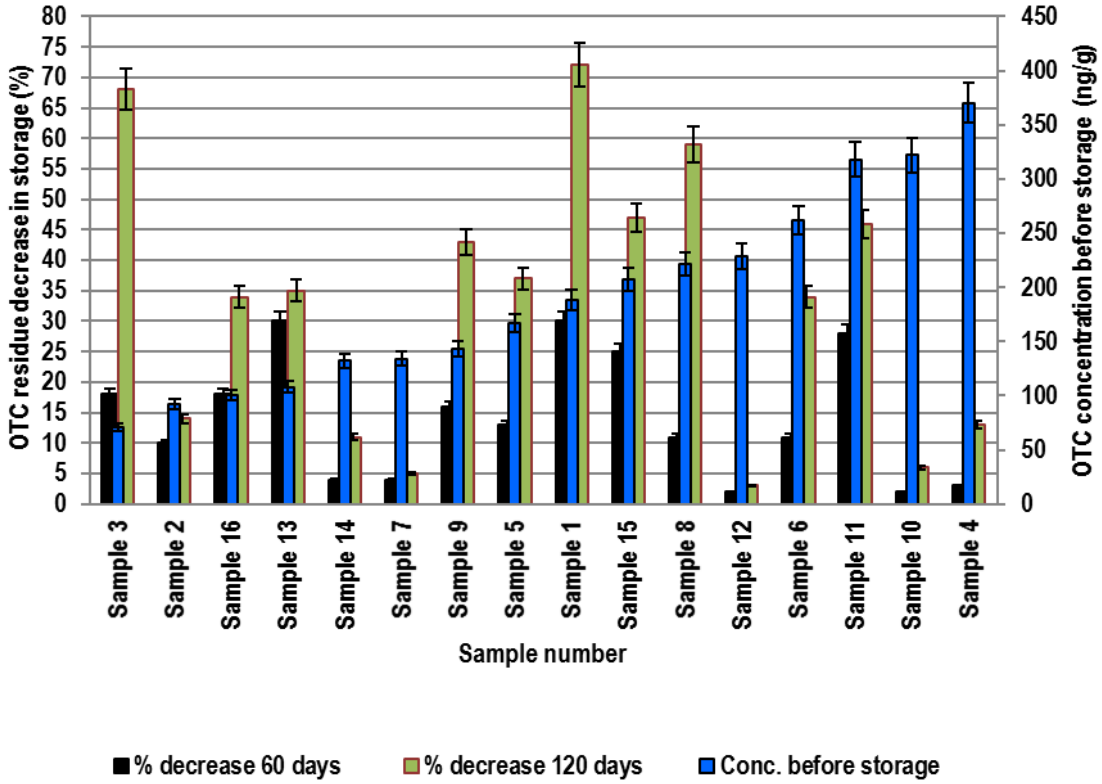

Figure 2. OTC concentration percentage decrease in relation to its increasing concentration before storage

DISCUSSION

Presence of antimicrobial residues in beef can pose hazards to human health. Among them are allergic reaction and imbalance of intestinal microflora, bacterial resistance to antibiotics in microorganisms and losses in the food industry through growth inhibition of food processing microorganisms (Cunha, 2001). Although drug manufactures always recommend withdrawal periods for drugs used in food producing animals, it is common to find oxytetracyline residues at concentrations above Codex Alimentarius Commission MRL in beef readily sold for human consumption (Kaneene and Miller, (1997). Various factors contribute to the presence drug residues in beef including; failure to observe withdrawal periods, age of the animal, disease status (Kaneene and Miller, (1997). This means that sometimes drug residues may be present in beef even when withdrawal period has been observed. Therefore, it is important to understand local factors that may lead to the presence of drug residues in beef, as well as factors that break down these residues to acceptable levels. Pavlov *et al.* (2005) found a decreasing level of tobramycin sulphate from chicken breast and thigh muscle during the period of cold storage. The drug showed initial higher levels in the liver, followed by breast and thigh muscles, with no residues in the muscles on the 30th day. A study by Pavlov *et al.* (1993) showed that freezing at -20 ºC caused a lower degradation than that caused by boiling. So neither boiling nor freezing could be used as reliable methods to get rid of amoxycline residues in meats.

In this study, HPLC was used to determine the concentration of oxytetracycline (OTC) in beef samples in order to determine whether cold storage has an effect on OTC residues. The mean concentration of OTC before cold storage was (191.71 ± 90.21.93 ng/g versus 166.40 ± 86.49 ng/g and 133.50 ± 83.24 ng/g after cold storage at -20 ºC for 60 and 120 days. The difference in OTC residue levels before cold storage and after freezing beef (Table 5.1) were statistically significant ($p<0.05$. The decrease in OTC concentration observed in this study is consistent with other studies in which cold storage decreased the concentration of oxytetracyline and antimicrobial drug residues in foodstuffs.

These results are consistent with a number of other studies that reported reductions in antimicrobial residue concentrations in meat following cold storage. O'Brien *et al.* (1981) used the diameter of growth inhibition zone to establish antibiotic concentrations and observed that the concentration of oxytetracycline decreased by 7.4%, sulphadimidine by 20.1% and ampicillin by 76.05%-100% in meat samples stored at 4 ºC for 6 weeks. Usually, the stability of antimicrobials is far higher during storage at –20°C in comparison with

4°C (Honikel *et al.,* 1978; O Brien *et al.,* 1981; Pavlor *et al.,* 2005).

A study conducted in Turkey indicated that residue levels decreased within days in drugs such as Florfenicol (a fluorinated synthetic analog of thiamphenicol and Florfenicol amine (major metabolite of the antibiotic florfenicol, a fluorinated derivative of chloramphenicol) without any significant difference between storage conditions at -20°C and +4 ºC (Ayhan *et al.,* 2015). This study is also in line with that by Tansel *et al.,* (2006) which showed that concentrations of gentamicin residues were retained for fourteen days at both refrigerated (+4°C) and room temperatures (15-20 °C), then started to lose strength on day 21 of storage. Another study by Papapanagiotou *et al.* (2005) reported that sulphamethazine (SMZ) residues were stable at -20 °C and −75 °C in all piglet's muscle tissue examined for at least 3 and 5 months, respectively. A study by Alfredsson and Ohlsson (1998) reported that levels of sulphamethazine spiked in beef and frozen at -20 °C for 3 months decreased by 35%.

The level of penicillin G kept in a deep-freezer for 10 days decreased by half in the gluteal muscles, and by 20% in the kidneys (Boison *et al.* 1992). Findings from other studies have shown that freezing of penicillin G, ampicillin, OTC, sulfonamide, quinolones and gentamicin have minor or no effect on the residues levels (Nouws and Ziv, 1976; Boison *et al.* 1992; Verdon *et al.* (2000); Baydan *et al.* (2002) and Sireli *et al.* (2006. The decrease in the quinolones activity in frozen stock solutions stored at –20 °C did not exceed 10%, whereas the levels of β-lactams did not change during 3 months of storage (Okerman *et al.,* 2007).

Although many studies have demonstrated a general decrease of OTC levels during cold storage of beef, our study shows that the decrease was not a result of the process of freezing but was rather due to individual sample factors prevalent at the initial stage of freezing. This is supported by the fact that despite all samples being stored under the same conditions, OTC residues continued degrading at different rates during the whole study period. Only two samples with OTC levels marginally above Codex Alimantarious MRL of 200 ng/g before freezing had their concentration reduce to levels below the MRL during the freezing period. Immediately after slaughter and before grading or freezing, beef must undergo the process of chilling (cold storage at 0 – 4 °C to achieve core beef temperature of 7 °C and below) in order to stop the growth of spoilage microorganism and improve the quality of meat (FAO 1991). However, variations in the speed of the chilling process can produce meat with varying quality factors such as color, pH and microbial growth (Aalhus *et al.,* 2001) which may have an effect on the stability of drug residues in meat. Therefore, more research is required in order to determine the effects of pre-slaughter and meat chilling factors on OTC residues in meat.

In conclusion, the results of this study show that OTC residues were detectable in frozen beef up to 120 days although on average, there was a significant decrease in concentration. The reduction of OTC residues was not dependent on the freezing process or the initial concentration but was rather due to unknown time dependent individual beef sample factors. Although OTC levels in beef decreased due to non-freezing factors, any residues above MRL may not be expected to reduce to acceptable levels as a result of freezing.

ACKNOWLEDGMENTS

The authors are grateful to the INTRA ACP MOBILITY Project for the financial support of this study.

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Studies on seroprevalence and risk factors for occurrence of Bovine brucellosis in cattle in Lindi district, Tanzania

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SUMMARY

Brucellosis is a contagious bacterial zoonotic disease of public health importance worldwide. A cross-sectional study was conducted from July to November 2017 in Lindi District to estimate the seroprevalence of brucellosis in cattle, assess farmers' knowledge and to identify risk factors for *Brucella* infection in animals. Questionnaires were administered to 60 livestock keepers and blood samples collected from 300 cattle for brucellosis analysis using Rose Bengal Plate Test and competitive-enzyme linked immune-sorbent (cELISA) assay tests. Results indicated that almost half (48.3%) of the households owned small herds which were mostly (58.7%) indigenous cattle. Proportions of positive reactors to brucellosis were 6.0% and 5.2% based on RBPT and c-ELISA respectively. Adult cattle were found to be frequently affected by brucellosis than young ones, and the difference was statistically significant ($p<0.05$). Comparable frequencies of infection were found in different sex groups and among cattle from different wards and different herd sizes. Most of the farmers lacked knowledge of brucellosis. History of abortion ($p=0.00$) and improper disposal of aborted materials ($p=0.04$) were found to be significantly associated with occurrence of bovine brucellosis in cattle. This study reports for the first time on occurrence of brucellosis in Lindi District and highlights on the possible risk factors for its transmission in cattle. Control efforts need to be put in place for this and other diseases with serious public and economic impacts the public.

Key words: Cattle, brucellosis, seroprevalence

INTRODUCTION

Livestock production in Tanzania forms an integral part of agriculture; the sector which contributes immensely to the economy of the country (John *et al*., 2010). Productivity of livestock is seriously affected by animal diseases some of which are naturally transmissible between animals and humans (Nonga *et al.*, 2009, Mellau *et al.*, 2010, 2011).

Brucellosis is an economically important disease in production animals worldwide. In cattle, the disease is usually caused by *Brucella abortus* and occasionally by *B. melitensis* and *B. suis*. Brucellosis is characterized by late term abortion; infertility and reduced milk production as a result of retained placenta, endometritis, and a varying degree of sterility in the males and cows. It is amongst the neglected zoonoses largely due to lack of public awareness and yet it is one of the most important zoonotic infections, especially in pastoral and mixed crop-livestock farming systems in Africa. Though it has been eradicated in many developed countries, brucellosis remains an uncontrolled problem in regions of high endemicity such as Africa, Mediterranean, Middle East, parts of Asia and Latin America (Faye *et al*., 2005; Karimuribo *et al*., 2007).

The risk of transmission of brucellosis to humans is largely lies on the presence of the disease in the susceptible animal populations. The effects on the disease are accentuated among marginalized communities as it is associated with poverty, poor farm management practices, and high levels of illiteracy; a feature of poor communities in developing countries. Bovine brucellosis, classified as one of the neglected zoonosis of interest by the World Health Organisation, has the potential to affect both public health and people's livelihoods, as it can perpetuate the cycle of poverty.

Brucellosis is a disease of greatest importance as it reduces production and reproduction performance of cattle and is of public health significance. Lindi region is an emerging livestock keeping area as it has been receiving cattle from different parts in Tanzania but have not been screened for important diseases like brucellosis. The proposed study intends to bridge this gap by exploring the status of brucellosis, its distribution, and the associated risk factors. The gathered information will contribute to database of brucellosis in Tanzania and can be used to devise appropriate national strategies for the control of the disease. The current study was meant to determine the seroprevalence of bovine brucellosis, assess the farmer's awareness on the diseases and possible factors for its transmission in cattle in Lindi district, Lindi region, Tanzania.

MATERIALS AND METHODS

Study design and population

This cross sectional study was conducted in Lindi rural District which is among the six districts of Lindi Region. The district is located on the southern part of Tanzania and it is bordered to the north by Kilwa District, to the south by Mtwara Region, to the west by Nachingwea District, and to the east by the Indian Ocean and Lindi Urban District. It has cattle population of 14, 900 and human population of 215,764 (National census, 2002). Only about 15% of people in Lindi district are involved in livestock keeping and subsistence farming. Cattle migrate from different parts of Tanzania to the district. The dominant pastoralists in the district are Maasai and Sukuma. The District is administratively divided into twenty-four wards. Chikonji, Chiponda, Kilolambwani Kitomanga, Kiwalala, Kiwawa, Mandwanga, Matimba, Milola, Mipingo, Mchinga, Mnara, Mtama, Mtua, Nachunyu, Nahukahuka, Namupa, Nangaru, Nyangamara, Nyangao, Nyengedi, Rutamba, Sudi and Tandangongororo. In most of these wards there are no livestock keeping activities instead they are involved in cashew nut production and fishing due to presence of Indian Ocean. The study was based on purposive selection of three wards where there are livestock keepers which include Kitomanga, Rutamba and Milola.

The population under study was considered to be heterogeneous comprising of varied gender and age groups. The inclusion criteria were; smallholder both women and men who keep dairy cattle or indigenous cattle, willingness to participate in the study and able to give information and accessibility of the place during data collection. The exclusion criteria included; unwillingness to participate in the study, unable to give information asked and living in inaccessible areas. Also those who had no time for interviews were excluded.

Sample Size determination and selection of study animals

The number of animals sampled was determined using the formula developed by Naing et al (1996); $n=1.96^2P\ (1-P)/d^2$. The expected prevalence (P) of 0.20, precision level (d) of 0.05 and confidence level of 95% resulted in a required sample size of 245 animals. Since multistage cluster sampling technique was adopted, in order to achieve the same precision, the original sample size was multiplied by the design effect (D) calculated using the formula D=1 + (b-1) roh (Otte *et al*., 1997). The average number of samples per cluster (b) was 7 and intra-cluster correlation coefficient or rate of homogeneity (roh) was set at 0.10. Number of clusters (c) sampled was determined by the formula $c=P\ (1-P)\ D/SE^2n$ and Standard error (SE) was calculated by SE=d/1.96 (Otte *et al*., 1997). Accordingly, the total number of animals bled was 300 and the number of clusters to be sampled was 56. Selection of villages and study herds was done by a simple random sampling technique. Individual animals in the herd were selected by a systematic random sampling technique. The study was conducted on both smallholder dairy herds and traditional herds comprised of indigenous cattle. In this study, a herd was regarded as a cluster, defined as a group of cattle (of both sexes) kept in one enclosure and constituting ten or more animals aging six months and above. Furthermore, herds were classified into two categories; large herds (>15 animals) and small herds (≥10≤ 15 animals) based on the classification used by the departments of Livestock and Fisheries Development in the study area. Cattle aging >2 years was representing adults and those aging ≤2 years was representing young animals adopting categorization of age groups used by Degefu *et al*. (2011). Also questionnaires were be administered to 60 randomly selected livestock keepers. In the 3 purposively selected wards i.e. Kitomanga, Rutamba and Milola, two villages were randomly selected from each ward, making 6 villages and from each village 10 questionnaire were administered to 10 households which were randomly selected in each village.

Ethical Consideration

Research permit was provided by Research, Publications and Ethics Committee of the College of Veterinary Medicine and Biomedical Sciences of Sokoine University of Agriculture (Appendix 2). Permission letters were obtained from Executive Director of Lindi District council. Verbal consent was obtained from each of the heads of study households, after explaining the purpose and importance of the study prior to commencement of interviews and sampling. Participation in the study was on voluntary basis. All the information collected from the participants and the laboratory results obtained after blood sample analysis were kept under the custody of the researcher as confidential and the study participants were treated anonymously.

Data collection

Recruitment of research assistants

Four research assistants were recruited; two livestock field officers who had a certificate in animal health were recruited to assist the researcher in data collection. These had good communication skills and experience on interaction with smallholder dairy farmers. The other two research assistants had Diploma in laboratory technology. Inclusion of local research assistants to the area helped in maximizing trust of respondents, interviewer- interviewee interaction and facilitation of laboratory work. The research assistants were briefed on the objectives of the study, data collection process, target respondents, selection criteria, approach during interview, understanding questions, elaborating why and how each question was asked, assuring the respondents confidentiality, how to record responses from interviewees, laboratory analysis procedures and data collection and recording.

Farmer interviews

Information about each herd and the animals kept was collected by means of a structured questionnaire, which was completed at all the selected herds on a single visit. The questionnaire was designed to comprise mostly closed ended (categorical) questions to ease data processing, minimize variation, and improve precision of responses. The questionnaires were pretested in the field and adjusted as required. Interviews were conducted to members of the households who were more conversant about the herds using Swahili dialect. Data on risk factors for *Brucella* seropositivity, livestock owner's knowledge, attitudes and practices regarding brucellosis was concurrently collected. Information on individual animal variables (age, sex, breed, management system, disease control programme eg. vaccination was recorded on separate sample data sheets. Other information on occurrence of reproductive events such as; history of abortion, retained placenta or other reproductive disorders was also collected. Herd personnel's knowledge and awareness of brucellosis and its transmission, disposal of placenta, aborted materials and history of raw milk consumption were also recorded. A total of 60 households were included in the study.

Blood sampling from cattle

The selected cattle for the study were properly restrained in a crash for those livestock keepers with crashes, but for those without a crash, ropes and manual restraint were used. About 5 ml of blood sample was collected from the jugular vein of each animal into plain vacutainer tube. Each tube was labeled by using codes describing the specific animal and herd. The samples were left to clot at room temperature overnight and subsequently centrifuged at 3000 rpm for 10 minutes to obtain clear serum. Thereafter about 2 ml of serum was collected into cryovials and stored at -20°C at Lindi Regional Hospital until serological analysis which was performed (a month later) at the College of Veterinary Medicine and Biomedical Sciences laboratories of Sokoine University of Agriculture.

Laboratory analysis of sera samples by Rose Bengal Plate Test

All collected sera samples were first screened using Rose Bengal Plate Test (RBPT) for *Brucella* antibodies according to the test procedure recommended by OIE (OIE, 2009). Briefly, 30 µl of RBPT antigen and 30 µl of the test serum were placed alongside on the glass plate and mixed thoroughly. After 4 minutes of rocking, any visible agglutination was considered as positive result (Omer *et al*., 2002).

Laboratory analysis of sera samples by competitive Enzyme Linked Immunosorbent assay(c-ELISA)

Rose Bengal Plate Test positive sera were then subjected to competitive enzyme linked immunosorbent assay (c-ELISA) as a confirmatory test, adopting a test procedure and interpretation of results as recommended by the manufacturer (Animal Health and Veterinary Laboratories Agency AHVLA, New Haw, Addlestone, Surrey, KT153NB, UK). Only animals positive on c-ELISA were regarded as *Brucella* seropositive. The spectrophotometer was adjusted at an absorbance of 450nm. A positive negative cut-off was calculated as 60% of the mean of the optical density (OD) of the four conjugate control wells. Any test sample that give OD equal to or below the value was regarded as positive. A herd was considered positive for the disease when at least a single animal tested positive for c-ELISA.

Data analysis

Data were first entered and cleaned in Microsoft Office Excel® 2007 (Microsoft Corporation, One

Microsoft Way, Redmond, 98052-7329, USA) and imported into Epi-Info version 7 (CDC Atlanta, USA) and MedCalc® version 13.0.2 (MedCalc software, Acacialaan 22, B-8400, Ostend, Belgium) for analysis. Frequencies were determined for proportions of positive animals and herds. Risk factor analysis adopted logistic regression in two steps i.e. univariate Multivariate analysis. The model was constructed by a forward stepwise selection of variables. The predictor variables were assessed for collinearity using Pearson's Chi-squared test. The potential confounding effect of those variables not retained in the final model was assessed using Mantel-Haenszel Chi-Squared test. The model was evaluated for goodness- of- fit using a Hosmer-Lemeshow test. The discrimination ability of final model was assessed using the receiver operating characteristics (ROC) defined as the area under the curve (AUC). The interaction term was introduced in the final model to assess effect of modification. For final analyses, a p-value of ≤ 0.05 was taken as significant.

RESULTS

Socio-demographic attributes of surveyed livestock farmers in Lindi rural District

In this study 60 farmers were reached out for interviews. Their socio-demographic attributes are summarized in Table 1.

Table 1. Respondent's socio-demographic characteristics and livestock keeping experience in Lindi rural (N=60)

Variable	Category	Frequency	Percent
Gender	Male	51	85.6
	Female	9	14.4
Age (years)	20-45	20	32.5
	46-65	36	60.1
	66 and above	4	7.5
Education level	Non formal	15	25.4
	Primary	32	54.0
	Secondary	7	12.0
	Graduates	0	0.0

Livestock production and health management practices among surveyed farmers in Lindi rural District

Of the visited farmers (n=60), 65.8% practice extensive, 9.2% practice intensive and 30.0% practice Semi-intensive production system. Majority of them had a 3-10 yrs experience in livestock farming. Around two thirds had an experience of 10-20 years and the rest kept animals for less than three years. Only 8.6% of the farmers had attended livestock production and/ or health related training. Only 5.8% of the herds were bred using artificial insemination, while 94.2% used natural breeding. Indigenous cattle produce between one and three litres of milk a day; whereas majority of the cross-bred dairy cattle (78.79%) produced between 5 and 10 litres a day. A small proportion of the dairy cattle (21.21%) produced between 10 and 15 litres of milk a day. All the respondents reported access to veterinary care for their animals, majority of them (86.7%) from the public veterinary services. None of the farmers vaccinated his/her animals against brucellosis. However, 31.8% of herds were vaccinated against other diseases such as Foot and Mouth Disease (FMD), Rift Valley Fever (RVF), East Coast Fever (ECF), Contagious Bovine Pleuro Pneumonia (CBPP), Anthrax and Lumpy Skin Disease (LSD). Records keeping for vaccination and other routine treatment were reported from 52.8% of the respondents. Most farmers reported other common diseases and they seem to be not aware of brucellosis as one of the most common zoonotic disease which can have a very big implication on human health.

Livestock owner's knowledge, attitudes and practices (KAPs) regarding brucellosis in the study area

Majority of the farmers in the current study (85.0%) were not aware of brucellosis. All of those who were aware of the disease acknowledged to have seen suspected case of brucellosis in animals; both in cattle and sheep. Abortion and unthrifty new born were mentioned as common signs of the disease in animals. Two of the respondents among those who were aware of the disease seemed to be aware of the zoonotic potential of the disease but couldn't figure out how human acquire infection. In this survey it was revealed that 22.2% of those who were aware of brucellosis attempted to treat infected animals and 55.5% sold infected animals at the live animal market. All the interviewed farmers left aborted cows with other animals. Unregulated animal movement was common among 100.0% of those who practices extensive farming. About 43.3% of respondents admitted to have observed abortion in their herd. The most common method of disposing off placenta, fetuses and dead calves was by burying in the ground as reported by 53.8% of respondents who had aborting cows in their farms. Other methods of disposal were throwing to the bush (15.4%), feeding to dog (23.1%) and disposal in pit (7.7%).

Prevalence of Bovine Brucellosis in indigenous and crossbred dairy cattle in Lindi District council

A total of 300 cattle from sixty herds in three wards were sampled in this study. Out of these 61 were males and 239 were females. At individual animal level, the proportions of positive reactors to brucellosis were 6.0% and 5.2% on RBPT and c-ELISA tests, respectively. At ward level the proportions of positive animals were 5.0% for Kitomanga (n=200), 6.0% for Milola (n=50) and 6.0% for Rutamba (n=50). The positive animals were detected in 15% of the sampled herds (n=60). Around 13.8% of the small herds (n=29) tested positive, whereas 16.13% of the large herds (n=31) tested positive. There existed no statistically significant differences in proportions of sero-positive animals between small and large herd sizes; as well as between wards. Table 1 below displays results of sero-positivity to brucellosis by different categories of sampled animals based on a confirmatory test.

Table 2. Prevalence of brucellosis among different categories of cattle in Lindi rural District based on c-ELISA (n=300)

Attribute	Categories	No. of animals tested	No. of positive animals (%)	Chi square (95 CI)	P-value
Sex	Male	61	2 (3.3)	0.24 (-5.9% to 7.36%)	0.63
	Female	239	14 (5.9)		
Genotype	Indigenous	201	10 (5.0)	0.008 (-4.5% to 8.1%	0.93
	Crossbred dairy	99	6 (6.0)		
Age	Young	108	1 (0.9)	5.24 (1.59% to 11.72%	0.02
	Adults	192	15 (7.8)		

Risk factors for occurrence of brucellosis in indigenous and cross-bred dairy cattle in Lindi rural District

Several factors were screened for association with occurrence of bovine brucellosis at herd level. These included cattle breed, age, sex, breeding method (natural service v/s artificial Insemination), herd size, production system (extensive, intensive, semi-intensive), history of abortion, contact with animals from other herds, contact with other animals, sharing of bulls, introduction of new animals into the herd and disposal of aborted materials. On univariate analysis; contact with pigs, contact with small ruminants, contact with wildlife, source of replacement from livestock market, improper disposal of aborted materials, use of bulls from other herds and use of dogs as guardians during grazing qualified ($p<0.10$). On multivariate analysis; history of abortion and improper disposal of aborted materials were found to be significantly associated with occurrence of bovine brucellosis in a herd.

Table 3. Results of multivariate logistic regression analysis of risk factors for occurrence of bovine brucellosis in indigenous and cross-bred dairy cattle in Lindi rural District

Risk factor	Category	OR	P-value
History of abortion in the herd	Yes	12.3	0.00
	No		
Disposal of aborted materials	Improper	2.97	0.04
	Proper		

*OR = Odds Ratio

DISCUSSION

We are reporting an animal level prevalence of brucellosis of 5.2% in Lindi rural District, which is slightly lower than those reported in previous studies in the country (Assenga *et al*., 2015, 6.8%; Chitupila *et al.*, 2015, 5.6%; Karimuribo *et al.*, 2007, 6.2%; Swai *et al.*, 2010, 7.3%). A study in Ethiopia, however, reported lower seroprevalence levels of 3.19% (Berhe *et al.*, 2007) than what is reported in the current study. The same two studies in Ethiopia, however reported higher herd level seroprevalences of 26.1% and 42.31% respectively;

contrary to 15.0% obtained in the present study. Similarly, in the country, Swai *et al*. (2010) reported a slightly higher (20%) herd level seroprevalence than what is reported in this study.

There is no controlled study that has been conducted on the relative susceptibility of female and male cattle to brucellosis. Similar to our finding, earlier studies (Berhe *et al.,* 2007; Mellau *et al.,* 2009; Desefu *et al.,* 2011) contend that there is no sex predisposition of cattle to *Brucella* infections. However, based on the reactor rates, some authors (Degefu *et al.,* 2011) propose that bulls are more resistant than sexually mature heifers and cows but less resistant than sexually immature heifers. A study by Ferede *et al.* (2011) discovered that *Brucella* infections limited to testes in male animals result into non reactors or reactors displaying low antibody titers. The same authors associated less susceptibility of male animals to *Brucella* infection with lack of erythritol (Ferede *et al.,* 2011). Seroprevalence may increase with age as a result of prolonged exposure to pathogens particularly in traditional husbandry practices where females are maintained in herds for long period of time than males (Blood and Radostitis 1990).

No statistically significant difference in proportions of positive reactors to brucellosis was observed between small and large herds. Our observation is consistent with those made by previous authors (Jergefa *et al.,* 2009; Chitupila *et al.,* 2015). Some other studies in the country (Swai *et al.,* 2010) and elsewhere (Berhe *et al.,* 2007) have reported a contrary finding to ours. The authors of these works have observed higher prevalence in large herds than in small herds. These different observations among studies may be influenced by the presence of varying exposure scenarios in the different study populations.

A significantly small proportion of young animals turned out to be positive for brucellosis in the present study. This observation underscores findings reported by previous authors (Berhe *et al*., 2007; Mellau *et al*., 2009; Swai *et al.,* 2010; Degefu *et al*., 2011; Assenga *et al*., 2015). It is argued that age is one of the intrinsic factors which influence the susceptibility to *Brucella* infection such that young and sexually immature animals are more resistant to primary infection and often clear infection although latent infections do occur (Degefu *et al*., 2011). Similarly, growth and multiplication of *Brucella* organisms are stimulated by sex hormone and erythritol which tend to increase in concentration with age and sexual maturity (Walker, 1999; Dinka and Chala, 2009; Ferede *et al*., 2011). Authors of a study conducted in the country associated higher sero-prevalence of brucellosis in adult animals with under nutritional, stress and lower immunity that develops following acute infection (Swai *et al.,* 2010).

Lack of knowledge on brucellosis was a feature in majority of the livestock keepers in the study area. This compromises disease prevention and control efforts. This jeopardizes the health of these farmers as lack of knowledge and awareness about the disease implies that they don't take required precautions when handling infectious products. Moreover, with these results it is obvious that no precaution is taken to prevent spread of the disease to other herds within or outside the study area. With the perception that brucellosis in animals can be cured and the practice of farmers disposing suspect animals into the food chain or selling them to others is likely to result into propagation of the disease. he use of antimicrobials against *Brucella* infections in animals causes L-transformation on the cell wall in a way creating carrier. Regarding disposal of infected animals, Holt *et al*. (2011) points out that, selling infected animals may increase transmission of brucellosis between households in a village, between villages and even to larger geographical areas. In its Regulation, 2007, The Animal Disease Act, 2003 indicates that animals should be tested for brucellosis, reactors separated from the healthy animals and destroyed. Isolation of anmals suspected of having brucellosis is not practiced by livestock keepers in the study area. Holt *et al*. (2011) asserts that failure to separate animals that abort increases the risk of susceptible animals acquiring brucellosis through contact with aborted materials.

In this study, history of abortion in the herd was revealed to have a positive association with the serological prevalence of bovine brucellosis in the study area. This could easily be linked by the possibility of most of the abortion occurring as a result of infection with *Brucella* organisms. Contamination of environment with aborted materials, lochia, urine and uterine discharges from *Brucella* positive cows have been documented to be major sources of infection to other incontact susceptible animals (Blood and Radostitis 1990).

Some few studies have reported on risk factors for bovine brucellosis in the country. A study by Chitupila *et al.* (2015) revealed that improper disposal of aborted materials is among the factors. This observation has surfaced again in this study whereby improper disposal of aborted materials has been linked with the likelihood of occurrence of

brucellosis. Susceptible animals can be infected via direct contact with aborted materials or by products of parturition. Similarly, improperly disposed aborted materials contaminate the environment with *Brucella* organisms and become major sources of infection to susceptible animals (Blood and Radostitis 1990). Such contamination of environment can lead into contamination of pasture and hence cattle become infected when grazing. Once embedded in protein like aborted materials and /or uterine exudates *Brucella* organisms can survive in dry conditions for 42 days to 75 days as they are protected from the effects of direct sun light (Blood and Radostitis, 1990). Some other authors report that under ambient temperature and relative humidity, *Brucella* organisms can survive in aborted fetuses in sheds and in liquid manure for up to 8 months (Blood and Radostitis, 1990). The organisms can also survive in the grass for up to 100 days depending on the season as suggested by Blood and Radostitis, (1990). In some cases dogs can acquire *Brucella* infection through access to infected aborted materials from cattle; and infected dogs shed the organisms into environment via urine, vaginal secretions, aborted materials or feces (Blood and Radostitis, 1990).

The present study provides evidence of presence of bovine brucellosis in an emerging cattle keeping area in Southern Tanzania. It also points out to associated risk factors with occurrence of the disease, all of which have been mentioned earlier. With the prevalent production system, poor bio-security and congregation of animals at grazing and watering points, it is likely that the disease can spread within and between herds. It is therefore advocated to institute control measure for the disease focusing more on preventing exposure of susceptible animals to the pathogen. Public education on the epidemiology and health impacts of the disease will help to prevent further spread of the disease and protect human health within and outside the study area. General principles of hygiene and mass vaccination of susceptible population to achieve herd immunity will also help to prevent the disease.

ACKONOWLEDGMENTS

The authors acknowledge to all people who participated in this research. Livestock keepers are thanked for accepting their animals to be screened for brucellosis. This study was funded by One Health Workforce project under OHCEA

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Studies of brucellosis in lactating cows in Babati district, Tanzania

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SUMMARY

The present cross-sectional study was carried out to determine prevalence and risk factors for transmission of brucellosis in lactating cows in Babati district. Rose Bengal plate test (RBPT), buffered acidified plate test (BAPA), competitive enzyme-linked immunosorbent assay (c-ELISA) and polymerase chain reaction (PCR) tests were used in this study to determine the presence of antibodies against *Brucella* and *Brucella* genome. Milk and blood samples from 192 randomly selected lactating cows were collected. Furthermore, questionnaires were administered to 66 milk producers to determine the risk factors for the transmission of brucellosis in between animal populations. The RBPT and BAPA results showed 4.7% (nine cows) and 5.2% (10 cows) seroprevalence, respectively. When RBPT and BAPA positive samples were tested using c-ELISA for serologic confirmation, only eight cows (4.2%) turned out to be positive. The milk samples from eight cows that were positive for Brucella antibodies using c-ELISA were tested for the presence of Brucella DNA using PCR. Three out of the eight milk samples were positive for *Brucella abortus* indicating shedding of Brucella in milk. Analysis of risk factors for transmission of brucellosis by Fisher's exact test or Chi-square showed that livestock mixing with different herds (P=0.0097, OR=11.3333), farming system of cattle (P=0.0400, OR=3.9474), breed of cattle (P=0.0284, OR=1.9860), herd size of cattle (P=0.0030, OR=1.9537) and movement of animals through selling and purchasing (P=0.0500, OR=5.0588) were statistically associated with Brucella positivity. This study provides evidence of brucellosis in lactating cows of Babati district and shedding of *Brucella* in milk. Institution of appropriate control measures including public health education, surveillance of animals accompanied with removal of positive cases according to laws and immunisations of cattle are highly recommended.

Key words: Seroprevalence, *Brucella*, Rose Bengal plate test,

INTRODUCTION

Brucellosis is well-documented by the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and also the World Organisation for Animal Health (OIE), as the most widespread bacterial zoonoses in the world posing serious public health problems and extensive economic losses (Lopes *et al.,* 2010; Neta *et al.,* 2010; Yasmin *et al.,* 2011). Brucellosis is one of the most important and well-known bacterial zoonoses in the world (Lopes *et al.,* 2010; Swai and Schoonman, 2010). The disease is additionally described as true zoonosis because all human infections are of animal origin (Kaoud *et al.,* 2010). Brucellosis is considered a re-emerging disease of special importance in East, North of the Mediterranean countries, South and Central Asia, Central and South America. Moreover, recent reports add zones as far apart because the Republic of Korea and Zimbabwe as foci representing the wide potential hazard. Brucellosis for the primary time was reported in 1859 in Malta (Lee *et al.,* 2009; Matope *et al.,* 2010).

Brucellosis is a disease that caused by Gram-negative coccobacilli, non-motile, non-spore forming, aerobic, non-toxigenic and non-fermenting bacteria of the genus *Brucella*. Brucella genus is divided into six classical species, namely *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*, is still widely used due to historical and clinical reasons but recently identified *Brucella species* isolated from marine mammals, *B. ceti* and *B. pinnipedialis*, are now included in classification (Mariana *et al.,* 2010). The pathogenic Brucella includes *B. suis, B. melitensis, B. abortus and B. canis* which infect swine, goats, cattle, and dogs, respectively (Jelastopulu *et al.,* 2008). However, infection with any of the three species of Brucella may occur in all domestic animals. *B. canis* is also a pathogen of human but is of lesser importance. In addition, two recently identified *B. species* isolated from marine mammals, *B. ceti* and *B. pinnipedialis*, can also cause human brucellosis (Mariana *et al.,* 2010). On the other hand *B. ovis* and *B. neotomae* have never been reported to cause disease to human being (WHO, 2006). Among the four *Brucella species* known to cause disease in humans, *B. melitensis* is thought to be the most virulent and causes the most severe and acute cases of brucellosis, while *B. abortus* is reported to be the most widespread (Yingst *et al.,* 2010).

Brucellosis imposes great economic loss to the farmers since its lead to abortions in newly infected herds, retention of placenta, leading to metritis and endometritis, increased infertility, still births, reduced milk production leading to early culling and replacement of animals (Xavier *et al.,* 2009; Shafee *et al.,* 2011). Also the most serious losses are the number of humans that suffers brucellosis leading to high cost of treatment, manpower incapacitation which affects person, family, community and national economic growth (Kunda *et al*., 2010; Wankyo, 2012). Control of brucellosis in animals tremendously reduces the burden of disease in human and veterinary charges. Most of the previous studies conducted in Tanzania involved parastatal farms and few indigenous cattle herds (Karimuribo *et al.,* 2007). Limited studies about brucellosis have been carried out in Babati district, like that of Mtui-Malamsha (2001) and Shirima (2005). The studies carried out in livestock in Babati did not find or quantify risk factors associated with transmission or spillover of infection between cattle which produces milk for human consumption and other livestock as well as wild animals. There was no report that provided useful information to public and professionals about prevalence, molecular diagnosis and the risk factors of brucellosis in lactating cows in Babati district. Babati district was selected in the present study because dairy cattle business and milk production demands increasing tremendously and none livestock owners in the study area were using Brucella vaccines for the control of Brucella infections.

The study was conducted in order to determine the prevalence of brucellosis in selected lactating cows. Molecular diagnosis was used to detect *Brucella species* DNA from positive milk samples that were initially screened and confirmed using serological tests. It is well known that serological methods are not always sensitive or specific and they have repeatedly been reported to cross-react with antigens other than those from *Brucella* species (Göknur *et al.,* 2010). Due to this reason, this current study used two selected screening serologic tests and one serologic confirmatory test. The purpose of this study was to establish epidemiological data for brucellosis in cattle and determine the performance of selected serological tests.

MATERIALS AND METHODS

Study area, design and animals

This study was conducted in Babati district which is formed by two councils, namely, Babati district (BDC) and Babati Town councils (BTC). The district is at an altitude that range between 950 to 2450 meters above sea level. Rainfall in Babati district ranges between 500 and1200 mm per annum. There are two rainy seasons; the short rains from October to December and the long rains between February and May. Average temperatures range from 22ºC to 25ºC, though it can be colder in the highlands around Bashneti and warmer in the lowlands around Magugu, Mwada and Nkaiti wards. Lactating cows were sampled from both Babati district and Babati town councils (Figure 1).

The present study was cross sectional that was carried out between July 2013 and August 2014. The target study animals were lactating dairy cattle which mostly were crosses of Friesian, Ayrshire and Jersey.

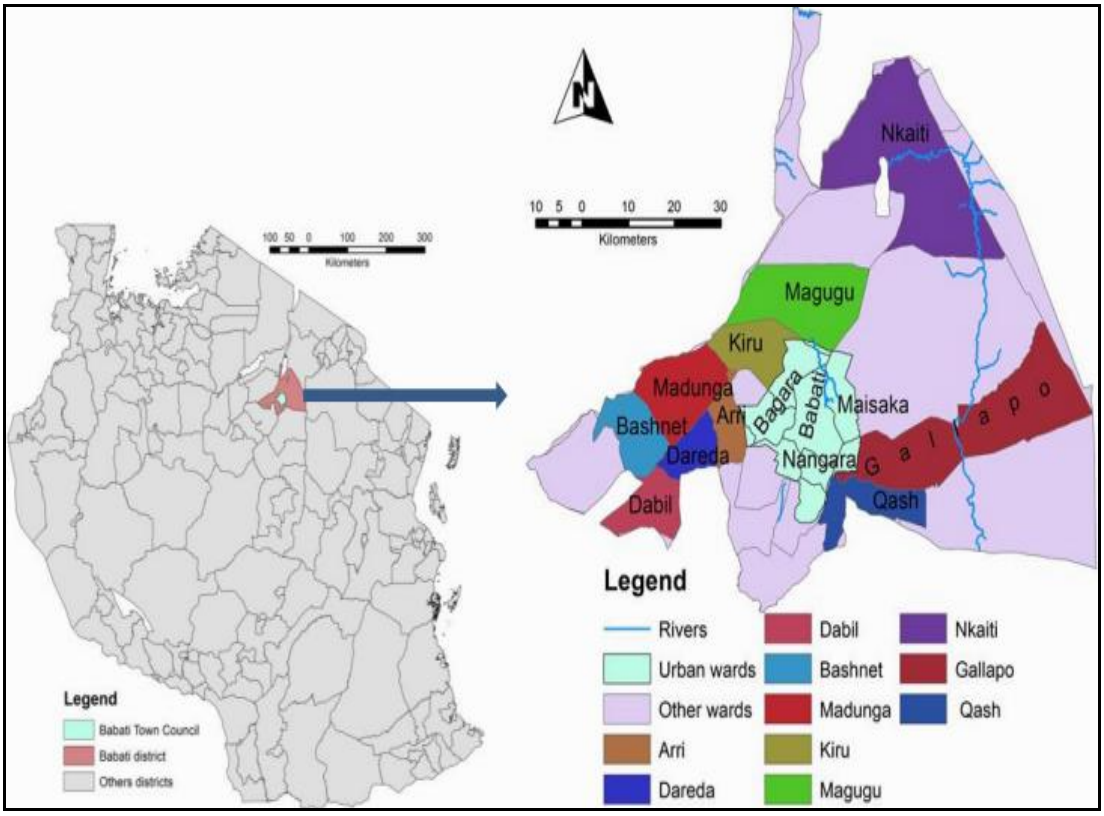

Figure 1. Map of Babati district showing sampling locations. Samples were collected from Babati district and Babati town councils. Sampling locations are indicated by names on the map.

Estimation of sample size

Each cattle keeping household was regarded as a cluster. The number of clusters (C), required for this study was calculated using the formula $C=\frac{P(1-P)D}{(SE^2)n}$, where D= 1+ρ (n-1), SE= $\frac{L}{Z_\alpha}$, P = estimate value for proportions, D = design effect, SE = standard error; n = average cluster size =3, ρ = intra-cluster correlation coefficient = 0.15 (Otte and Gumm, 1997). The average herd size was three animals per herd (BDC and BTC annual reports, 2012), the expected prevalence of brucellosis in dairy cattle was assumed to be 12.2% (Swai *et al.,* 2005). The statistical confidence level was decided to be 95%, and desired absolute precision was 5%. Accordingly, using the formula and the values above, the sample size required was determined to be 66 households (cluster), and 192 lactating cows.

Animal selection and data collection

This study included 66 households owning 192 lactating cows. Based on cattle concentration, the households were sampled from BDC and BTC. The list of heads of the households with at least three lactating cows was obtained from BDC and BTC's Livestock and Fisheries Departments and they were used as sampling frame. The household was a sampling unit and the lactating cows for study were selected randomly. Age of animals was classified as young, middle age and adult lactating cow with less than three years, three to five years and above five years, respectively. There was no history of vaccination against brucellosis in cattle of Babati district.

Sample collection and handling

Before milk and blood sampling was undertaken, the head of selected household was interviewed with the questionnaires which focused on general livestock husbandry, epidemiology, ecological factors and assessment of knowledge and awareness of transmission of brucellosis. Thereafter, the selected lactating cows were restrained using ropes, crush and bull ring depending on what restrain technique was favourable on specific herd situation found. Approximately 10 ml of whole blood was collected from the jugular veins by venipuncture into plain vacutainer tubes (Griner Bio-One GmbH Kremsmunster, Austria). In addition, from the same animal, 10 ml of milk was collected from washed and dried udder into Falcon tubes. Blood samples were stored in cool box and later on transferred to

Babati District Veterinary Office where they were allowed to clot in a slant position on a table and serum was harvested into Eppendorf tubes after 24 hours. Serum and milk samples were kept in ice box during transfer to laboratories at Sokoine University of Agriculture. Both sera and milk samples were stored at -20°C until used for serological and molecular screening of brucellosis.

Laboratory analysis of samples

Serological detection of *Brucella* antibodies

Serum samples from cattle were tested for antibodies against Brucella. In the present study, screening of Brucella antibodies was done using RBPT and BAPA while c-ELISA was used as confirmatory test, according to protocols for *Brucella species* detection described by OIE, 2009. Laboratory analysis of samples for all tests was carried out at the Faculty of Veterinary Medicine (FVM), SUA. A village was considered positive if there was at least one animal in herd in that village responding positive to c-ELISA confirmatory test while a herd was considered positive if at least one animal was detected to have Brucella antibodies in that herd.

Sera were tested for antibody against *Brucella species* using rapid Rose Bengal plate test (RBPT) using the Rose Bengal stained antigen (Central Veterinary Laboratory, UK). Briefly, 30 µl Rose Bengal antigen (Weybridge standard**)** was placed on glass-plate and followed by mixed with an equal volume of test serum. Afterwards, the antigen and test serum were thoroughly mixed using stirring stick and the slide was gently rocked for four minutes at room temperature. The reactions were then examined for agglutination by naked eyes. The sample was considered positive if serum in the glass plate agglutinated and the test was repeated for samples with weak agglutination.

Sera were tested for antibody against *Brucella spp.* using Buffered acidified plate test (BAPA). Briefly, 80 µl of serum followed by 30 µl of antigen were placed onto a clear glass plate and mixed with a stirrer to cover a circle with approximately 27 mm. Positive and negative control serum were also separately included during the testing. Afterwards, the plate was rotated four times, covered and the antigen-antibody mixture incubated for four minutes at room temperature. Then, the plate was again rotated for four times, followed by incubation of the antigen-antibody mixture for four minutes. The reactions were then examined and scored for agglutination by naked eyes. The sample was considered positive if serum in the glass plate was agglutinated and test was repeated for samples with weak agglutination.

Competitive enzyme-linked immunosorbent assay

The confirmation of the presence of *Brucella* antibodies was performed using c-ELISA following a protocol described by Veterinary Laboratory Agency (VLA), Surrey, United Kingdom. Briefly, the conjugate solution was prepared and diluted to working strength and 100 µl added onto each well of 96 well ELISA plate. Afterwards, 20 µl of each test serum sample was added per well. After addition of the conjugate, plate was vigorously shaken to allow mixing of the serum and conjugate solution. The plate was covered with the lid and incubated at room temperature (21°C ± 6°C) for 30 minutes on rotary shaker, at 160 revolutions per minutes to allow the interaction between antibodies and the antigen coated on the plate. After incubation, the contents of the plate were discarded and the plate rinsed five times with washing solution and thoroughly dried by tapping on absorbent paper towel. The ELISA reader was switched on and the unit was allowed to stabilize for ten minutes. Before the unit was used, the substrate and chromogenic solution was prepared by dissolving one tablet of urea and hydrogen peroxide in 12 ml of distilled water. Afterwards, 100 µl of substrate and chromogenic was added to each well. The plate was left at room temperature for minimum of 10 minutes and maximum of 15 minutes. The reaction was stopped by addition of 100 µl of acetate buffer and condensation on the bottom of plate was removed by absorbent paper towel. Reading of the plate was made at 450 nm by using Thermo Labsystems Multiskan R.C. The lack of colour development indicated that the sample tested was positive. A positive /negative cut-off was calculated as 60% of the mean of the optical density (OD) of the four conjugate control wells. Any test sample that gave OD equal to or below the value was regarded as positive.

Molecular diagnosis

DNA extraction from milk samples

The collected milk was thawed and used for DNA extraction using ZR Genomic DNA™ Tissue MiniPrep kit (Zymo Research, USA). To 3 ml of milk samples, 200 µl of water, 200 µl of 2x digestion buffer and 20 µl proteinase K were added and incubated overnight at 55^{o}C. After overnight incubation 500 µl lysis buffer was added and mixed thoroughly by vortexing. Then samples were centrifuged at 10 000 *g* for one minute to pellet precipitated proteins. Thereafter, supernatant was transferred to a Zymo-Spin™ IIC column and DNA allowed to bind to the column by centrifugation at 10 000 *g*. DNA bound to the column was washed using buffers to remove PCR inhibitors. DNA was eluted into a micro-centrifuge tube. The eluted DNA was stored at -20^{o}C until PCR.

Amplification of *Brucella* species DNA by PCR

Detection of the presence of *Brucella* spp. genome in milk samples was done using PCR. The components of the PCR mix and the primers used for the detection of different *Brucella* spp. are shown in Table 1 and 2, respectively. PCR was performed using a DNA polymerase from Bioneer, Korea. The amplification conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 55°C) and extension (90 seconds at 72°C), and a final extension at 72°C for 10 minutes on a thermal cycler (TaKaRa, Japan). After PCR, 5 µl of the PCR products was mixed with a 6x loading dye. The PCR products were then electrophoresed in one per cent agarose gel in buffer containing Gel Red (Biotium, USA) and a marker of one kilo base pairs ladder was used. Electrophoresis was performed at 80 Volts for 45 minutes. Finally the results were read and image captured using a gel documentation system (Gel doc EZ Imager, BioRed, USA).

Table 2. Preparation of PCR mix for the detection of *Brucella* in milk samples

Component	Volume for one reaction (µl)
PCR premix containing DNA polymerase and dNTPs	0.5
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Nuclease free water	13.5
DNA template	5

Table 3. Primers used for the detection of *Brucella* in milk samples

Primer	Sequence 5'→3'	Target *Brucella* spp.	PCR product size (bp)	Reference
IS711	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-TGC-CAG	Forward primer for both *B. melitensis* and *B. abortus*		Bricker and Halling, 1994
abortus	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	Reverse primer for *B. abortus* biovars 1, 2 and 4	495	Aggour *et al*., 2013
melitensis	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	Reverse primer for *B. melitensis* biovars 1, 2, and 3	730	Aggour *et al*., 2013

Livestock owner's cross-sectional questionnaire survey

A pre-designed structured questionnaire with both close and open ended questions was used to collect information on herd level risk factors we believed to be associated with *Brucella* prevalence. Pre-testing of the questionnaire was carried out in one of the study areas to ten dairy owners to detect any lack of clarity of questions was noted and later revised and few changes were made before final version was developed. A questionnaire form comprising of variables such as herd size, source of their cattle, mixing of cattle with cattle from other herds, purpose of his/her dairy farm, milk and meat consumption habit and presence of brucellosis patient in their family. In addition, data on individual animal such as sex and age were recorded. Questions related to general livestock husbandry, livestock and wild animals feeding pattern, contacts between wild and domestic animals and livestock movement. The revised questionnaire

was then administered to households where animal blood and milk samples were taken. The interviews were conducted by the author alone after harvesting blood and milk from livestock and one member of family was involved. The selected respondent was the one knowledgeable about the herd, usually the head of the house. When the head of house was absent, other members of the household like the wife, child, parents/parents in law of head of house or other specified member with knowledge of herd under investigation were interviewed. The interview took about 30-40 minutes.

Data analysis

Data was entered in Microsoft Excel 2010 and analysed by Epi Info (Epi Info™ 7.1.3, Atlanta, USA). Individual animal level prevalence was defined as the number of positive reactors per 100 animals tested. Herd level prevalence was computed as the number of herds with at least one-reactor cattle divided by the total number of herds tested. Descriptive statistics such as frequencies and percentages were calculated. A confidence limit of less than 5% ($P< 0.05$) was used to indicate a significant level. Chi-square test and Fisher's exact were used to compare the statistical significance in prevalence of brucellosis in livestock using Epi Info statistical software. Logistic regression analysis was used to assess strength of association of different factors to the occurrence of brucellosis in cattle and its potential risks. A multivariate logistic regression model of risk factors was fitted by backward stepwise selection of variables (McDonald, 2009). The variables were retained in the model based on likelihood test ratio p-value ($p<0.25$ for the first model and $p<0.05$ for the final model). The goodness of fit of the model was tested by Hosmer and Lemeshow test (MedCalc version 13.1.1). Furthermore, the agreement of the tests RBPT, BAPA and c-ELISA used in the diagnosis of bovine brucellosis were analysed using kappa statistic (κ).

RESULTS

Seroprevalence of Brucella in lactating cows of Babati district

A total of 192 of lactating cows that produce milk for public consumption were involved in this study. All animals were tested for the presence of antibodies against *Brucella* spp using RBPT, BAPA and c-ELISA. The number of animals that tested positive is indicated in Table 3. Out of 192 animals, nine animals were positive for Brucella antibodies after screening using RBPT and 10 animals were positive for *Brucella* antibodies after screening using BAPA. The agreement between the RBPT and BAPA to detect brucellosis was good (κ = 0.94). When the 10 positive animals were tested using c-ELISA, eight of these animals were confirmed to be positive (Table 3). The agreement between the c-ELISA and BAPA to detect brucellosis was good (κ = 0.88). Out of the eight animals confirmed to be positive for Brucella antibodies, all of them were found within animals originating from traditional cattle farmers and large scale dairy farmers and no positive animal originated from small and medium scale dairy farmers. There was no statistical significance difference between the prevalence of Brucella and the breed of animals, location of cattle, farming system, herd size or age.

Molecular detection of Brucella species DNA by PCR

Eight milk samples from animals that were confirmed to be positive by c-ELISA were screened for the presence and type of *Brucella* spp. using PCR. Primers that specifically amplify *B. abortus* and *B. melitensis* were used in the PCR (Table 2). When PCR was performed using primers that specifically amplify the IS711 gene of *B. abortus*, three out of eight cows were positive, producing an expected PCR product of 495 bp (Figure 2). No PCR product was observed when PCR was performed using primers that specifically amplify the IS711 gene of *B. melitensis*.

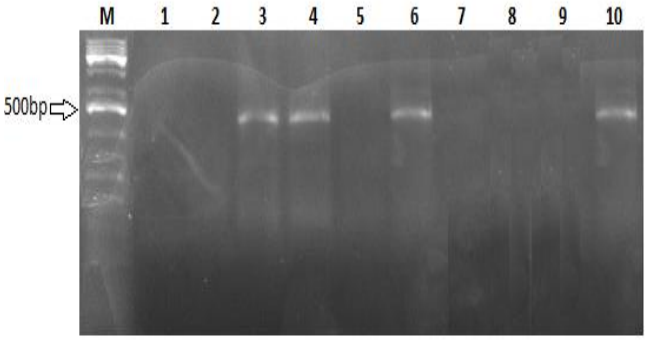

Figure 2: Amplification of *B. abortus* IS711 gene using PCR. A 1% agarose gel electrophoresis of *B. abortus* IS711 gene amplicon of 495 bp from total DNA of cow milk. Lane M; 1kb DNA ladder; Lane; 3, 4 and 6 are positive milk samples; Lane; 1, 2, 5, 7 and 8 are negative milk samples; Lane; 9 negative control containing water; Lane 10; positive control containing DNA of *B. abortus.*

Table 4. Seroprevalence of brucellosis in lactating cows of Babati district

Parameter	Category	Number of cows screened	Number (%) of positive cows by different serological tests		
			RBPT	BAPA	c-ELISA
Production system	Dairy	142	6 (4.2)	7 (4.9)	5 (3.5)
	Traditional	50	3 (6.0)	3 (6.0)	3 (6.0)
Cattle breed	Friesian	94	2 (2.1)	3 (3.2)	2 (2.1)
	Ayrshire	48	4 (8.3)	4 (8.3)	3 (6.3)
	TSHZ	50	3 (6.0)	3 (6.0)	3 (6.0)
Age	≥ 3 years	28	0 (0.0)	0 (0.0)	0 (0.0)
	≥ 5 years	152	8 (5.3)	9 (5.9)	7 (4.6)
	≤ 6 years	12	1 (8.3)	1 (8.3)	1 (8.3)
Herd size	Small (1-5 animals)	104	0 (0.0)	0 (0.0)	0 (0.0)
	Medium (6-10 animals)	24	0 (0.0)	0 (0.0)	0 (0.0)
	Large (≥ 10 animals)	64	9 (14.0)	10 (15.6)	8 (12.5)
Location	BDC villages tested	24	5 (20.8)	5 (20.8)	5 (20.8)
	BTC villages tested	12	1 (8.3)	1 (8.3)	1 (8.3)

Risk factors associated with transmission of brucellosis between lactating cows and other animals in Babati district

Total number of 66 respondents was administered with questionnaire for assessing risk factors of transmission of brucellosis. Majority (68%) were adult men above the age of 25, among 66 respondents, 53 (80.3%) were dairy cattle owners, 13 (19.7%) traditional cattle farmers, in whom 59.1% had knowledge on brucellosis. The results related to awareness about brucellosis (Table 4).

Risk factors of transmission of brucellosis basing on statistical analysis

Analysis for risks factors of brucellosis transmission in the study area reported by household respondents through questionnaire revealed some variables which are potential risk factors that were considered to be associated with brucellosis were based on individual cattle and herd level. At herd level factors where farming system, herd size, mixing of cattle from different herds (livestock contact), sale or purchase of animals from and to unknown farm and cattle breed to be associated with increased risk of brucellosis transmission. These variables were subjected to univariate logistic regression analysis (Table 4). Univariate analysis indicated that herd size (OR=1.9537, P=0.0030), mixing of cattle (OR=32.5000, P=0.0027), selling and buying of cattle from and to unknown farms (OR=5.0588, P=0.0500), farming system (OR=3.9474, P=0.0400) and cattle breed (OR=1.9860, P=0.0284) were significantly associated with risk for having brucellosis, hence were subjected to multivariate analysis.

Table 5. Risk factors of transmission of brucellosis in cattle

Variable	Category	Frequency	Percentage
Number of cattle	1-5	55	83.3
	6-10	6	9.1
	11-above	5	7.6
Sale or purchase of animals	Yes	21	31.8
	No	45	68.2
Livestock mixing	Yes	13	19.7
	No	53	80.3
Grazing in communal pasture	Yes	14	21.2
	No	52	78.8
Livestock-wild animals contacts	Yes	9	13.6
	No	57	86.4
Brucellosis knowledge	Yes	39	59.1
	No	27	40.9
Farming system	Dairy	53	80.3
	Traditional	13	19.7
Type of cattle kept	Indigenous	10	15.2
	Exotic	41	62.1
	Both	15	22.7

Table 6. Univariate analysis of risk factors of transmission of brucellosis

Term	Odds ratio	95%CI	P-value
Cattle type	0.7998	0.2022-3.163	0.7502
Communal pasture	2.0000	0.3268-12.238	0.4533
Communal water	1.1333	0.1186-10.833	0.9135
Farming systems	3.9474	1.0645-14.638	0.0400*
Herd size	1.9537	1.2546-3.042	0.0030*
Brucellosis knowledge	3.8235	0.4210-34.727	0.2335
Livestock contacts (mixing)	11.3333	1.8016-71.294	0.0097*
Livestock wildlife contact	3.7857	0.5828-24.591	0.1632
Livestock-wild common water	3.7857	0.5828-24.591	0.1632
Placenta into bush	3.2500	0.5094-20.737	0.2126
Sale or purchase animals	5.0588	0.8466-30.231	0.0500
Veterinary services	0.7143	0.1331-3.835	0.6948
Cattle breed	1.9860	1.0752-3.669	0.0284*

Note: * statistically significant

The multivariate analysis indicated no statistical significant association between cattle breed (OR= 1.9516, P=0.1533), farming systems (OR=0.5724, P=0.5633), herd size (OR=1.7773, P=0.0729), mixing of cattle (OR=1.8513, P=0.7190), selling and buying of cattle (OR=0.6213, P=0.7530) with having brucellosis. The study showed mixing of cattle from different herds (OR=1.8513), cattle breeds (OR=1.9516), and herd size (OR=1.7773) had higher chances of contracting brucellosis compared to those who do not mix their cattle and have small herd size (Table 5).

Table 7. Multivariate analysis of risk factors of brucellosis transmission

Terms	Odds ratio	95% CI	P value
Cattle breed	1.9516	0.7795-4.886	0.1533
Farming system	0.5724	0.0863-3.796	0.5633
Herd size	1.7773	0.9479-3.333	0.0729
Livestock mixing	1.8513	0.0646-53.045	0.7190
Sale or purchase	0.6213	0.0321-12.044	0.7530

Hosmer and Lemeshow test showed the model fit the data (P=0.6018)

DISCUSSION

Seroprevalence of brucellosis in lactating cows

The present cross-sectional study was carried out to determine the prevalence and the risk factors associated with transmission of the brucellosis in lactating cows in Babati district. The tests used in the study were RBPT, BAPA, c-ELISA and PCR. The selected serologic screening tests were RBPT and BAPA and had good agreement for detecting brucellosis when compared by kappa statistics (κ = 0.94), (Table 3). The RBPT is capable of detecting infected animals earlier due to its ability to detect presence of IgG1, which is produced early after exposure. False positive reactors are normally due to residual antibodies from vaccination history of the herd, colostral antibodies in calves, cross-reaction with certain bacteria and laboratory errors. The positive predictive value of this test is low and a positive result is required to be confirmed by some other more specific test like ELISA, SAT or CFT (OIE, 2009; Megersa *et al.*, 2011). Due to the fact that, RBPT can give up false-negative reactions mostly due to prozoning (OIE, 2009; Göknur *et al.*, 2010), BAPA was used as second screening test in this study. The agreement between the screening tests and confirmatory test (c-ELISA) was good in detecting brucellosis when compared by kappa statistics (κ = 0.88), (Table 3). The c-ELISA was chosen to be used in this study due to its several diagnostic merits which include high sensitivity and specificity, ability to differentiate vaccinated animals from naturally infected ones, or those infected with cross reacting organisms and its use in areas where disease prevalence is low (OIE, 2009). The results from this study consequently are reliable and indicated that brucellosis is prevalent in Babati district.

The overall seroprevalence of brucellosis in lactating cows in Babati district is 4.2% (8 of 192). The seroprevalence of brucellosis according to cattle type are as follows; 3.5% (5 of 142) for dairy cattle, 6.0% (3 of 50) for traditional cattle (Table 3). These levels of seroprevalence observed in this study are in close agreement with previous studies in which the seroprevalence of brucellosis was found to be high in traditional cattle and low in dairy cattle. Other documented prevalence of brucellosis include, 4% in dairy animals and 15% in traditional cattle in northern zone of Tanzania (Swai *et al.*, 2010), 14.3% in traditional cattle in Mikumi-Selous ecosystem (Temba, 2012), 1.5% for smallholder dairy cattle and 17.9% for indigenous cattle in Iringa and Tanga (Karimuribo *et al.*, 2007), 4.9% in traditional cattle in Arusha and Manyara (Shirima *et al.*, 2005), and 3.2% in dairy cattle in Arusha (Minja, 2002). However, it is lower than the seroprevalence reported by Mtui-Malamsha (2001) and Swai *et al.* (2005) who reported 12.2% in dairy cattle in Manyara and Kilimanjaro, respectively.The difference in seroprevalence reported by Mtui-Malamsha (2001) and Swai *et al.* (2005) and the current study could be due to the difference in animal population, sample size and differences in livestock management practices.

The low seroprevalence in the smallholder dairy animals is likely due to stall feeding that minimises contacts between herds and other animals (Karimuribo *et al.*, 2007). Management practices such as breeding bulls, artificial insemination and intensive husbandry system, which involve confinement, are among elements that have an effect on the number of seropositive animals in an area or herd (Minja, 2002). The cut and carry feeding system of animals that is practiced by many dairy smallholders help to control brucellosis, however, can serve as a potential risk factor when fodder is collected from areas used by indigenous traditional cattle which encroach the peri-urban and urban settings especially during the dry season (Karimuribo *et al.*, 2007). The observed lower sero-prevalence in dairy cattle can also be explained by the altitude of farmers to consider their dairy cattle as enterprises and tend to control brucellosis or take some precautions when purchasing their replacement stock (Chimana *et al.*, 2011; Wankyo, 2012).

Traditional cattle farmers use free-range management system in which they share communal pasture and water points which leads into mixing of cattle and this has shown in this study as an important risk factor (P = 0.0097) for exposure to *Brucella species*, also Matope *et al.*, (2010) and Chimana *et al.*, (2011) found the same. Also traditional cattle farmers frequently purchase cattle from other herds or common livestock markets (*Minadani*) where screening of these cattle for brucellosis is not carried out due to limited availability of veterinary services, this further increases chance of contact with infected herds (Chimana *et al.*, 2011).

Results from this current cross-sectional study revealed, uncontrolled movement, purchasing of livestock (P = 0.0500) from unknown disease status farms and intermixing of pastoralists, agro-pastoralists and smallholder dairy cattle from different regions upcountry migrated to the study area recently could perhaps account for no statistical significance difference in seroprevalence observed

(Chimana *et al.,* 2011; Temba, 2012). In this study found large herd size to be a risk factor (P = 0.0030) due to farmers with large livestock herds with no enough owned pasture and water sources, are forced to use free-grazing farming system to find pasture and water where mixing of different herds occurs and leads to health animals contracting brucellosis (Kohei *et al.,* 2011). Type of management system was a potential risk factor (P=0.0284) for seroprevalence of bovine brucellosis rather than breed despite being potential risk factor. Since all seropositive were from the group of cattle kept in the free-range and semi-extensive management system and none from intensive management system (Table 3). This finding is in agreement with the report of (Karimuribo *et al.,* 2007; Matope *et al.,* 2010) with regard to cattle management systems.

In *Brucella* infection, prevalence increases with age, probably because of greater exposure to infection, time female animals spent in herds is longer than male for breeding reason (Omer *et al.,* 2000; Mohammed *et al.,* 2010). Female animals usually have high brucellosis prevalence due to the presence of the *eri* gene which is essential for erythritol in allantoic fluid which stimulate the growth and multiplication of Brucella organisms and tend to increase in concentration with age (Mellau *et al.,* 2009; Aggour *et al*., 2013). Older animals have higher seroprevalence rate than young animals (Table 3), the effect of age on Brucella infection is related to sexual maturity of animals. Being sexually mature female is a known risk factor to Brucella infection (Minja, 2002; Temba, 2012).

Furthermore, the results of this current cross-sectional study indicate that among 36 villages sampled, six out of 36 (16.7%) had Brucella seropositive in cattle while 30 (83.3%) had none (Table 3). Consequently, it can be inferred from the results that brucellosis is localized among villages in Babati district. The results additionally indicates that out of 66 herds sampled six (9.1%) had Brucella seropositive while 60 (90.9%) had none of the animals reacting to any of the tests. The results therefore indicate that, although brucellosis is localised among villages also it is less distributed among herds. The nature of distribution of disease in villages poses danger of further spread among herds and individual animals because most of pastoral, agro-pastorist and few dairy herds use communal grazing grounds and watering points especially during dry season as reported by Karimuribo *et al.* (2007). It is common to see overcrowding of animals at water points, especially during the dry season, and probably infected aborted animals, recently calved infected animals and infected animals with retained placenta may grossly contaminate the water source resulting in the disease (Matope *et al.,* 2010). Grazing in communal land contribute to build up of Brucella organism in the environment due to uterine discharges, urine and lochia from infected animals which have been found to be major sources of infection to other animals (Shafee *et al.,* 2011). *Brucella* organism can survive in grass for varying period of time with infectivity up to 100 days depending on season and this poses risk of infection to other animals during grazing. The rate of dissemination of brucellosis in the herds and among villages will largely depend on management system practised, animal population density and patterns of movements (Kunda *et al.,* 2010). It is likely that the routine animal's movement in search of water and pasture during dry season and intermixing of herds from different households and from different villages may exacerbate the rate of spread of disease into non infected areas (Karimuribo *et al.,* 2007).

Poor aborted materials disposal systems as seen in this study because of lack of community knowledge about the zoonotic implications of the disease. Collapse of the animal health services in Tanzania as result of the privatisation of veterinary services may also contribute to the perpetuation of the disease in the study areas (Karimuribo *et al.,* 2007; Mellau *et al.,* 2009). As it is well known that during abortion, large numbers of Brucellae are released which may, in turn, cause the infection to other animals and humans (Turatbek *et al.,* 2006; Kunda *et al.,* 2010). Tarangire and Manyara National Parks wild animals have been interacting with livestock and humans from the villages bordering the parks for several decades could perhaps suggest cross transmission of infection at interface where animals share grazing pastures and water especially during dry seasons. The higher prevalence in domestic ruminants is the coexistence of livestock and wild animals which facilitates survival and translocation of the disease causing agent (Mellau *et al.,* 2009).

Several studies have shown that buffaloes and wildebeest were most affected among African wild animals (Shirima, 2005). Seroprevalence reported in wildlife are 67% in buffalo Tarangire National Park (Anderson, 1988), 28% in buffalo in Ngorongoro-Serengeti ecosystem (Shirima, 2005), 24% and 17% in buffalo and wildebeest, respectively in Serengeti ecosystem (Fyumagwa *et al.,* 2009). Therefore, the presence of brucellosis in both domestic and wildlife animals as well humans emphasizes the need for collaboration between livestock owners, livestock experts and wildlife experts. The importance of wildlife brucellosis is based on the

difficulties in eradication, disease dynamic between wild animals, livestock and human being, and conflicts between farmers and wildlife experts (Shirima *et al.,* 2005; Mellau *et al.,* 2009).

Molecular detection of Brucella species DNA in cattle milk by PCR

Applications for PCR strategies vary from the identification of the illness to characterization of field isolates for epidemiologic functions together with classification studies (Poester *et al.,* 2010; Vivek *et al.,* 2014). Based on these facts, PCR amplification targeting the species-specific genetic element IS711 in the Brucella chromosome was performed to determine and confirm the presence of Brucella DNA in milk samples (Poester *et al.,* 2010). Only positive samples tested by serologic methods were used to determine and confirm the presence of Brucella DNA in milk samples. The results from this study indicated that only three milk samples (out of eight) had *Brucella abortus* but not *B. melitensis*. As is well known, *B. abortus* can be shed in the milk of infected animals intermittingly, in cattle and other species (Capparelli *et al.,* 2009). So it possible that the other five milk samples was taken when animals were not shedding bacteria into milk. The difference also can be due to long termpersistence of anti-Brucella antibody without presence of the disease agent in milk. Furthermore, it can be due to relatively low detection limit of PCR, because it is possible that some milk samples contained bacteria less than the detection limit and hence failed to be found as positive (Göknur *et al.,* 2010; Kechagia *et al.,* 2011). The PCR has shown in this study that it is a technique that enables for speedy and correct identification of brucellosis (Baddour, 2012). Another advantage of PCR technique is that detection can be done without necessarily culturing the bacteria that are infective to humans (Göknur *et al.,* 2010).

From this study, there is a proof that brucellosis is present within the population of milk producing cattle in Babati district. Routine screening of animals or surveillance for brucellosis is incredibly necessary in brucellosis control. It might facilitate to notice positive cases as early as doable thus on scale back the chance of cross contamination to different animals at intervals the herds or flocks and take correct measures on time. More attention should be paid towards implementing a proper control program for brucellosis and more efforts should be directed towards improving the animal health biosecurity program. Build-up immunity of animal population against brucellosis is possible approach to all livestock which can suffer from brucellosis. Mandatory vaccination of cattle with Brucella vaccine like S19 and RB51 which are present in the markets and applied into heifers of 3-8 months of age in dairy and traditional cattle. In addition, controlling brucellosis in small ruminants is done mainly by Rev-1 vaccination and will indirectly reduce the prevalence of this disease in other animal species especially cattle. Control progress should be monitored serologically and evaluated epidemiologically.

ACKNOWLEDGEMENTS

The authors are indebted to the livestock keepers in Babati district for accepting to participate in the study together with their cattle. The Bababti district authority is thanked for the permission to do research and ward livestock officers are thanked for their assistance during data collection. The laboratory assistance given by Mr. E. Rugaimukamu and Ms. M. Makange of the College of Veterinary Medicine and Biomedical sciences is highly valued. This study was funded by Tanzania Commission For Science and Technology (COSTECH).

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Knowledge, perceptions and practices regarding brucellosis in pastoral communities of Kagera Region, Tanzania

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SUAMMARY

A cross-sectional study was conducted in June 2017 to assess the knowledge, perception and practices of brucellosis by pastoralists of Kagera ecosystem, Tanzania using qualitative methods. Five focus group discussions of six participants were conducted with livestock farmers, administration leaders, religious representatives and youth. In addition, discussions with three key informants were conducted, involving officials of livestock, wildlife and public health department in each district. Data were analyzed using content analysis with inductive and deductive methods. This study revealed low knowledge regarding brucellosis by interviewees. Although participants recognized brucellosis as a zoonotic disease, they seemed to consider it of less importance. In addition, participants had low knowledge on causes, symptoms and mode of transmission of this disease. However, they perceived the interactions between humans, livestock and wildlife together with the neighborhood with other countries to be potential risks for introduction of brucellosis in their communities. Moreover, their habit of drinking unpasteurized milk, the lack of protective gears assisting animals giving birth and poor vaccination program need to be improved by community health education. A coordinated one Health approach is needed and further studies are suggested to reveal the status of brucellosis in Kagera ecosystem to guide its control and prevention.

Key words: Knowledge, practices, brucellosis, pastoral communities, Kagera, Tanzania.

INTRODUCTION

Brucellosis is a worldwide zoonotic disease for both public health and economic importance, affecting humans, livestock and wildlife. Brucellosis has a worldwide distribution where Africa continent is one of the endemic areas (Corbel, 1997). This infectious disease causes important losses and human burdens in infected zones (Boschiroli *et al.*, 2001). Different *Brucella* species are identified as causative agents of brucellosis and some of them are known to be pathogenic to humans: *B. abortus, B. canis, B. inopinata, B. melitensis, B. pinnipedialis, and B. suis* (Tiller *et al.*, 2010; Zheludkov and Tsirelson, 2010; Whatmore *et al.*, 2014). It is known that brucellosis is endemic in several areas in East African region (Chota *et al.*, 2016) and it reduces productivity through abortions and weak offsprings; causing a major threat in national and international livestock trade.

In Tanzania, some previous studies have reported existence of risk factors for brucellosis transmission in pastoral communities such as occurrence of abortions in herds, assistance of animals during parturitions, individuals living in close proximity with livestock and animal slaughtering occupation (Swai and Schoonman, 2009; John *et al.*, 2010; Assenga *et al.*, 2016). Brucellosis is associated by many communities to people who drink raw-milk/ animal blood, consume raw meat; or to those who share a bed or utensils with brucellosis patients (Mubyazi *et al.*, 2013). Lessons learnt from public and local knowledge, perceptions and practices regarding infectious diseases and other communicable diseases in selected areas of Tanzania demonstrated higher understanding by pastoralist of the existences of a number of certain diseases transmitted between humans and animals (Swai *et al.*, 2010; Mangesho *et al.*, 2017). Moreover, livestock keepers could recognize abortions, emaciation, a drop in milk production and fever as clinical signs associated with brucellosis (Shirima, 2005). Despite the good perception and knowledge of common diseases circulating in their area, livestock farmers needs to improve their practices to control those diseases, which most of the times leads to failure at individual and national levels (Chengula *et al.*, 2013). The activities undertaken for controlling brucellosis, may involve the capacities for detection of the disease, the participation of the stakeholders for mass vaccination or culling; the epidemiosurveillance system based on the perception of the risk for the humans, livestock and wildlife in the ecosystem. Despite their knowledge and perception of the threat caused by certain diseases in their communities, pastoralists adopt some cultural behaviors which

could favor the transmission of infectious disease in the localities (Musallam *et al.*, 2016).

The understanding and the eradication of brucellosis, needs a characterization of the disease, the multidisciplinary actions from different stakeholders in the infected zones (Zinsstag *et al.*, 2005). Also, the transboundary transmission of zoonotic diseases may be considered and be evaluated from the local understanding of communities; this reinforced by cross-border molecular tracing which can confirm brucellosis as a zoonosis of concern for regional public health (Gwida *et al.*, 2012). Little is known about the local understanding of brucellosis by pastoralists in Kagera, Tanzania. This study was conducted to assess the knowledge, perception, and practices regarding brucellosis of different stakeholders in the pastoral communities of Kagera Region; an ecosystem located on borders between Tanzania, Burundi, Rwanda and Uganda.

MATERIALS AND METHODS

Study area

This study was conducted in two districts namely Karagwe and Ngara, of Kagera Region, in north-western part of Tanzania. Livestock contributes significantly to the economy of Kagera region, and animals are exported to neighboring countries (Ministry of Livestock and Fisheries Development, 2011). According to National Sample Census of Agriculture report of 2012, cattle population in Kagera region was 837,204. Other animals were 593,607 goats, 53,061 sheep, 44,402 pigs, 1,005,415 poultry, 2 water buffaloes, 15,627 rabbits, 40,471 dogs, 159 donkeys, 18 horses and 6,629 cats. Dairy farming is spread throughout the Region with an estimated 21,438 dairy cattle. Kagera ecosystem is subdivided into three agro-ecological zones (Lake Shore and Islands, Plateau Area and Low land) in which crops grown are mainly bananas, cassava, beans, maize, coffee and tea. The area has game reserves such as Kimisi and Burigi in which zebras, impalas, buffalos, elephants, giraffes, leopards, hippos and crocodiles can be found. Health facilities are distributed in all districts and various transport means link Kagera to other regions and neighboring countries particularly Burundi, Rwanda and Uganda. The climate is equatorial with temperatures ranging between 20°C and 28°C. Kagera Region, in general has rainfall ranging between 900 - 2,000 mm per annum.

Study design

A cross-sectional study design was used to assess the knowledge, perception and practices of brucellosis by the pastoral communities of Kagera in June 2017, using a qualitative research method.

Participants selection and data collection procedure

Two focus group discussions (FGDs) and one Key Informants Interview (KIIs) were conducted in Ngara district, while three FGDs and one KII were done in Karagwe district. Each FGD involved a minimum of six persons selected purposively: three farmers, one person from local administration, youth representative, and one person from religious confessions. Discussions in KIIs involved three persons of government officials from animal health, public health and wildlife departments at each district level. Participants were originated from five villages selected purposively (urban, peri-urban and rural areas) to get a variation of insights on brucellosis from different people according to their location. FGDs and KIIs approaches were combined to get coverage of information from experts and non-experts regarding brucellosis disease.

The FGDs and KIIs were conducted in the respective communities of the participants; i.e, ward executive and district official's offices (hospitals). Digital recording by mobiles phones was used to record discussions and to take pictures. FGDs were conducted in Swahili language by a facilitator, while interviews with KI were conducted in English by the researcher. The interview guide was structured around four main themes as follows:

(i) Perception of brucellosis by the population in Kagera ecosystem: Participants were asked about the local name of brucellosis, existence of the disease in their locality. The knowledge on the causes, the presenting symptoms, and the mode of transmission of brucellosis were also assessed. Furthermore, the socio economic impact and the prophylactic approach of this zoonotic disease in the ecosystem were discussed.

(ii) Risk factors for brucellosis prevalence in Kagera ecosystem,

(iii) Potential for transmission of brucellosis in Kagera ecosystem due to neighboring with other countries

(iv) Roles of different stakeholders in the ecosystem in the control of brucellosis.

The facilitator introduced the aim of the study, explaining each theme clearly to participants. The discussions lasted for approximately 45 minutes. For the KIIs, the interviews were conducted in English by the researcher and both FGDs and KIIs groups were asked the same questions.

Data analysis

Data recorded from FGDs were transcribed verbatim to Microsoft Word and later translated from Swahili to English. The coding of the categories was done manually using Microsoft Excel inasmuch as, the data were small and themes and sub themes were identified. Later the content analysis was done with inductive and deductive methods based on the categories grouped in different themes and subthemes as well as emerging themes. Themes and subthemes were analyzed in their chronologic order of inquiry.

Ethical considerations

This study was approved by institutional review board of Sokoine University of Agriculture, and ethics clearance was also obtained from the Medical Research Coordinating Committee of the National Institute for Medical Research (ref: NIMR/HQ/R.8a/Vol.IX/2456). Verbal consent was obtained before conducting each FGD by all the team members. For confidentiality matter, participants were ensured for none use of their names during analysis, report or publication. Approval by participants for recording and taking pictures was requested before starting this activity.

RESULTS

Socio-demographic description of the participants

Thirty seven participants from six villages of Karagwe and Ngara districts were recruited to participate into Focus group discussions. The mean age of the participants was 49 years with a standard deviation of 10.55 and 30.55% of participants were females. The focus group discussions involved farmers, youth, religious leaders and local administration representatives. Four of the participants had no formal education, 21 had primary education, and 12 had secondary school or higher education. Key informants interviews were conducted in group of three individuals from public health, livestock and wildlife departments at district level (Table1). All the key informants were degree holders.

The knowledge and perceptions on brucellosis by the pastoral communities of Kagera ecosystem

The understanding brucellosis among the study participants in Kagera Region was not direct because some of them confused this disease with the "abortion process". In Tanzania, brucellosis is normally known in Kiswahili as "ugonjwa wa kutupa mimba" meaning the "disease of abortion". The facilitator had difficulties to get the right words in local language to signify "brucellosis". Describing the disease to participants, the term abortion was used as a prominent symptom; but, it wasn't enough to differentiate brucellosis from other abortive diseases which people are accustom to see or to report in humans and their livestock. Explanations and discussions were needed to make participants understand the phenomena of abortions, which was influencing much the answers given in the focus group discussions conducted in the two Districts.

Participants gave different local names of brucellosis: Amakole, Omwizi, Entandago, Kuramburura, and Kururumura. However, the most common local name of brucellosis used in the two districts was "Kutoroga".

Also, the existence of brucellosis in their locality, as well as the zoonotic nature of the disease was recognized by all the groups who participated in this study. However, participants provided different causes of brucellosis. Five groups out of seven believed that brucellosis is caused by seasons (three groups mentioned dry season and two groups mentioned rain seasons), while three groups said that brucellosis is caused by other diseases (malaria, foot and mouth disease. One group mentioned vectors (tsetse flies, mosquitos), age, contaminated water, drought and famine as causes of this disease.

> "*..... Few days ago, I was a farmer this disease can occur when the cattle drink contaminated water with bacteria. Also dry season causes abortion because of high temperature. There are so many causes' including different diseases. That's what I know*." (FGD Bweranyange- Karagwe District).

Six out of seven groups mentioned abortion as a symptom of brucellosis in humans and livestock. Other symptoms in humans mentioned were fever, tiredness, skin changes, and the two key informants groups insisted on the fact that brucellosis may have a resemblance of symptoms with other febrile diseases such as malaria.

In livestock, participants mentioned additional symptoms of brucellosis such as fever, hygroma, vaginal discharges, skin changes, inappetence, orchitis, tiredness, general weakness and coughing. Only one group of key informants mentioned hygroma as symptom of brucellosis observed in wildlife.

"The signs are the same, cattle can feel like fever, then hair rise up and blood start to come out, and abortion can occur almost within two days. We as farmers are very accustomed to the problems of cows than those riches (cattle owners) who give us the cattle to graze for them" (FGD conducted in Nyakasimbi-Karagwe District - farmer).

Table1. FGDs per District and locations

District	Village	Characteristic	Participants		No. of FGDs conducted
			Female	Male	
Karagwe	Bweranyange	Rural area participants	1	6	1
	Nyagasimbi	Rural area participants	1	5	1
	Nyakahanga	Urban and peri-urban area participants	2	4	1
	Kayanga	Officials from Livestock, Public health and wildlife department	1	2	1
	Benako	Rural area participants	3	3	1
Ngara	Ngara mjini	Urban and peri-urban area participants	2	4	1
	Ngara	Officials from Livestock, Public health and wildlife department	1	2	1
Total			1	26	7

Mode of brucellosis transmission also retained the attention of participants when they mentioned different ways in humans: consumption of uncooked meat and unpasteurized milk, sexual intercourse and unprotected assistance of their animals during parturition. In addition, they mentioned the poor disposal of aborted materials and placentas. In livestock, participants centered the transmission of brucellosis on the sharing of pasture and water between domestic animals and wildlife, the physical and sexual contact between animals, vectors (mosquitos and tsetse flies) and contact with vaginal discharges of infected animals. Two groups mentioned the interactions between animals and the dissemination of vaginal discharges as source of contamination of brucellosis in wildlife.

"In animals, the transmission can be due to the increase of the number of cattle in the same area where self-infection between those cattle can occur; or, if the livestock is not well vaccinated, also during the sharing of pastures with those cattle which are not vaccinated well. It may happen that you perform vaccination very well but the problem becomes on sharing pastures with others who have some diseases. This may result in the transmission of some diseases which you cannot recognize" (FGD - Nyakahanga-Karagwe District Farmer1).

Throughout the discussions, the participants talked about the social impact of brucellosis in their localities. Three out of seven groups believed that brucellosis could affect their willing of raising animals and could lose their faith in their marriage. In addition, the economic impact of brucellosis was pointed on the issue of the loss of milk production, unnecessary expenditure to cover the treatments (incomes decrease), which could also contribute to the inability to pay school fees for their children. All the groups converged to say that brucellosis decreases the total number of livestock. Furthermore, participants highlighted the negative impact of brucellosis on their health through the loss of pregnancies, the deaths and the nutrition problems due to the decrease of milk production.

"Maybe on medical aspect, first of all, if you fail to diagnose brucellosis timely, you will not treat correctly and result into an avoidable death, if you treat wrongly the patient, thinking that maybe is malaria or typhoid while is not, the outcome of improper

> *treatment is bad consequences to the patient, like death; and misuse of medicines."* (KII1- Ngara District).
>
> *"... but this problem can cause the fall of production for both animals and humans."* (Cheikh - FGD Ngara District).

Regarding the prophylactic approach for brucellosis, focus group participants agreed that women actually are seeking in general for medical care in health centers and hospitals. Key informants groups specified the use of antibacterial drugs such as doxycycline and rifampicin as treatment options in case of suspicion of brucellosis, even if according to them, some of these drugs particularly rifampicin were commonly used to treat tuberculosis. For livestock sector, farmers in all groups attested to call for veterinary services, also they confirmed buying drugs themselves and rarely applying for vaccination program. The use of traditional medicine to treat brucellosis in livestock and humans in case of abortions was mentioned by two groups during discussions.

> *"Ah no, when you suspect something even if it is not yet confirmed, but if you see that it is likely to be, you start to treat. So alternatively, we use doxycycline; even if it is not available in the hospital, in the pharmacies, it is available."*(KII1 Ngara District).
>
> *"Here the government has never provided such vaccine or medicine but ourselves when the problem happens, we go to the pharmacy to buy some medicines for treating our livestock. But about prevention cases from the government; we didn't receive any."* (FGD Bweranyange- Karagwe District farmer2).

Risk factors for brucellosis in humans, livestock and wildlife

The important risk factors for brucellosis mentioned by participants (five groups) were: a movement of livestock and wildlife in the ecosystem, the sharing of pastures and watering points between wildlife and livestock.

> *"….because most of the people who are living here close to this Kimisi game reserve are involved in movement in game reserve. They take their livestock to graze inside the game reserve. So, their interaction with wildlife can increase the disease. So, these animals can cunningly increase the risk."*(KII- Karagwe District-wildlife official).

KII groups recognized the existence habits of drinking unpasteurized milk, poaching and the poor disposal of aborted material (placentas and aborted materials are thrown in the environment or given to dogs) as major risk factors for brucellosis in humans in the communities. Climate change, consumption of uncooked meat and sexual intercourse (favored by the movement of people in the ecosystem) were also reported as risk of introduction of brucellosis in the study area by two groups.

The risk for transmission of brucellosis in Kagera ecosystem due to neighboring with other countries

Six groups stated that the interactions observed on borders between livestock and wildlife and the existence of games reserves on borders constitute a risk for transmission of brucellosis from others countries. Furthermore, the existence of movement of people crossing borders for pastoralism and business (commercial activities), the migration of people (sites for refugees) were evidenced by different groups as potential risks for the introduction of brucellosis from neighboring countries.

> *"During the conflicts war in Rwanda and Burundi I was here and during that time I was keeping goats but this disease was already there before the refugees came here. But during that period there are some refugees who brought some cattle and used to sell it to indigenous people but there were no any benefit from this, because all of them died. We are not sure if those cattle died because of this disease or if the problem was the climate changes they faced once they arrived here. But I think the problem was the environment, they were not support the weather. After the refugees returned to their home, the process of keeping animals increased in high percent and lobbers of cattle increased also."* (Pastor in FGD in Benako- Ngara District).

During the discussions, five groups mentioned also the uncontrolled movement of wildlife on borders (wild animals don't know borders) to be a risk of introduction of brucellosis from a country to another.

The role of different stakeholders in the ecosystem in the brucellosis control

Actually brucellosis is not controlled in the pastoral communities of Kagera. Little is being done for the effective surveillance of this zoonotic disease. All the groups confirmed that few farmers were vaccinating their animals.

Otherwise, participants from all groups requested the government to apply for the community health education (trainings and seminars) and they shared the opinion about the necessity of mass vaccination program against brucellosis as it is done for others diseases (Foot and mouth disease, east coast fever CF). Two groups implored the improvement of the equipment in health facilities, also solicited the reinforcement of livestock service in the local communities (increase of the field livestock officers).

> *"….so, it's better if the government can bring the service near and if possible every village should have an animal health center."*(Farmer2- FGD Bweranyange-Karagwe District).

The Key informants proposed to build a laboratory for the diagnosis of brucellosis, to conduct research for mapping brucellosis in the area and they advocated for multisectorial collaboration (sharing information between livestock, wildlife and public health department) about brucellosis.

> *"I think there is a need of having a research to be sure if really brucellosis is existing or not? We are assuming and assumption can be possible, but from what is happening. It is likely that brucellosis exists. To be sure of that, we need to have a research to confirm, to see the magnitude of the problem."* (KII-Ngara District).

DISCUSSION

This study revealed low knowledge, poor perception and practices regarding brucellosis in pastoral communities of Kagera Region, northern Tanzania. Previous studies in Tanzania informed on the magnitude on brucellosis in some areas of the country (Assenga *et al.*, 2016; Kiputa *et al.*, 2008; Kunda *et al.*, 2005; Roug *et al.*, 2014), indicating the disease being one of important threats to both veterinary and public health in the country. Qualitative research studies like the current study are limited and provide further understanding of the problem, and hence, contribute to improved surveillance and management of brucellosis (Mangesho *et al.*, 2017) in affected communities.

All participants described brucellosis as a zoonotic disease while most admitted existence of the disease in their localities. Nevertheless, the presence of a disease can't be confirmed from mere perceptions of people. For example, some local names like "Okutoroga" didn't mean exclusively brucellosis as a disease, but they were indicating the process of abortion in general, which could be attributed to the existence of other abortive diseases in the area. The knowledge of brucellosis by population in Kagera as a zoonotic disease resorted in this study. On the other hand, a study conducted in Tanga and Arusha revealed that rabies, tuberculosis and anthrax were considered the three most common zoonotic diseases(Swai *et al.*, 2010). After all, farmers understand the possibility of transmission of infectious diseases from animals to humans without much consideration for their threat (Mangesho *et al.*, 2017). A study conducted in Kenya showed a high level of knowledge of brucellosis in pastoral communities where respondents reported brucellosis to be a zoonotic disease and abortion as its common symptom (Obonyo and Gufu, 2015). But, in Ethiopia, none of the respondents to a study reported the zoonotic importance of brucellosis (Tesfaye *et al.*, 2013). Brucellosis was perceived by the participants in this study to be caused by others diseases such as malaria in humans, FMD in livestock; which indicates that brucellosis could be less considered among the principal threats in the study area. In addition, if brucellosis is one of the causes of losses in their communities, this situation could lead to ignore its real contribution, and to attribute abusively its burdens to other diseases. Although abortion was mentioned by all FGD groups as common symptom of brucellosis in humans and livestock, women who participated in this study affirmed not to observe a big number of abortions in humans nowadays and, according to them, the rare cases which can occur could not be associated to brucellosis. Studies also documented that *Brucella* species occasionally are causing spontaneous human abortions, but the contribution of brucellosis to abortions in women is still controversial (Khan *et al.*, 2001; Kurdoglu *et al.*, 2015).

Although participants confirmed to have a habit of consumption of unpasteurized milk and non-inspected meat in the area, these animal products were only reported by key informants among the modes of transmission of brucellosis. Others studies in Tanzania reported findings in which pastoralists did not perceive the products from animal origin to be dangerous (Bashaka, 2015; Mangesho *et al.*, 2017; Swai *et al.*, 2010). However, participants had a focus on the interactions between wildlife and livestock, when they share pasture and water, as a privileged mode of transmission of brucellosis in Kagera Region. Respondents in a study conducted in Uganda also believed that the proximity of livestock to wildlife contributes to the presence of brucellosis (Kansiime *et al.*, 2015).

Participants perceived in this study the impact of brucellosis by underlining the losses of pregnancies in humans and the loss of willing for raising animals after abortion cases. They had also knowledge of the impact of brucellosis on the decrease of milk production and its negative implications on nutritional health problems at the moment of paying the tuition fees for their children. In fact, zoonotic diseases like brucellosis can cause losses with far-reaching social impacts (Ducrotoy *et al.,* 2014). Losses particularly due to brucellosis are remaining to be quantified through epidemiological studies, because abortions due to brucellosis in humans and livestock are not much understood Furthermore, studies on the economic impact caused by brucellosis in livestock are reasonably consistent in different production systems in Africa and Asia (McDermott *et al.,* 2013). Economic burden in pastoralist areas are also due to others infectious diseases, but generally in Africa, in regions where the infection rate can reach 30% for bovine brucellosis, the economic losses are calculated to 5.8% of gross income per animal reared (Domenech *et al.*, 1982).

During the discussions, participants explained their prophylactic practices regarding brucellosis. The key informants from public health were treating suspected cases of brucellosis with doxycycline, specifically those patients who demonstrated long febrile periods; but, they affirmed to do it without any diagnosis protocol. A systematic review on treatment of brucellosis in human for the recent twenty years, concluded with similar data where doxycycline-aminoglycoside combination was the first choice with doxycycline- rifampin and the study recommended doxycycline-cotrimoxazole to be the alternative regimens (Alavi and Alavi, 2013). Treating suspected cases combined with self-medication by people suggests that population of Kagera Region could be exposed to an antimicrobial resistance threat in humans and their livestock. In fact, Tanzania is placed among countries which are in need of standard surveillance of antimicrobial resistance in human and livestock pathogens (Mshana *et al.,* 2013). Diseases can be misdiagnosed in the population because of the absence of diagnostic tools. Furthermore, sound control of diseases requires relevant skills and information about their causes, symptoms and mode of transmission (Lindahl *et al.,* 2015). The results from this study showed that few farmers are individually vaccinating their animals. Despite the difficulties to eradicate brucellosis in field conditions, different vaccines have been applied to control this disease. Efforts are need to sensitize people for mass vaccination against brucellosis which could lead to the control of it zoonotic transmission (Olsen and Stoffregen, 2005). Some participants' reported to use local medicines to treat brucellosis in humans and animals. This practice is shared by smallholder dairy farmers in Pakistan (Arif *et al.,* 2017). Actually, 193 plants are documented in East Africa region to be used by farmers for treating diseases of their livestock including brucellosis (Katerere and Luseba, 2010). However, these practices are sometimes kept jealously by farmers and are transmitted from generation to generation. Moreover, traditional medicine are valuable resources for new agents against antibiotic-resistant strains, and studies have been conducted in this sector (Motamedi *et al.,* 2010; Noudk *et al.,* 2017; Sheng, 1993).

Practices of assisting animals during parturition without any protection and the disposal in the nature of placentas and aborted materials could be associated to the lack of community health education. Moreover, protective gears during assistance of parturition could not be available in pastoral areas; and the limited incomes from small farmers could perpetuate such poor practices. In addition, this behavior can be related to the low risk perception of brucellosis in the communities. Small scale farmers in Tajikistan didn't use any protection when handling cows having an abortion or when dealing with aborted materials (Lindahl *et al.*, 2015). The results from this study pointed also the interactions between wildlife and livestock, poaching activities as potential risk factors for brucellosis infections to humans and livestock. In fact, scholars have documented the presence of brucellosis in wildlife (Fyumagwa *et al.,* 2009; Godfroid, Nielsen *et al.,* 2010; Muma *et al.,* 2010; Waghela and Karstad, 1986; Williams *et al.,* 1993). However, the role played by wild species in spillover of brucellosis to livestock remains to be cleared. In addition, in this study, little was discussed by participants about the mode of transmission, the risk factors or the impact of brucellosis in wildlife in their communities. In reality, experts from wildlife sector could increase the diagnosis and surveillance of prevalent diseases and share the information with the rest of stakeholders in the communities. These interactions may be controlled to minimize the risk as the main reservoirs of brucellosis in the ecosystem are domestic and wild animals which may carry *Brucella* regardless of infection prevalence in the main hosts (Zheludkov and Tsirelson, 2010).

The existence of game reserves on borders of all the neighboring countries with Tanzania was seen as a risk for transmission of brucellosis. In fact there are games reserves like Burigi, Kimisi on part of Tanzania, Ruvubu national Park in Burundi, and Akagera National Park on part of Rwanda where an uncontrolled movement of wildlife species can be observed on borders between those countries. Even though the introduction of brucellosis in Kagera region is not documented, observations from a study stated that the potential impact of a disease outbreak can be amplified by interactions of drivers (Suk *et al.,* 2014). Participants of this study mentioned also the movement of refugees with their livestock in the area, together with an increase of sexual intercourses, consequent to cross border exchanges as potential drivers of brucellosis in their communities. Moreover, the increase in animal product demand can favor the spread of transboundary animal diseases (Otte *et al.,* 2004), including brucellosis.

Some recommendations were addressed specifically to the Government to control brucellosis in their communities. Even though the request of infrastructures for diagnosis of brucellosis were prominent, farmers should act through associations or in their cooperatives where indeed mass vaccination programs can be implemented. Participants converged to solicit the community health education for integrating the management of zoonotic diseases, brucellosis included. Other studies recommended also the increase of knowledge of local communities as a strategy for prevention and control of brucellosis (Bashaka, 2015; Obonyo and Gufu, 2015). Key informants in general advocated for the multidisciplinary collaboration, to establish the status of the disease in the area. Indeed, the health education on zoonosis was indicated in a study, as one branch of collaboration between veterinary and public health services (Ward *et al.,* 1993). A reinforcement of livestock personnel skills at community level was proposed. A study conducted in Uganda underlined the training and recruiting more health personnel, education of the communities about brucellosis diagnosis and vaccination as important gaps for the prevention of this disease in the communities (Kansiime *et al.,* 2015). The exchange of information between the neighboring countries at multidisciplinary level could also increase the risk management and control of brucellosis in the ecosystem.

Study limitations

This study has some limitations based on the fact that the discussions were not directly conducted by the researcher because of language barrier. Even though the facilitator recruited was trained, he got problems to translate to the participants "brucellosis" as a disease and "abortion" as a symptom, because in Swahili, brucellosis is called "Ugonjwa wa kutupa mimba"= "Disease of abortions". During discussions, there were confusions to understand the difference between brucellosis and others abortive diseases in the area. Participants were requesting for more clarifications to understand difference between abortions as symptom and brucellosis as disease. Discussions with key informants were made in groups of three persons instead of independent interviews due to their lack of time. With such approach, participants could influence each other's during their responses. However, the information collected from the Key Informants complemented the knowledge from the rest of participants to this study. In fact, the results from this qualitative study can't be extrapolated to the rest of the population in the Region because, participants were not randomly selected. However, this research was conducted in pastoral communities, where exist strong interactions between humans, livestock and wildlife in an ecosystem located on borders between four countries (Tanzania, Burundi, Rwanda and Uganda), which is the strength for this study.

Conclusion

This study assessed the knowledge and perception regarding brucellosis in pastoral communities of Kagera Region, Tanzania. Focus group discussions and interviews with keys informants revealed a low knowledge, perception and practices of brucellosis in the study area. Participants possessed low knowledge on causes, symptoms and mode of transmission of brucellosis. However, people from these pastoral communities attributed different local names to brucellosis and they were aware that it is pertaining to zoonotic diseases. Despite of their knowledge on the existence of strong interactions between humans, domestic animals and wildlife in the bordering ecosystem, their risk perception of brucellosis is poor due to the neglected and cultural behavior of people in their communities. The improvement of the knowledge and practices regarding brucellosis request a clear community health education program and should involve cross border collaboration with stakeholders in neighboring countries. More researches are needed to elucidate the status of this transboundary disease in the pastoral areas of Kagera Region.

ACKNOWLEDGEMENT S

The authors would like to thank the funders for this work (Government of Burundi, Government of Tanzania through Intra-ACP program). The collaboration from local Government and pastoralists of Kagera Region is also recognized in this paper.

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Porcine Cysticercosis – An emerging neglected food-borne parasitic zoonosis in urban settings in Tanzania: Need for immediate control strategies

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SUMMARY

Porcine cysticercosis (PCC) caused by larval stage of *Taenia solium* is a neglected parasitic zoonosis with significant economic and public health impacts worldwide. Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) ranked it first on the global scale of food borne parasites. In endemic countries, it is an emerging food borne parasitic zoonosis in urban centres where infected pigs from rural areas are slaughtered and consumed. The public is at risk of acquiring the infection due to the fact that meat inspection and control is inadequate in most low income countries. A survey was conducted to establish the status of PCC in pigs slaughtered in Arusha, Dar es Salaam and Mbeya cities; and to assess the existence of possible risk factors for its transmission in Dar es Salaam city. Meat inspection records from official slaughter slabs for the year 2016 and 2017 for Arusha and Mbeya and meat inspection findings in Dar es Salaam for a period of October, 2017 to January 2018 were used. The results revealed PCC infection rate of 1.74% (n= 957) in Arusha, 6.3% (n=766), in Dar es Salaam and 0.27% (n= 35418) in Mbeya, and overall infection rate was 0.68% (=45761). All infected pigs originated from rural areas. Possible risk factors for *T. solium* cysticercosis transmission in Dar es salaam city, as determined through a questionnaire survey, included lack of centralized slaughtering facilities for pigs, inadequate meat inspectors, lack or inadequate meat inspection and control, poor knowledge among butchers and pork vendors about the parasite and possible public health implications. The findings indicated that the public was at high risk of acquiring infection if immediate control measures were not taken. In view of this, it is recommended that pig slaughtering should be centralized for effective inspection and pork control. Lastly, disease control programmes should be formulated using one health approach targeting all actors in pig value chain.

Key words: Arusha, Dar es salaam, Mbeya, *T. solium*, pigs, one health

INTRODUCTION

Taenia solium cysticercosis (TSC) ranked first on the global scale of food borne parasites (FAO and WHO, 2014). High demand for pork in populated centres leads to increased production and transportation of pigs from rural areas where porcine cysticercosis (PCC) is known to be endemic (Satterthwaite *et al*., 2010; Mkupasi, 2017). This creates a room of transporting and slaughter of pigs with PCC in urban areas leading to food borne parasitic zoonosis if enters the food chain (Mkupasi *et al* 2011; Kimbi *et al.,* 2016). The disease causes great economic losses to pig farmers and morbidities and mortalities among humans.

The life cycle of *T. solium* involves human and pigs. Pigs are the principal intermediate hosts, they become infected when ingesting human faeces that contain viable *T. solium* eggs. Humans get infested with the adult tapeworm when eats raw or undercooked pork with infective *T. solium* larvae (Shey-Njila *et al.,* 2003). Human can also act as intermediate host following ingestion of *T. solium* eggs and cause cysticercosis/neurocysticercosis. In the infected person, the eggs develop into cysticerci that are located in the skeletal muscles, subcutaneous and nervous tissues (Schantz *et al*., 1993). Clinical signs of cysticercosis in human depend on anatomical location of the parasite. Neurological and usually fatal clinical manifestations occur when cysticerci lodge in central nervous tissues (Garcia and Del Brutto, 2005).

Porcine cysticercosis is reported more frequently in countries with low socio-economic development, in particular those with low standards of personal hygiene, poor environmental sanitation, traditional pig management, lack of and/or inadequate meat inspection and limited knowledge regarding the life cycle and public health implications of *T. solium* (FAO and WHO, 2014). In Tanzania PCC is prevalent in most pig producing areas. It has been reported in the northern highlands with prevalence ranging from 0.3 - 13.3% in slaughter pigs (Nsengwa and Mbise 1995; Boa *et al.,* 1995) and 3.2 - 46.7% in smallholder pig-farming villages based on tongue examination (Ngowi *et al*., 2004). In southern highlands regions (Mbeya, Iringa and Ruvuma) of the country, prevalence ranging from 5.5 - 16.9% have been reported based on tongue

examination (Boa *et al*., 2001; Boa *et al*., 2006) and 30-32% based on Ag-ELISA (Komba *et al*., 2013). Porcine cysticercosis is also prevalent in Dodoma, the central part of the country with prevalence of 14.9% (Ngowi *et al*., 2014). As in many developing countries, in Tanzania the extent of PCC may be under estimated due to limited disease surveillance, absent or unreliable data and lack of diagnostic facilities (Zoli *et al.,* 2003; Willingham III *et al*., 2010).

Dar es Salaam city and other populated areas are important market for pigs from rural disease endemic areas (Mkupasi *et al.,* 2011). If not monitored, TSC may become a problem in these areas. Infection in human is increasingly being reported also in developed countries, possibly due to increased human mobility (Carabin *et al*., 2011). However, in developing countries data about the disease in human is scant, many cases possibly goes undiagnosed.

Risk factors for PCC transmission in rural area have been extensively studied. Free ranging of pigs and limited use of latrines in a given area are strongly associated with endemicity of PCC *(*Komba *et al.*, 2013*;* Mkupasi and Shonyela, 2017*)*. Poor knowledge of the parasite life cycle may lead farmers to practices like open defecation and selling or eating infected pork which perpetuates disease transmission (Kungu *et al*., 2017). In urban areas where currently pork is highly consumed the status of human infection and possible risk factors have not well described.

Meat inspection is conducted to ensure only disease free meat, wholesome and of no risk to human health enters the food chain. This is accomplished through ante-mortem and post-mortem inspection by qualified personnel. Adequate meat inspection and control is one of the proposed control measures of TSC as it will interrupt the parasite life cycle (Pawlowski *et al.,* 2005). This work was conducted to establish current status of PCC in pigs slaughtered in Arusha, Dar es Salaam and Mbeya cities and assessed possible risk factors for parasite transmission in Dar es sa laam city; the information needed for formulating country wide disease control strategies.

MATERIALS AND METHODS

Risk factors assessment

Information on risk factors for taniasis – cysticercosis transmission was gathered using a check list questionnaire and direct observation.

Study areas and pig population

The study focused on PCC in pigs slaughtered in Arusha, Dar es Salaam and Mbeya cities, Tanzania. Dar es Salaam city is located between latitude $6^{o}46`$ and $6^{o}51`$ S and longitude $39^{o}14$ and 39°18' E. It occupies an area of 162.5 km^2 being the most populated city in the country with 4,364,541 people (NBS, 2013). The city was included due to the reason that it is among the main market of pigs from rural areas all over the country. Arusha city is located in northern highland of Tanzania, with a population of 416,442 plus 323,198 in the surrounding Arusha District; and Mbeya city located in the southern highland of the country with 385279 people (NBS, 2013). The two cities were chosen because they are surrounded with high pig production rural areas which were reported to have PCC and most slaughtered pigs are coming from these endemic areas.

Data collection

Retrospective survey was conducted to establish the status of PCC in pigs slaughtered in Arusha, and Mbeya cities, while in Dar es Salaam prospective survey was performed. To establish status of PCC all official slaughter places in the three study areas were included. In Dar es Salaam city the study was conducted from October 2007 to January 2008. Pigs slaughtered in the dates of visit were inspected and important information was collected for each pig, including the place of origin, sex and inspection findings. In Arusha and Mbeya cities the abattoir records for two consecutive years (2016 and 2017) were retrieved and analysed.

Meat inspection

After the pig carcass was dressed, routine inspection of the head and carcass was done **v**isually followed by incisions made according to the Tanzania and OIE general guidelines for inspection of pig carcasses, which recommend incision of the following muscles and organs; tongue, masseter, heart and triceps brachii to search for *C. Cellulosae* (FAO, 1994).

Butchers (person who slaughter and sell pork) and pork vendors in Dar es Salaam city were interviewed. During interview, questions were translated from English to Swahili. A total of 24 butchers and 40 pork vendors were interviewed in three Districts (table 3 and 4) after received their

oral consent. Targeted pork vendors were those who purchased pork and prepared for public consumption in different pork centers known as "*kitimoto*". The assessed information included the knowledge of TSC transmission, pig carcass inspection, familiarity with PCC and reasons for pig carcass condemnation.

Data analysis

The proportional of PCC infected pigs was determined by computing the number of infected pigs divide by total number of examined pigs in Microsoft Excel 2007.

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RESULTS

Status of PCC in slaughtered pigs

Most pigs examined in the cities came from rural areas. For instance only 12% of slaughtered pigs in Dar es Salaam city were raised within the area. The statuses of examined pigs in the three cities in relation to PCC were as presented in Table 1.

Risk factors assessment

The possible risk factors evaluated in Dare es salaam city for butchers and pork vendors are presented in Table 1 and 2, respectively

Table 1. Total pigs examined using routine meat inspection in the three cites showing PCC infection statuses

City	Pigs examined	Positive for PCC	% positive cases	Period	Type of data
Arusha	9577	167	1.74	2016 & 2017	Retrospective
Dar Es Salaam	766	48	6.3	2007/2018	Prospective
Mbeya	35418	94	0.27	2016 & 2017	Retrospective
Total	45761	309	0.675		

PCC – porcine cysticercosis

Table 2. Participants characteristics on potential risk factors for *T. solium* cysticercosis transmission in Dar es salaam city (butchers n= 24)

Risk factors	Yes (%)	No (%)
Completed formal education	16 (67)	8 (33)
Selling inspected pork	12 (50)	12 (50)
Encountered total condemnation of pig carcass	19 (79)	5 (21)
PCC the cause of total condemnation	12 (50)	12 (50
Heard about PCC	20 (83)	4 (17)
Seen cysticercus cellulosae	20 (83)	4 (17)
Understand how pig acquire the infection	8 (33)	16 (67)
Understand the link between pig and human infections	4 (17)	20 (83)
Knows clinical signs in human	5 (21)	19 (79)

Legend: PCC – Porcine cysticercosis

DISCUSSION

This study has established the prevalence of porcine cysticercosis and possible risk factors for *T. solium* cysticercosis transmission in pigs slaughtered in Arusha, Dares Salaam and Mbeya cities suggesting serious public health risk. In Tanzania, urban centres are the main market for pigs raised in rural areas where PCC is known to be endemic (Kimbi *et al.*, 2016). Importantly, all infected pigs slaughtered in the three cities (Table 1) originated from rural areas. The observed prevalence of PCC in slaughter pigs despite of serious screening of live pigs by tongue examination method done at farm level by traders; indicate high disease prevalence at the source. It has been reported that heavily infected pigs are slaughtered and consumed locally in rural

areas hence perpetuating the parasite life cycle (Mkupasi and Shonyela, 2017).

This underscores the importance of public health education in rural endemic areas for disease control. Although low overall occurrence of PCC has been recorded in this survey, many infected pigs might be entering food chain in urban areas through clandestine market.

Table 4. Participants characteristics on potential risk factors for *T. solium* cysticercosis transmission in Dar es salaam city (pork vendors n=40)

Risk factors	Yes (%)	No (%)
Completed formal education	18 (45)	22 (55)
Buying inspected pork	28 (70)	12 (30)
Heard about porcine cysticercosis	5 (13)	35 (87)
Seen cysticercus cellulosae	3 (8)	37 (92)
Understand how pig acquire the infection	2 (5)	38 (95)
Understand the link between pig and human infections	1 (3)	39 (97)
Knows the clinical signs in human	1 (3)	39 (97)

Again, considering the low sensitivity of the meat inspection method, the observed prevalence should be regarded as an underestimation of the actual prevalence of the infection as also reported in a study conducted in Kenya (Thomas *et al*., 2016). Similarly, abattoir surveys conducted elsewhere have reported the prevalence of PCC in slaughtered pigs with infected pigs originating from rural areas (Kisakye and Masaba, 2002; Phiri *et al.,* 2003 and Zirintunda and Ekou 2016). This reflects the traditional pig management system practiced in rural areas where pigs are raised by smallholder pig farmers facilitating diseases transmission. This means that for effective and sustainable disease control, rural areas should be targeted.

Regarding risk factors, one of the important observed possible risk factor was lack of centralization of slaughtering of pigs. In Tanzania, establishment of pig slaughter slabs / houses has not been given due priority. Emphasis has been given to cattle and small ruminants probably due to religion disease transmission in rural endemic area will continue to occur. In addition, infected pigs may pass through clandestine markets to enter the food chain. To be effective and sustainable control influence as it has been observed in livestock markets establishment (Kimbi *et al.,* 2016). As a result slaughter slabs in the study areas were many, in wrong locations and in poor conditions. The number and scattered locations of slaughter slabs observed in this study affected monitoring and control of slaughtered pigs by few available meat inspectors. This makes implementation of routine meat inspection difficult and should be considered to be an important predisposing factor for taeniasis-cysticercosis transmission in study areas. This will lead to an urban foci or point source of infection, as infected pork may enter the food chain as also reported in Nigeria (Edia-Asuke *et al*., 2014).
Due to these limitations, it was noted that some pork was sold without being inspected because of the difficulties for the few available meat inspectors to be present at each slaughter slab all the time as it was observed in Dar es Salaam city. Consequently, this created opportunities for some butchers to sell infected pork or hide and sell it after the meat inspector has left. This study also revealed that ante mortem inspection was rarely done for the same reason. Difficulties in monitoring slaughter pigs compromise efforts to control meat-borne zoonoses in developing countries as also reported elsewhere (Phiri *et al*., 2003; Zoli *et al*., 2003). If pork preparation before consumption is inadequate it may pose health risk to consumers and public as whole (Mkupasi, 2017).

Furthermore, limited knowledge about *T. solium* cysticercosis to both butchers and pork vendors observed in this study subjected them to practices which promote transmission of the parasite like selling or buying uninspected pork (Table 2 & 3). Similar observations were reported from pig butchers in Nigeria (Edia-Asuke *et al*., 2014). Although were not targeted, few pig traders who were interviewed found to be more knowledgeable about *T. solium* cysticercosis compared to other groups. This means that traders also could be important in controlling the disease as they frequently interact with pig farmers. Education has been reported to be effective in controlling the parasite and other food borne zoonoses (Ghimire *et al.,* 2013). However, targeting slaughterhouses and consumers as the primary intervention point may not be effective in developing countries because inspection and condemnation measures are inefficient as observed in this study. Also the measures should involve all actors in the pig value chain starting form pig keepers. Public engagement and use of one health approach is necessary for effective and sustainable control of TSC.

The present study clearly shows that PCC is common to pigs slaughtered and consumed in the three cities posing high risk to the public health. This indicates that predisposing factors for disease transmission do exist in rural areas where infected pigs originated. High number of slaughter slabs and of poor standards limited pig inspection and pork control by few available inspectors. To safeguard the public health pig slaughtering should be centralized. Lastly, TSC control programs using one health approach targeting all actors in the pig value chain is essential for effective and sustainable control of the parasite to safeguard the public health.

ACKNOWLEDGEMENTS

The financial support by CYSTINET – Africa project (Tanzania I) is highly appreciated. We are also thankful to meat inspectors, butchers and pork vendors for accepting to participate in this study.

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Relationship between faecal egg count and chronic status of liver fasciolosis of cattle in slaughtered in Dar es Salaam, Tanzania

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SUMMARY

Fasciolosis is an economically important parasitic disease of cattle in tropical and subtropical countries that limit productivity of animals. It is caused by *Fasciola hepatica* and *Fasciola gigantica.* Conventional diagnosis of chronic status of liver fasciolosis during postmortem or meat inspection is not a better method for screening the disease. Biochemical diagnostic can be used for screening but it is very expensive to afford the cost. The current sudy aimed to establish the relationship between faecal egg count and chronic status of liver fasciolosis of cattle in Tanzania. A total of 198 cattle were screed for fasciolosis and found to have fasciolosis. During postmortem meat inspection it was found that 183 (93.2%) livers sampled were lightly affected, 6 (3.1%) livers were moderately affected and 6 (3.1%) livers severely affected. Only *F. gingatica* was detected in the moderate and severely affected cattle. The chronic status of fasciolosis based on gross pathological lesion and intensity of lesions of affected liver showed that 183 (93.2%) livers sampled were lightly affected, 5 livers were moderately affected and 4 livers severely affected. In moderate and severely affected livers, the number of *Fasciola* eggs was 9-12 and ≥13, respectively. Cattle from Dodoma were more affected (76.7%) compared to those of from Shinyanga 33 (16.7%) and Singida 13 (6.6%). It is therefore concluded that the relationship faecal egg count and chronic status of liver fasciolosis of cattle could become a better test for screening of the disease of fasciolosis.

Keywords: Chronic liver fasciolosis, soap sedimentation, relationship, diagnosis, feacal egg count.

INTRODUCTION

Fasciolosis is an economically important parasitic disease of cattle in tropical and subtropical countries that limit productivity of animals. It is caused by helminthes in the family fasciolidae, which are trematode of the genus *Fasciola* (Terefe *et al.,* 2012). The two most important species of this genus are *Fasciola hepatica* and *Fasciola gigantica* (Urquhart *et al*., 1996; Keyyu*et al.,* 2005).

The most suitable intermediate host for *Fasciola g*igantica and *Fasciola h*epatica are both species of snails *Lymnae natalensis* and *Lymnae truncatula.* Fasciolosis is a disease of cattle herds and small ruminant flocks that are grazed in wet marshy land area (Brown, 2005). Both species of *Fasciola* have been reported in Tanzania, *Fasciola gigantic*a being the most common (Keyyu *et al.,* 2006) while *Fasciola hepatica* has been reported to occur at Kitulo plateau in southern highlands of Tanzania (Walker *et al.,* 2008).

Essentially fasciolosis is divided into an acute and chronic form (Nzalawahe *et al.,* 2013). Acute fasciolosis is more common in sheep and goats (Mungube *et al.,* 2006). The affected sheep and goats clinically are characterized by anorexia, reluctant to move, distended abdomen which is painful to touch and sudden death (Soulsby, 1982). On the other hand, chronic fasciolosis is the most common form in cattle and is the one detected during the course of meat inspection (Kambarage *et al.,* 1995; Nonga *et al.,* 2009; Komba *et al.,* 2012).

Fasciolosis is commonly recognized as liver flukes infections which cause morbidity and mortality in cattle characterized by weight loss, anemia and hypo-proteinemia, reduced production of meat, milk, and wool, and expenditures for anthelmintics. Fasciolos is remains economically significant parasite of livestock and is emerging zoonotic infection. The total global economic loss attributed to fasciolosis has been estimated earlier to be more than US$3 billion per year (FAO, 1994). In Tanzania, the previous studies reported the prevalence of bovine fasciolosis in Iringa region to range from 17.8% in small scale dairy farms to 94% in traditional cattle farms. This cause an impact of liver condemnations rates for fasciolosis to reach 100% in some slaughter slabs in Iringa region (Keyyu, 2004; Keyyu *et al.,* 2005; 2006). It causes highly economic losses due to liver condemnations (Mellau *et al.,* 2010).

The diagnosis of fasciolosis is comprised with a lot of challenge and some limitation. Essentially fasciolosis has been divided into an acute and chronic form (Nzalawahe *et al.,* 2013). Acute fasciolosis is more common in sheep and goats (Mungube *et al.,* 2006). It is characterized by hepatic fibrosis caused by healing of the liver from

traumatic destruction of liver parenchyma due to migration of immature flukes and hyperplasic cholangitis caused by the presence of adult flukes in bile ducts. The acute and subacute forms of the disease are primarily due to mechanical damage caused by simultaneous migration of immature flukes in the hepatic parenchyma (Müller, 2007). Chronic fasciolosis develops when the adult parasites migrate to the bile ducts and cause cholangitis,biliary obstruction, and fibrosis (Radostits *et al*. 2007).

The conventional diagnosis of chronic fasciolosisis during postmortem by observing gross pathological lesion of the liver is not palatable for screening the disease in live animal. Another method is biochemical diagnostic, also it is very expensive, the farmers cannot afford to pay its diagnostic cost. This study aimed to establish the relationship of feacal egg count and chronic status of the liver fasciliosis in cattle slaughtered in Dar es Salaam, Tanzania.

MATERIALS AND METHODS

Study area, design and animals

This study was conducted in slaughterhouses of three municipalities of Dar es Salaam city namely Kinondoni, Ilala and Temeke. Processing of sample was carried out in Parasitology Laboratory, Tanzania Veterinary Laboratory Agency, Dar es Salaam. The study design was cross-sectional study whereby samples were collected only once. The slaughter cattle originated from different parts of the Tanzania and mostly were local breeds that included Tanzania short horn zebu.

Sample collection

Samples were collected from slaughterhouses in Dar es Salaam city as from June to August, 2017. A total of 198 cattle were sampled. During postmortem examination, livers were taken carefully and examined for the presence of chronic status and or intensity of fasciolosis. The liver flukes species were also being collected for laboratory identification. The chronic status of fasciolosis was being categorized based on gross pathological lesion of the liver described by Ogunrinade and Adegoke (1982). The categories involved: (a) Lightly affected: a quarter of the organ is affected, and only one bile duct is prominently enlarged on the visceral surface of liver; (b) Moderately affected: half of the organ is affected and two or more bile ducts are hyperplastic and; (c) Severely affected: almost the entire organ is involved, liver is cirrhotic and triangular in outline as the right lobe is often atrophied.

Immediately after removal of liver from abdominal cavity of the carcass, the bile duct and gall bladder were incised and the flukes recovered, then each liver was sliced into 1 cm thick and soaked overnight in saline 0.15MNaCl in special jars (45 ml) at room temperature then poured in the Petri dishes ready for examination under stereo microscope. Identification of the fluke species were based on the morphological features and classified in to *F. hepatica, F. gigantica,* mixed and unidentified or immature forms of liver fluke as described by Soulsby (1982).

Coprological examination

Fluke egg counts: Five grams of faecal sample were collected directly from rectum by groved hand then put in the falcon tube, then well packed and transported to laboratory in cool box packed with ice packs. Then two grams of cattle faeces was grinded with mortar and pestle in 40 ml of 5% detergent solution. Then the mixture was filtered through a strain of 300 nm sieve into glass tube and allowed to sediment for 3 minutes. The supernatant was removed by vacuum aspiration and the sediment material rewashed two to three times with the detergent solution and finally with tap water. The sediment stained with one or two drops of 1% methylene blue then examined in gridded Petri dish under dissection microscope (4 x magnifications). Fluke eggs were appeared yellow in colour against the feacal debris, which stained blue. *Fasciola* eggs found were counted and recorded number as EPG (Khallaayoune *et al.*, 1991).

RESULTS

The summary of results is shown in Table 1. During postmortem meat inspection it was found that 183 (93.2%) livers sampled were lightly affected, 6 (3.1%) livers were moderately affected and 6 (3.1%) livers severely affected (Figure 1). Only *F. gingatica* (Figure 2) was detected in the moderate and severely affected cattle. The chronic status of fasciolosis based on gross pathological lesion and intensity of lesions of affected liver showed that 183 (93.2%) livers sampled were lightly affected, 5 livers were moderately affected and 4 livers severely affected. In moderate and severely affected livers, the number of *Fasciola* eggs was 9-12 and ≥13, respectively. Cattle from Dodoma were more affected (76.7%) compared to those of from Shinyanga 33 (16.7%) and Singida 13 (6.6%).

Table 1. Relationship of chronic status and number of eggs count (n=198)

Category of liver infection rates	Number (%) of cattle examined	Number of *Fasciola* egg	Species of *Fasciola*
Light affected	186 (93.8)	No egg seen	-
Moderate affected	6 (3.1)	9-12	*F. gigantica*
Severely affected	6 (3.1)	≥13	*F. gigantica*

Table 2. Relationship original of cattle with type of *Fasciola* species

Original of samples	Dodoma	Shinyanga	Singida
Numbers of cattle	152	33	13
Species of *Fasciola*	*F. gigantica*	*F. gigantica*	*F. gigantica*

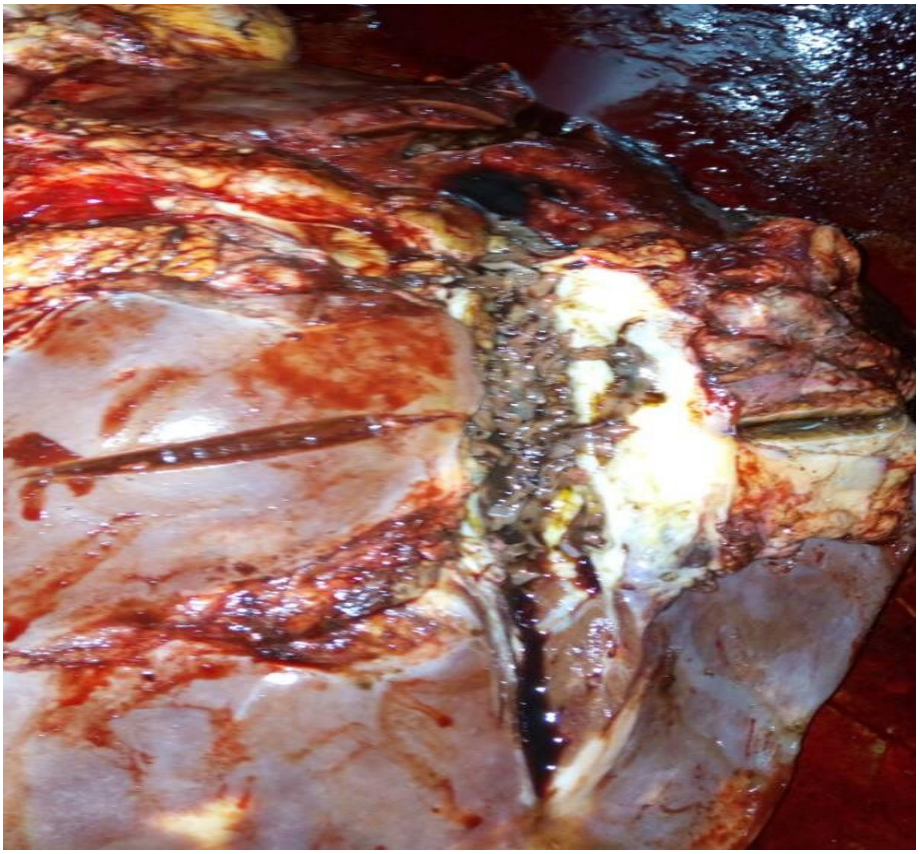

Figure 1. Liver with a chronic form of fasciolosis which is generalized

Figure 2. Recovered *Fasciola* from severely affected liver which were identified as *F. gingatica*

DISCUSSION

Fasciola gingatica was recovered in many of the slaughtered cattle suggestive that fascilosis is still a big problem to cattle. The chronic status of fasciolosis based on gross pathological lesion of the liver, was detected in the liver as described by Ogunrinade and Adegoke (1982). In the lightly affected liver, none of the flukes were recoverd. Most of the flukes were recovered from Moderate and severity affected livers. The average egg fluke count in moderately affected livers had less as compared with the severely affected suggestive that the eggs increase gradually from lightly affected

liver up to, moderately and severity affected of liver. Comparison of diagnostic efficiency of faecal and liver examinations in bovine is direct proportional, in which at low number of eggs, liver is acquire lightly affected while at high number of eggs liver acquired the moderately and severity chronic status.

The sensitivity and specificity of the soap sedimentation technique were calculated from the numbers of positive and negative tests in cattle with and without flukes in their livers, respectively (Smith, 1995). Taking postmortem examination as a gold standard technique for diagnosing *Fasciola* species infection, the similar study the sensitivity and specificity of bovine faecal examination were found to be 69.54% and 100% (Okaiyeto *et al.*, 2008). The presence or absence of *Fasciola* species eggs in the faeces of cattle it can results presence of fluke or in the liver.

The problem of *Fasciola* in Africa because of negligence disease, but it shows the high impact in economic loss due to bovine liver condemnation. The cost associated with condemnations of *Fasciola*-infected bovine livers at Arusha, Tanzania in cattle and sheep, respectively has been reported (Mellau *et al*., 2010). Infected cattle livers were condemned more often than those of sheep. This study also witness high amount of liver condemned in slaughterhouse of Dare es Salaam especially at Vingunguti. At this slaughter facility, liver condemnations are very high, the number of cattle slaughter per day is up 1000 and fasciolosis cases per day are betwen 100 and 200. Normally, cattle are more prone to infections than sheep because of their grazing patterns in wet marshy areas favored by the fluke snail, but sheep prefer to graze away from marshy pastures, if feed is available elsewhere (Blood *et al*., 2007; Boray, 2007).

It is concluded that the current study revealed that the pathological lesions in chronic fascioliasis were proportional to the number of eggs counted by soap sedimentation. The level of infected cattle observed in this study suggests the existence of favorable climatic conditions throughout the year for the development and survival of the parasite in the area of origin. The results of this study indicate that the relationship between faecal egg count and chronic fasciolosis of cattle could become better method for screening of the disease. It is suggested that more study to check the chronic liver fasciolosis relationship with other sedimentation for *Fasciola* have to be undertaken.

ACKNOWLEDGMENTS

The authors are grateful to all staff if different salughter facilities in Dar es Salaam for their assistance during data collection. The laboratory technicians at Central veterinary laboratory, Tanzania Veterinary Laboratory Agency, Dar es salaam are thanked for their assistance during laboratory analysis.

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Comparative effectiveness of Aloe vera aqueous crude extracts and ivermectin for treatment of gastrointestinal nematodes infection in goats

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SUMMARY

The current study was undertaken to determine the effectiveness of Aloe vera aqueous crude extracts in comparison to Ivermectin in treatment of gastrointestinal nematodes infections in goats at Sokoine University of Agriculture in Morogoro. Goats were examined for GIT nematode infections using modified Mc master technique and those with EPG ≥ 150 were recruited for this trial. Furthermore, the recruited animals were randomly allocated into three groups (@10 animals) that included one control group and two experimental groups. The control group was left untreated while the remaining experimental groups were treated with Aloe Vera aqueous crude extracts and Ivermectin respectively. Faecal samples were collected at day of treatment (day 0) and days 14 and 21 post treatment. The effectiveness of the Aloe Vera and Ivermectin was assessed using Feacal Egg Count Reduction Test (FERT). The anthelmintic was considered to be effective when the calculated FECRT% was ≥ 95% and 95% Lower Confidence Limit (LCL) was ≥ 90%. The day 14 post treatment results of FERT% and LCL for Aloe vera were 97% and 74% while for Ivermectin were 96% and 69% respectively. However, the FERT% and LCL results at day 21 post treatment were 100% for both products. The findings of this study indicate that Aloe vera aqueous crude extracts were effective as Ivermectin in treatment of GIT nematodes infections in goats.

Keywords: Aloe vera, Ivermectin, GIT nematodes, Goats

INTRODUCTION

Gastrointestinal nematodes are responsible for causing huge economic losses in goat productivity worldwide. The GIT nematode parasites that have been reported to infect goat in Tanzania include; *Haemonchus contortus*, *Trichostrongylus* spp, *Oesphagostomum* spp (Connor *et al.*, 1990, Keyyu *et al.*, 2002). *Haemonchus controtus* is ranked as the major constraint to goat productivity in Tanzania (Connor *et al.*, 1990). Control of helminthes infections in domesticated ruminants in the country; largely depend on prophylactic or therapeutic use of broad spectrum anthelmintics (Keyyu *et al.*, 2008). The most commonly used anthelmintics for control of GIT nematode infections in goats by farmers in Tanzania include benzimidazoles (Albendazole), macrocyclic lactones (Ivermectin and imidazothiazoles (levamisole).

The use of anthelmintics as a major means of controlling GIT nematode infections in small ruminants is threatened by development of anthelmintic resistance worldwide (Kaplan 2004; Wolstenholme *et al.*, 2004) including Tanzania where albendazole resistant to *H. contortus* in sheep has been reported (Keyyu *et al.*, 2002). The development of anthelmintic resistance necessitates the searching of new effective alternatives against GIT nematodes (Amhed *et al.*, 2013) and traditional medicinal plants are considered as one of the most promising alternatives (Maphosa *et al.*, 2010).

Worldwide, there are several medicinal plants including Aloe vera are tested for their anthelmintic activity (Eguale *et al.*, 2007). In vitro studies on the anthelmintic activity of aloe vera extracts to the GIT nematode infections in sheep and goats have been reported elsewhere in the world (Maphosa *et al.*, 2010, Ahmed *et al.*, 2013). The later studies indicated that aloe vera extracts had larvicidal and egg hatching inhibition effects for *H. controtus* and the authors recommended for invivo studies on the efficacy of the plant. This study was designed to determine the effectiveness of the aloe vera aqueous extracts on treatment of GIT nematode infections in goats compared to ivermectin as a positive control.

MATERIALS AND METHODS

Preparation of aqueous aloe vera extracts

A crude extract was prepared as described by Kaingu *et al.* (2013) whereby fresh aloe vera leaves were chopped using machete and placed in a rotary blender and blended to slurry. The slurry was then squeezed to give out the crude extract viscous juice which was placed in the glass bottles and stored in a refrigerator.

Experimental design

An experimental study design was adopted in this study. In order to get animals that were used in the selection of groups, all goats at Sokoine University Farms were screened for GIT nematode infections using quantitative floatation method (Modified Mc Master technique). The goats with EPG ≥150 were randomly selected and divided into three groups (control group, aloe vera group and ivermectin group) of 15 animals each.

The control group was left untreated and the remaining two groups were treated with aloe vera aqueous extracts and ivermectin respectively. The concentration of the amount of aloe vera aqueous that was administered to the goats was not established but goats were drenched 5 mls of the prepared extracts. The ivermectin was administered subcutaneously at dosage of 0.2 mg/kg body weight.

Data collection and processing

Faecal samples from each group were collected at day of treatment (day 0) and at days 14 and 21 post-treatment). Collection of samples was per rectum using gloved hand fingers that followed by labeling the samples with permanent maker and eventually the samples were kept in cool and then transported to laboratory for further analysis. In the laboratory the samples were processed by Modified Mc Master method and recovered eggs were examined and counted under compound light microscope so as to establish faecal egg counts for each sample. Identification of eggs aided by using standard morphological keys of GIT nematode eggs of goat (Bowman, 2009). Pre-treatment (day 0) and post-treatment (days 14 and 21) faecal samples were pooled for each group and faecal culture, harvesting and identification of larvae was performed as described by Hansen and Perry (1994).

Data analysis

The analysis of the data was conducted using Faecal Egg Count Reduction Test (FECRT %) as described by Coles *et al*., (1992). In this analysis the post-treatment faecal egg counts of treated groups are compared with that of the control group to compute the percentage reduction of faecal egg counts. The anthelmintic considered to be effective when the percentage reduction is ≥ 95% and 95% Lower Confidence Limit is ≥ 90%.

RESULTS AND DISCUSSION

The pre-treatment faecal culture results indicated that goats at Sokoine University Farms were infected with the following species of GIT nematodes: *Haemonchus contortus* (48.4%), *Trichostrongylus* (21.2%), *Cooperia* (14.8%) species, *Oesophagostomum* (14.4%) and *Strongyloides* (1.2%). Similar results have been recorded in the previous studies at SUA farms by Keyyu et al. (2002, 2003).

This study has indicated that aloe vera aqueous leaves extracts was effective as ivermectin in treatment of GIT nematode infections in goats, as the calculated FECRT% at day 21 was > 95% and 95% LCL was > 90% (Table 1). These findings concur with the previous in vitro studies that reported aloe vera extracts were effective against *Haemonchus contortus* (Maphosa *et al*., 2010, Ahmedi *et al.,* 2012) and *Ascaridia galli* (Kaingu *et al.,* 2012).

Table 1: FECRT% and 95% LCL for aloe vera and ivermectin

Treatment group	Days post treatment	FECRT%	95% LCL
Aloe vera	14	97	74
	21	100	100
Ivermectin	14	96	69
	21	100	100

Moreover, the current study has indicated that ivermectin is still effective for treatment of GIT nematodes infections in goats at SUA Farms. These results agree with previous study at SUA farms indicated that ivermectin was effective against GIT nematode infections in sheep and goats (Keyyu *et al*., 2003). However, GIT nematodes resistance to ivermectin in domesticated ruminants has been reported elsewhere in the world (Geurden *et al.,* 2015). This study clearly indicates that aloe vera aqeous crude extracts had good effects on GIT nematode infections as ivermectin. However, more studies are recommended to evaluate the efficacy of the aloe vera, before it recommended for use to the farmers.

ACKNOWLEDGMENTS

The authors would like to acknowledge Higher learning Student Loan Board and the Late Prof. A.A Kassuku for funding the project. Moreover, authors appreciate the great technical assistance from Mr. L.P Msalilwa and R. Manyesela.

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Prevalence of *Leptospira interrogans* in free range domestic duck (*Cairina moschata*) from selected areas of Morogoro Municipality, Tanzania

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SUMMARY

Leptospirosis is described as the most common and universal zoonotic bacterial disease around the world caused by *Leptospira interrogans* which affects different species of domestic and wild animals. The bacteria may occurs worldwide especially in subtropical, tropical, and wet environment with slightly alkaline soil. The current study was conducted between November 2016 and July 2017 in the selected areas in Morogoro aimed at estimating the prevalence and establishes the common serovars of *Leptospira* in free range ducks. A total of 30 ducks from 12 households were used. Before blood sample collection, the duck biodata was recorded and the owner was asked on the general management system including the scavenging areas and any veterinary intervention in place. The ducks were restrained and blood samples were collected from the branchial vein and left to clot before harvesting the serum. Multiple Agglutination Tests was performed using four serovars namely *Kenya*, *Grippotyphosa*, *Pomona* and *Hebdomadis* as reference serovars. The results indicated that all the ducks were local breeds (*Cairina moschata*) which scavenged for fed around homestead. The ducks accessed dumping areas; stagnant water, animal houses and the mud around. No veterinary intervention to ducks was reported by all 12 respondents interviewed. Laboratory results indicated that 5 samples (16.7%) were positive at 1:20, 1:40 for Hebdomadis and Kenya serovars which implies that the ducks had been exposed to the specific *Leptospira* serovars. This study reports for the first time the seroprevelence of leptosirosis in ducks in Tanzania. Because of the zoonotic nature of *Leptospira* and sharing of common environment between ducks and humans, the diseases can easily get access to human. Therefore, in the efforts of surveillance and control of leptosisis in humans and animals, ducks should also be involved.

Key words: Leptospirosis, *Leptospira interrogans*, MAT, serovars, ducks

INTRODUCTION

Leptospirosis is the most common and universal zoonotic bacterial disease around the world caused by pathogenic *Leptospira* called *Leptospira interrogans* (Abela- Ridder *et al*, 2010). In Tanzania *Leptospira* infection has been reported in many different animal species including domestic animals such as cattle, sheep, pigs, horses and dogs (Machang`u *et al*, 1997; Mgode *et al.*, 2006). Leptospirosis is transmitted from animal to human through direct and indirect contact with urine, abortion products and other material contaminated with fluids from infected animals (Bharti *et al.*, 2003). A wide variety of wild animal hosts including rodents, bats, possums, deer, mongoose and small insectivores also have been reported to habour the infection (Bharti *et al.*, 2003; Ellis, 2010). Ducks may come into contact with the *Leptospira* in infected stagnant water and in mud while swimming, passing through contaminated water, drinking contaminated water and coming into contact with urine from an infected animal (Mwachui *et al.*, 2015) and outbreaks of disease mainly occur after heavy seasonal rainfall (Lau et *al.*, 2010).

Leptospira are aerobic, Gram-negative spirochetes, with periplasmic flagella, resembling a question mark when viewed through a light microscope and are slow growing. Traditionally, *Leptospira* were divided into two groups; the pathogenic *Leptospira* were all classified as members of *Leptospira interrogans*, and the saprophytic *Leptospira* were classified as *Leptospira biflexa*, (Mohammed *et al*, 2011; Mety and Dikken, 1993). The first step in the pathogenesis of leptospirosis is penetration of tissue barriers to gain entrance to the body. Potential portals of entry include the skin via a cut, genital tract, and the mucous membranes of the conjunctivae or oral cavity swallowing while swimming in contaminated water (Corwin *et a.l*, 1990; Lingappa *et al.*, 2004; Stern *et al.*, 2010). Thereafter, there is hematogenous dissemination: Pathogenic leptospires make their way into the bloodstream and persist there during the leptospiremic phase of the illness (Stern *et al.*, 2010) there after the spirochetes multiply in the

organs mostly the central nervous system, kidney and liver. The symptoms of leptospirosis develop around 7 to 14 days after exposure to *Leptospira* with mild clinical signs namely; chills, high temperature, sudden headache, nausea, vomiting and loss of appetite, muscle pain and conjunctivitis (Beran *et al.,* 1994).

Diagnosis of leptospirosis in human is difficult based on clinical signs, such that may be misdiagnosed and mismanaged as being mistaken for malaria with similar clinical presentation that might contribute to a high rate of infection in the study area. Methods used in detection of leptospires depend on the availability of resources. Detection of *Leptopisra* infection can be done by the use of Microscopic Agglutination Test (MAT) where dark field microscope is used to detect the agglutination. Other methods are culture, Enzyme Linked Immunosorbent Assay (ELISA), staining method and the Polymerase Chain Reaction (PCR).

Morogoro is among the regions with many livestock in Tanzania. The region is also bordered with several wildlife conservations areas which give lot interactions between humans and animals especially in the interface areas. This gives some possibilities for transfer of different diseases causing agents from wildlife to the domestic environment where domestic animals and humans can easily be infected. The average annual rainfall varies between 600 mm and 1800 mm. The eastern part of Uluguru mountain receive high rainfall of about 2850 mm annually, the leeward side of the mountain are generally dry and receive rainfall of less than 600 mm per year (Msanya *et al.*, 2001**).** The nature of the soil in the valleys of Morogoro is nearly neutral to alkaline (Msanya *et al.,* 2001). Residents of Morogoro Municipality also keep a number of animals including ducks which scavenge all over the home environment in search for food. Studies on Leptospira infection in animals has been done in other animals (Machang`u *et al,* 1997; Mgode *et al.*, 2006) but no any study in domestic birds. The purpose of this study was to estimate the prevalence and establish the common serovars of *Leptospira* infection in free range ducks reared in Morogoro Municipality, Tanzania.

MATERIALS AND METHOD

Study area and population

The study was conducted in Morogoro Municipality which is 190 km west of Dar es Salaam. The study flocks were from different suburbs namely Magadu, Falkland, Kididimo and Vibandani. Selection of study site was based on the convenience of accessibility from Sokoine University of Agriculture (SUA) laboratory and generally represented backyard farming of ducks in urban areas in Tanzania. The study population was Muscovy ducks (*Carina moschata*) managed in the backyard and allowed to scavenge freely during the day within the homestead.

Research design and sampling

The purposive sampling method was used during this cross sectional study design. Sampling involved only farmers who willingly agreed to participate in the study. A total of 12 backyard duck flocks were involved in this study. Before sampling, information on the duck biodata, general duck management system including the scavenging areas and any veterinary intervention in place were gathered from the owners. The ducks selected for sampling were manually restrained and 2 ml of blood sample was collected from the brachial vein on the inside of either wings using syringes and needles. The blood samples in the syringes were left at room temperature for one night to clot. The second day serum was harvested into cryovials and stored until analysis.

Laboratory diagnosis of the *Leptospira interrogans*

Microscopic Agglutination Test (MAT) which is considered as a gold standard method for leptospirosis serodiagnosis was carried out. This test was conducted at the Pest Management Center, SUA, and Morogoro, Tanzania. The MAT test was performed using standard laboratory procedure that aimed at detecting the antibodies reaction with the respective local antigens. Live leptospira representing 4 serovars including Kenya, Grippotyphosa local isolate from domestic animals and rodents found in Tanzania, were used. Other serovars that were used as references were *Leptospira* serovar Hebdomadis and Pomona. These serovars were cultured into fresh *Leptospira* EMJH (Ellinghausen and McCullough, modified by Johnson and Harris) culture medium incubated at 30 ^{o}C for 4 to 10 days before using as live antigen in MAT. Antigen density of 300×10^{8} leptospires/ml was used for MAT.

Serum samples were diluted to 1:10 –1:80 with phosphate buffered saline (pH 7.2) in U–bottomed microtiter plate. Live leptospires antigen (50 µl) were added to diluted serum to give final dilutions of 1:20–1:160. The plates with serum–antigen mixture were incubated at 30 ^{o}C for 2–4 hours

before examining for agglutination of leptospires and antibodies under dark field microscope.

RESULTS

General results

A total of 30 adult ducks from 12 households in four streets were sampled for analysis of *Leptospira* infection (Table 1). The ducks appeared apparently healthy. Interviews with owners indicated that all the ducks were local breeds (*Cairina moschata*). There was no formal feeding, rather ducks are left to scavenge for food and in rare occasions they were given kitchen leftovers. The ducks were scavenged for fed around homestead and accessed dumping areas; stagnant water, animal houses and any kind of muddy environment. No veterinary intervention to ducks was reported by all 12 respondents interviewed.

Serological results

The Microscopic Agglutination Test results are shown in Tables 1 and 2. The seropreplevelence of leptospirosis in ducks was 16.7%. Five sera samples ducks were reactive at 1:20, 1:40 for Hebdomadis and Kenya serovars which implies that the ducks had been exposed to the specific *Leptospira* serovars.

Table 1. Number of ducks from each street

Street	Number of households visited	Number of ducks	Number (%) of positive ducks
Folkland	4	6	2 (6.7)
Kididimo	3	10	3 (10.0)
Vibandani	3	8	0 (0.0)
Magadu	2	6	0 (0.0)

Table 2. Seroprevelence of *Leptospira* infection in ducks

Title	Number (%) of positive sera to different serovars				
	Hebdomadis	Kenya	Pomona	Grippotyphosa	Total number (%)
1:20	2 (6.7)	1 (3.3)	0 (0.0)	0 (0.0)	3 (10.0)
1:40	2 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.7)
Total	4 (13.3)	1 (3.3)	0 (0.0)	0 (0.0)	5 (16.7)

DISCUSSION

This study was carried out to determine the prevalence of leptospirosis in ducks from Morogoro Municipality. The overall prevalence of leptospirosis in ducks was 16.7%. This shows that a relatively high number of ducks were infected with *Leptospira* in the study areas. There has been a belief that leptospirosis is a disease of mammals only but this study shows that birds are also infected. It is still not clear as to whether the ducks were sick from leptospirosis of were just carriers of the infection since all the screened ducks were apparently healthy. Nevertheless, Beran *et al.* (1994) reported that birds like ducks do not develop clinical leptospirosis when are infected with *Leptospira* but rather develop antileptospiral antibodies. Everand *et al.* (1985) reported a seroprevalence of 11% in chickens. This suggests that it is true that poultry are susceptible to leptospira infections. Whatever the case, ducks in Tanzania are always left to scavenge for food around homestead areas and shed their faecal droppings all over and if infected, *Leptospira* can find their way into the food chain and affect humans. This study showed for the first time the role of ducks as reservoir hosts of *Leptospira* in Morogoro Municipality.

It was further found that 10% the agglutination was observed at the titre of 1:20 and 6.7% the agglutination observed at the titre of 1:40. The titres were extended up to 1:160 but the agglutination was not observed implying that the observed positive results indicated acute infection. The ducks had been recently infected or exposed to *Leptosira* and this shows that the pathogen exists either in the soil, other animals around or in humans. In this case, the ducks can be used as bioindicators of existence of *Leptospira* in the locality.

Of the four serovars tested, Hebdomadis and Kenya reacted positive to some samples. This is the

indication that ducks are more likely to be infected by Herbidomadis and Grippotyphosa serovas. Previous studies in Tanzania have reported the two serovars in humans, fish, domestic and wild animals (Machang`u *et al.*, 1997; Mgode *et al*., 2006). Since the two serovars are already in the surroundings, different animal species will be exposed as has been with the case of ducks. The emergence and endemicity of *Leptospira* in Morogoro Municipality may have resulted from the regular high seasonal rain to the area and due to an increase of pastoral population over the recent years (Machang`u *et al.*, 1997; Mgode *et al*., 2006). The nature of the soil in the valleys of Morogoro is nearly neutral to alkaline (Msanya et al., 2001) which give optimal condition for the survival of the *Leptospira* in the environment.

The questionnaire study further supports the laboratory observation on seropositivity of ducks on *Leptospira*. It was observed that ducks scavenge around areas of homestead and also easily access to dumping areas where every kind of rubbish is thrown including domestic animal manure. Ducks were exposed to muddy environment and wetland areas which may potentially serve as sources of *Leptospira* infections in these birds. Therefore, the intensive management system of ducks can help to minimize the unnecessary exposure of ducks to the contaminated areas which will make them to be safe but also for the affected birds, the chances of contaminating the environment becomes minimal.

It is concluded that ducks have been observed to be seropositive of Leptospira infection in Morogoro municipality which further give evidences of existence of this zoonotic pathogen. Efforts should be put in place to confine the ducks so that to minimize environmental shedding of the pathogens and also to protect the birds from *Leptospira* infection.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from the Higher Education Student Loan Board HESLB. Sincere thanks are extended to the farmers for accepting to use their ducks for this study samples. Also grateful to laboratory technicians at SUA Pest Management Center for advice and helping in analysis of duck sera samples.

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Citrobacter as a gastrointestinal pathogen, its prevalence and molecular characterization of antimicrobial resistant isolates in food-producing animals in Morogoro, Tanzania

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SUMMARY

Citrobacter is a gastrointestinal commensal of man and animals. The zoonotic *Citrobacter* spp. infection can occur if food products of animal origin are not hygienically handled. Therefore, the prevalence, antimicrobial resistance profile and resistance transmission mechanism of *Citrobacter* spp. in food-producing animals in Tanzania needs to be understood. *Citrobacter* isolates were recovered from 2.4% of the total of 1099 samples from apparently healthy animals. *Citrobacter* isolates were detected in 3.0% and 1.9% of the swine and the cattle samples, respectively. Over 80% of food products contamination with *Citrobacter* isolates originated from slaughtered cattle carcasses just before meat is transported to retail stores. About 62% of the isolates detected were resistant to at least one antimicrobial, whereas, 38.5% of the resistant isolates were exhibiting resistance to three or more antibiotic classes. All 26 presumptive *Citrobacter* isolates were screened for *invA, intI1* and *16S rRNA*. None of the isolates carried *invA*. Nearly 19% of the MDR *Citrobacter* isolates were found to carry an *intI1*. All *intI1*-positive isolates contained resistance gene cassettes *dfrA1, dfrA7* and *dfrA15*. Detection of resistance gene cassettes in the MDR *Citrobacter* isolates in animals and animal products represents a potential source for horizontal transfer of antimicrobial resistance genes and mobile genetic elements between pathogenic and non-pathogenic bacteria in the microbial population. The findings indicate that animal feces could one of the potential sources for contamination of animal products along the food production chain.

Keywords: MDR *Citrobacter*, Food-producing animals, Antimicrobial resistance, Class 1 integrons, Gene cassettes

INTRODUCTION

Gastrointestinal infection is among the most common infections experienced by humans including the urinary tract and respiratory infections. These infections are among the most common infections in both outpatients as well as hospitalized patients (Sami *et al*., 2017). *Citrobacter* spp. is associated with several infections, such as severe diarrhoea, urinary tract infections and pneumonia in humans with the neonatal meningitis can be fatal with up to 50% mortality rate among infants (Hossain *et al*., 2017).

The members of genus *Citrobacter* are Gram-negative, aerobic or facultative and rod shaped bacteria of the *Enterobacteriaceae* family, widely distributed in water, soil and food (Metri *et al*., 2013). They are known commensals of the intestinal tract of the higher vertebrates including humans and the animals (Liu *et al*., 2016). *Citrobacter* isolates are reported to be the third most common organisms causing urinary tract infections (UTI) in humans after *Escherichia coli* and *Klebsiella* species accounting for nearly 10% of all isolates (Valencia *et al*., 2009).

Detection of *Citrobacter* isolates from hospitalized patients is a common phenomenon. They are emerging as the commonest health care associated multi-drug resistant (MDR) pathogens accounting for 35-40% of the total health care infections, thus posing a serious public health threat (Ranjan and Ranjan, 2013). As reported by Bonadio *et al*. (2001), these organisms are acquired nosocomial pathogens in the health care facilities whose sensitivity to the array of common antimicrobials is dwindling as a result of the antimicrobial resistance due to uncontrolled and indiscriminate antimicrobial usage.

Antimicrobial resistant *Citrobacter* spp. might be introduced into food-producing animals through consumption of contaminated water and feeds, and the microbes may in due course enter the human food supply (Boonyasiri *et al*., 2014). Previous study conducted elsewhere reported a high load of members of *Enterobacteriaceae* family on the carcasses which are a clear indication of failure to realize proper preventive measures in producing safe meat for public consumption (Carrasco *et al*., 2012). The major source of contamination of the cattle carcasses is attributable to poor handling of fecal matters during evisceration, bacterial load on the animal skin, the slaughterhouse personnel and

the equipment used during the slaughter process (Hald *et al*., 2004; Teklu and Negussie, 2011).

The members of the Enterobacteriaceae family such as *Citrobacter* spp. have been reported to acquire antimicrobial resistance through different mechanisms, including mutations in genes and acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer by means of plasmids, transposons, bacteriophages and integrons (von Wintersdorff *et al*., 2016). The growing trend of antimicrobial resistance encountered in human is partly due to acquisition of already resistant pathogenic and commensal bacteria from food-producing animals and the human companion animals (Ohnishi *et al*., 2013).

Use of antimicrobials in livestock farming selects for drug-resistant bacteria and such use of antimicrobials creates ideal selective pressures for the propagation of resistant strains. The presence of *dfr* gene cassettes within class 1 integrons is partly an example of potential source of horizontal spread of resistance among bacteria present in different environments, including livestock, where antimicrobials are used for antimicrobial therapy and prophylaxis of food-producing animals (Mathew *et al*., 2007; Prescott, 2008). Therefore, the purpose of this study was to determine the prevalence, antimicrobial resistance profile and resistance transmission mechanism of *Citrobacter* isolates in food-producing animals in Tanzania which are not well understood.

Study area and sample collection

The study was carried out in Morogoro Municipality, Tanzania which lies between latitudes $5^0$7' to 10^0 0^0 S and between longitudes $35^0$6' - $39^0$5' E and at an elevation of 500 - 600 m above sea level and is about 200 km west of Dar es Salaam. The Municipal has a mixture of warm and cool temperatures ranging between 27 - 33.7^{0}C in the dry and warm season and 14.2 - 21.7^{0}C in cool and wet season. In addition, it experiences a sub-humid tropical climate with a bimodal rainfall pattern characterized by two rainfall seasons in a year with a dry season separating the short rains and long rains (Kashoma *et al*., 2015). Samples were collected from households (farms) with cattle and swine and slaughterhouses in Morogoro Municipal from February 2013 to March 2014. Fecal (n = 304), carcass (n = 148) and milk (n = 187) samples from cattle originating from the agro-pastoral communities, as well as from the dairy cattle farms were collected. Other samples from live animals included swine feces (n = 460) from farms with and without mixed farming. All samples were kept in a cool box before transporting to Sokoine University of Agriculture for further processing.

***Citrobacter* isolation and identification**

Isolation of *Citrobacter* isolates in this study followed the same conventional methods as for isolation of *Salmonella* spp. as described previously (Gebreyes *et al*., 2004). This protocol was used for a reason that the two bacteria namely, *Citrobacter* and *Salmonella* spp. have little dissimilarity in terms of the methods of isolation and identification. Briefly, a 10g portion of each fecal sample were pre-enriched in 90 ml of buffered peptone water (BPW; Becton Dickinson, Sparks, MD) and in addition about 90 ml of BPW was added to each Whirl-Pak bag containing individual carcass and floor drag swabs, and incubated at 37°C for 24 h. A 100 µl of each pre-enriched suspension following overnight incubation was added into 9.9 ml of Rappaport-Vassilliadis (RV) enrichment broth (Becton Dickinson, Sparks, MD) and incubated at 42°C for 24 h. Following overnight incubation at 42°C, a 10 µl of each of the enriched suspension was inoculated onto Xylose-lysine deoxycholate (XLD) agar (Himedia, Mumbai, India) plates and incubated at 37°C for 24 h.

Three isolates from colonies with black centres were selected from each positive sample for biochemical tests. Each selected isolate from colonies with black centres were inoculated onto triple sugar iron (TSI) agar (Becton Dickinson, Sparks, MD) slants, Lysine iron agar (LIA) slants (Becton Dickinson, Sparks, MD) and urea broth (Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 h. Other additional biochemical tests included, citrate utilization, oxidase and catalase tests. As a result of biochemical indeterminacies, the presumptuous isolates were stored at -80°C until further testing using *invA* Polymerase Chain Reaction (PCR) and *16S rRNA* gene sequencing to discriminate *Salmonella* from *Citrobacter* isolates.

Phenotypic characterisation

Citrobacter isolates were tested for antimicrobial susceptibility to a panel of 14 antimicrobials using Kirby-Bauer disc diffusion method (CLSI, 2002). The antimicrobial agents used and their respective disc potencies were as follows: ampicillin (Am; 10 µg/ml), amoxicillin-clavulanic acid (Ax; 30 µg/ml), amikacin (An; 30 µg/ml), ceftriaxone (Ce; 30 µg/ml), cephalothin (Ch; 30 µg/ml), chloramphenicol (Cl; 30 µg/ml), ciprofloxacin (CIP;5 µg/ml), gentamicin (Gm; 10 µg/ml),

kanamycin (Km; 30 µg/ml), streptomycin (S; 10 µg/ml), trimethoprim (TMP; 5 µg/ml), sulfisoxazole (Su; 250 µg/ml), and tetracycline (Te; 30 µg/ml). *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. *Citrobacter* isolates showing resistance to three or more antimicrobial agents were classified as multi-drug resistant (MDR) and those isolates with intermediate resistance profiles were considered susceptible.

Determination of *16S rRNA* and *invA* genes in Presumptuous isolates

Isolates were tested for the carriage of *invA* gene (*Salmonella* invasion gene) using a PCR. Absence of *invA* gene suggests that the isolates are tentatively *Citrobacter* isolates until tested for *16S rRNA* gene. Briefly, the isolates were inoculated onto tryptic soy agar (TSA) plates and incubated at 37°C for 24 h. The genomic DNA was extracted using the Qiagen DNeasy tissue kit according to the manufacturer's instructions (Qiagen Ambion, Austin, TX, USA). Primers used to amplify the *invA* gene included Forward (5'-TCGTCATTCCATTACCTACC-3') and Reverse (5'-AAACGTTGAAAAACTGAGGA-3'). The thermocycling conditions included Hot Start *Taq* activation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and amplification was done in 35 cycles. The reaction mixture was kept at 72°C for 10 min after the final cycle (Hoorfar *et al*., 2000). A total of 26 presumptuous *Citrobacter* isolates were selected for *16S rRNA* sequencing. Primers used for amplification of the *16S rRNA* included 27F (5'-AGAGTTTGAT**YM**TGGCTCAG-3') and 907R (5'-CCGTCAATTC**M**TTTGAGTTT-3') (Mao *et al*., 2012). The PCR amplification conditions were initial denaturation at 95°C for 4 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, and then the amplification cycle was repeated for a further 35 cycles and final extension was done at 72°C for 7 min. Ten microlitres of the PCR product of each isolate tested was electrophoresed on a 1% agarose gel stained with 5 µl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5X Tris borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

Detection of class 1 integron and resistance gene cassettes

The presence of class 1 integron and gene cassettes integrated between conserved segments (5'-3'CS) of class 1 integrons were detected by PCR. Primers used for amplification of the *IntI1* included IntI1-F (5'- GCCTTGCTGTTCTTCTACGG-3') and IntI1-R (5'-GATGCCTGCTTGTTCTACGG-3') (Levesque *et al*., 1995) and those for conserved segments included 5'CS (5'-GGCATCCAAGCAGCAAG-3') and 3'CS (5'-AAGCAGACTTGACCTGA-3') (Ploy *et al*., 2000). The PCR temperature profile included Hot Start *Taq* activation at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and then a final extension step at 72^0C for 7 min (Lindstedt *et al*., 2003). About 10 µl of PCR product of each isolate tested was electrophoresed on a 1% agarose gel stained with 5 µl of 10-mg/ml ethidium bromide for 1 h at 120 V using 0.5X Tris borate-EDTA (TBE) as running buffer. A 1-kb Plus DNA ladder was used as a molecular size marker.

***16S rRNA* and Gene cassettes sequencing**

The PCR products generated for sequencing of *16S rRNA* and variable regions of gene cassettes of class 1 integrons were purified using ExoSAP-IT PCR clean-up method. Briefly, a 5 µl of each of the post-PCR reaction products and a 2 µl of ExoSAP-IT reagent (Miles Road, Cleveland, OH) were mixed together, followed by incubation at 37°C for 15 min and 80°C for 15 min. Following clean-up, a 10 µl of each purified PCR products were pre-mixed separately in the same tube with 5 µl of 5 pMol/µl of each sequencing primers. The pre-mixing and the submission were done according to the organization guidelines (GENEWIZ, South Plainfield, NJ).

DNA sequences analysis

All DNA reverse sequences were converted to match the complement DNA forward sequences using the online Reverse complement software (www.bioinformatics.org/sms/rev_comp.html). The two DNA sequences for each isolates namely, forward and the reverse complement) were aligned using ClustalW2 (www.ebi.ac.uk/clustalw) and trimmed to obtain the consensus DNA sequences. sequence evolution (Kimura 1980; Saitou and Nei, 1987).

Statistical analysis

Data were entered and validated in Microsoft excel 2007 (Ms Corp., Redmond, WA, USA) and analyzed using MedCalc® Version 12.7.1.0 (8400 Ostend Belgium). Descriptive statistics were computed to determine the prevalence of *Salmonella* from different animal species and sample types. In addition, the MedCalc® was used to compare the frequency of antimicrobial resistance of *Salmonella* spp. recovered from different animal species and sample types. A value of $P \leq 0.05$ was considered significant.

RESULTS

Prevalence of *Citrobacter* isolates in Food-producing Animals

Citrobacter isolates were detected from 26 (2.4%) of the total of 1099 feces samples from apparently healthy animals and animal products. The results showed that *Citrobacter* isolates were detected in 3.0% (14 of 460) and 1.9% (12 of 639) of the swine and cattle, respectively. A total of 2 *Citrobacter* isolates (0.7%, 2 of 304), 2 (1.1%, 2 of 187) and 8 (5.4%, 8 of 148) were recovered from the cattle feces, milk samples and the dressed cattle carcasses of Tanzania short-horned Zebu at the slaughterhouses, respectively. *Citrobacter* isolates recovery from swine and cattle samples was not significantly different between swine versus cattle group ($P = 0.3754$).

The proportion of *Citrobacter* isolates detected from cattle fecal samples was significantly lower than in the animal products (milk and carcass) (% difference = 8%, 95% CI, 4.2 to 11.9; $P = 0.0001$). The findings have shown that the two-thirds (66.7%, 8 of 12) of the detected *Citrobacter* isolates originated from the animal products. The odds of recovering *Citrobacter* isolates from animal products were 4.65 times higher than if the isolates were recovered from the cattle fecal samples (95% Confidence Interval [CI] for an odds ratio [OR] of 1.001 to 21.377, $P = 0.049$), indicating that one of the major sources of the contamination of the milk and cattle carcasses is partly attributable to poor handling of fecal matters during evisceration, bacteria on the animal skin, the personnel and the equipment used during milking and slaughter process.

The consensus nucleotide sequences were chimera checked using online DECIPHER software (Wright *et al*., 2012). None of the 28 nucleotide sequences deciphered chimeras, were compared with available databases using the GenBank BLASTN to determine approximate phylogenetic affiliations (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic relationships were inferred by MEGA 6.0 (Tamura *et al*., 2013) using the Neighbor-Joining (N-J) method and the Kimura 2-P model of

Antimicrobial resistance and detection of resistance cassettes in class 1 integrons

According to the disc diffusion, the most common antimicrobial resistance was to cephalothin (46.2%), followed by amoxicillin-clavulanic acid (34.6%), ampicillin (26.9%), tetracycline (19.2%), trimethoprim (15.4%), streptomycin (15.4%), and sulfisoxazole (15.4%). There was no antimicrobial resistance found to amikacin, kanamycin, gentamycin, ciprofloxacin, chloramphenicol, ceftriaxone and ceftiofur. Of the 16 (61.5%, 16 of 26) *Citrobacter* isolates resistant to one or more antimicrobials, 50% (8 of 16) and 31.3% (5 of 16), 12.5% (2 of 16) and 6.3% (1 of 16) of the isolates were detected from the swine feces, dressed cattle carcass swabs, cattle feces and milk, respectively.

Over one-third (37.5%, 6 of 16) of the resistant *Citrobacter* isolates were detected from the animal products (carcasses and milk), 38.5% (10 of 26) of the isolates were pansusceptible and 38.5% (10 of 26) of the isolates were MDR *Citrobacter*. In addition, about 3 of 16 (18.8 %) of the resistant *Citrobacter* isolates amplified *intI1* gene and 100% (3/3) of *intI1*-positive isolates contained resistance gene cassettes known as *dfrA1-orfC*, *dfrA7* and *dfrA15* of size 1250 bp, 800 bp and 700 bp, respectively. The MDR *Citrobacter* isolates which contained the cassettes *dfrA1-orfC*, *dfrA7* and *dfrA15* showed the resistance types AmAxCfSuTeTMP, AmCfSSuTeTMP and AxCfSuTMP, respectively (Table 1).

Characterization of *16S rRNA* of *Citrobacter* isolates

Using MEGA 6 software, the phylogenetic affiliations of the *Citrobacter* isolates [n = 25], from cattle and swine sample types and a few members of the *Enterobacteriaceae* family as out-group members (n = 8) (*Salmonella* Typhimurium strain LT2 [NC003197.1], *Enterobacter asburiae* strain LF7a [NC015968.1], *Citrobacter rodentium* strain ICC168 [NC013716.1], *Salmonella* Anatum strain 315 [JQ694223.1] and *Escherichia coli* strain 64% similarity index. The other members of the *Enterobacteriaceae* family used in this study for comparison were found to cluster as out-growers forming the second clade.

UMN026 [NC011751.1] from the GenBank and two *Salmonella* spp. from a parallel study were inferred. Based on *16S rRNA* sequence analysis, *Citrobacter* isolates and out-group members formed two distinct clades. All *Citrobacter* isolates (n = 26) in the first clade further aggregated to form two clusters. *Citrobacter* isolates (n = 14) were shown to cluster to one of the two cluster at 99% sequence similarity index and the eleven other *Citrobacter* isolates (n = 11) with the out-group members [*Salmonella* Anatum, E. *coli* strain UMN026 (NC011751.1)] at

Table 1. Sequencing of resistant cassettes and 16S rRNA of Citrobacter isolates

Sample ID	Animal	Sample type	R- profile	Accession number	Gene sequenced
C.16746	Swine	Feces	Pansusceptible	KM986871	*16S rRNA*
C.16704	Swine	Feces	Pansusceptible	KM986865	*16S rRNA*
C.16741	Swine	Feces	Pansusceptible	KM986851	*16S rRNA*
C.16744	Swine	Feces	S	KM986852	*16S rRNA*
C.16699	Swine	Feces	AmCfSSuTeTMP	KM823525	*dfrA7*
C.16700	Swine	Feces	AmCfTe	KM986863	*16S rRNA*
C.16708	Swine	Feces	Pansusceptible	KM986872	*16S rRNA*
C.16756	Bovine	Carcass swab	Pansusceptible	KM986870	*16S rRNA*
C.16806	Bovine	Carcass swab	TMP	KM986855	*16S rRNA*
C.16694	Bovine	Carcass swab	AmAxCf	KM986848	*16S rRNA*
C.16702	Bovine	Carcass swab	AxCf	KM986849	*16S rRNA*
C.16693	Bovine	Carcass swab	Pansusceptible	KM986854	*16S rRNA*
C.16715	Bovine	Carcass swab	Pansusceptible	KM986856	*16S rRNA*
C.16790	Bovine	Carcass swab	AmAxCf	KM986859	*16S rRNA*
C.16791	Bovine	Carcass swab	Ax	KM986858	*16S rRNA*
C.16748	Bovine	Feces	AxCfSuTMP	KM823524	*dfrA15*
C.16696	Swine	Feces	Cf	KM986861	*16S rRNA*
C.16739	Swine	Feces	TeSSu	KM986850	*16S rRNA*
C.16679	Swine	Feces	AmAxCfS	KM986847	*16S rRNA*
C.16712	Bovine	Feces	AmAxCf	KM986853	*16S rRNA*
C.16697	Swine	Feces	Pansusceptible	KM986862	*16S rRNA*
C.16774	Swine	Feces	AxCfTe	KM986868	*16S rRNA*
C.16819	Swine	Feces	AmAxCfSuTeTMP	KM823521	*dfrA1*
C.16720	Swine	Feces	Pansusceptible	KM986860	*16S rRNA*
C.16775	Bovine	Milk	Cf	KM986869	*16S rRNA*
C.16778	Bovine	Milk	Pansusceptible	KM986867	*16S rRNA*

Antimicrobials: Ax, amoxacillin-clavulanic acid; Am, ampicillin; Cl, chloramphenicol; CIP, Ciprofloxacin; An, amikacin; Gm, gentamycin; Km, kanamycin; S, streptomycin; Su, sulfisoxasole; TMP, trimethoprim; Te, tetracycline; XLN, ceftiofur; Ce, ceftriaxone; Cf, cephalothin

DISCUSSION

Antimicrobial resistance is becoming one of the global devastating event ever recorded in human history (MacGowan and Macnaughton 2013). It poses a serious public health threat worldwide. The level of occurrence of resistant foodborne pathogens of the *Enterobacteriaceae* family including the *Citrobacter* isolates is increasingly high in developing countries. Since the *Citrobacter* isolates are gastrointestinal commensals of the food-producing animals they can contaminate food products of animal origin if are not hygienically handled (Nayar *et al*., 2014). Thus, the emergence of multidrug-resistant (MDR) foodborne pathogen such as *Citrobacter* isolates in food-producing animals is of public health concern because of the risk of transfer of antimicrobial resistant isolates or the resistance determinants to consumers through the food chain (Baquero *et al*., 2008).

Although *Citrobacter* spp. are less commonly isolated, they are emerging as a common nosocomial multidrug-resistant (MDR) pathogens in Tanzania. In a study conducted by Mshana *et al*. (2009), *Citrobacter* spp. were reported at a

prevalence of 1%, whereas in this study, the *Citrobacter* isolates were detected at a prevalence of 2.4% and a bit higher prevalence than that reported by Ayoyi *et al*. (2017) from pregnant women. Other previous studies in Tanzania and East African region have also reported the prevalence of *Citrobacter* isolates in range of 1-2.5% in humans (Nabbugodi *et al*., 2015; Sekharan *et al*., 2017). This study reports the higher rates of prevalence of *Citrobacter* isolates in the animal products [milk (1.1%) and the dressed cattle carcasses (5.4%)] than Arguello *et al*., 2012). Thus, the strict preventive measures need to be instituted to limit possible contamination of the milk and the dressed carcasses by foodborne pathogens (Funk *et al*., 2001; Kich *et al*., 2011).

Detection of class 1 integrons carrying resistance gene cassettes, namely: *dfrA1-orfC*, *dfrA7* and *dfrA15* of size 1250 bp, 800 bp and 700 bp from *Citrobacter* isolates is certainly one of the very indispensable findings in the current study. The *Citrobacter* isolates from which the three resistance gene cassettes were detected were multi-drug resistant (MDR) isolates containing the resistance types AmAxCfSuTeTMP, AmCfSSuTeTMP and AxCfSuTMP. The involvement of resistance gene cassettes are first reports from *Citrobacter* in food-producing animals in Tanzania. The involvement of class 1 integrons in carrying other resistance gene cassettes for horizontal transmission of resistance genes in bacteria was also reported in MDR *S.* Kentucky in Tanzania. This *aac(3)-Id–aadA7* gene, contained a single cassette array of 1500 bp which was shown to be transmitted by the class 1 integron-mediated MDR *S.* Kentucky (Sato *et al*, 2009). Detection of class 1 integrons in Tanzania was also reported from *Escherichia coli* and *S. enterica* subsp. *arizonae* from a new flock of lesser flamingoes imported from Tanzania to Hiroshima Zoological Park, Japan (Sato *et al*., 2009).

Nearly 62% of the *Citrobacter* isolates detected from the food-producing animals were resistant isolates and almost 40% of the isolates were MDR isolates implying that the magnitude of the antimicrobial resistance in *Citrobacter* isolates is increasingly high and the general public should be concerned of the resistant foodborne pathogens in the food chain. MDR *Citrobacter* spp. is becoming an importance nosocomial pathogen in health care settings. Any entry of such pathogens in immunocompromised and elderly persons, children may lead to life threatening conditions and the treatment options in many cases fail (Scallan *et al*., 2011; Kozak *et al*., 2014; Guerra *et al*., 2016.

in the cattle feces (0.7%). These higher prevalence of *Citrobacter* isolates on the cattle carcasses and milk than in the feces is a clear indication of failure to observe high levels of hygiene in producing safe meat and milk for public consumption. The major source of contamination of the dressed cattle carcasses could be attributed to contaminated slaughterhouse floor, improper handling of fecal matters during evisceration, bacterial load on the animal skin, the personnel and the equipment used (Hald *et al*., 2003; Teklu and Negussie, 2011;

Conclusions

In summary, this study was able to show the level of contamination of food products and the possible attributable causes for such contamination in the food products (dressed cattle carcasses and the milk) if no strict preventive measures are taken to limit the entry of foodborne pathogens in the food chain. This study also reports the involvement of class 1 integron in food products of animal origin and its significance in horizontal transmission of resistance genes among the pathogenic and non-pathogenic bacteria in the animal body or in the environment.

ACKNOWLEDGMENTS

This study was financially supported by Innovative Agricultural Research Initiative (iAGRI), International Programs, College of Food, Agricultural and Environmental Sciences, The Ohio State University.

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Causes of organ condemnations and financial losses in cattle slaughtered at Mahenge slaughter facility in Ulanga District, Morogoro, Tanzania

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SUMMARY

This study was conducted to identify major causes of organs condemnation in cattle slaughtered at Mahenge facility and to estimate the direct financial losses. Study involved antemortem and postmortem examinations to 155 cattle from January to February 2017, and 5 years (January 2012 – December 2016) retrospective data on meat inspection records. During antemortem examination, out of 155 cattle examined, 30 (19.4%) had various abnormalities that included lacrimation (5.8%), pale mucus membrane (3.9%), nasal discharge (2.6%), hernia (1.3%), salivation (1.3%), lameness (5.8%), emaciation (3.9%), depression (1.3%), blindness (1.3%), local swelling (1.9%) and rough hair coat (1.9%). Postmortem examination revealed that 23 (14.8%) liver, 21 (13.6%) lungs, 22 (14.2%) kidneys, 29 (18.7%), 12 (7.7%) were condemned due to various causes. Fasciolosis (13.5%) was the main causes of liver condemnation followed by calcified cysts (6.4%), peritonitis (4.2%) and abscess (2.3%). The major causes of lung condemnation were emphysema, haemorrhages, pneumonia, calcified cysts, abscessation and congestions accounting for 10.0, 7.4, 4.9, 1.6, 0.2 and 0.08%, respectively. The major causes for condemnations of kidneys were congenital cysts (2.6%), hydronephrosis (3.9%) and nephritis (3.3%). About 3,567,000.00 Tanzanian shillings (1621.4 USD) were lost from organs condemnation during the active survey of two months. From the retrospective data it was observed that intestine, liver and lung were the most condemned organs with condemnation rate of 18.7, 14.8 and 13.6%, respectively. The main causes of organ condemnation were (fasciolosis (14%), Emphysema (10%), pimply gut (7.2%), calcified cysts (6.4%), haemorrhages (7.4%) and jaundice (5.5%) respectively. Consequently, the overall direct financial loss during the five years was estimated to be 26,477,000.00 Tanzanian shillings (12,035 USD). This study identified various causes of organ condemnation and their economic importance in the area. Therefore, it is necessary to establish appropriate strategy for prevention and control.

Keywords: Slaughter facility, cattle, organ condemnation, financial loss

INTRODUCTION

Parasitic diseases are considered as a major health problem and cause a significant economic loss in countries where livestock production is an important segment of the agricultural practice. Developing countries have about two third of the world's livestock population but their meat and milk production is less than a third of the world (FAO, 1995). Tanzania ranking the third country with large livestock population in Africa with an estimated of 28.44 million cattle, 16.67 million goats, 5.01 million sheeps,1.85 million pigs, 37.5 million local chickens, 34.5 million exotic chickens, and 0.57 million donkeys respectively (TLMP, 2016). However, there are constraints that hindered the potential of livestock production include; traditional management system, limited genetic potential, lack of appropriate disease control policy and veterinary services. In Tanzania there is no centralized system of carrying out estimations to indicate significant economic losses resulting from condemnation of edible organs and carcass from different slaughter facilities of the country. Nevertheless, some studies are conducted by different researchers but not consistently. Studies done at Dodoma slaughter facility by Tembo and Nonga (2015) estimated financial loss due to organ/carcass condemnations to be Tanzania Shillings (TZS) 15 233 400, equivalent to US$ 9 892. Monitoring and other conditions at slaughterhouse have been recognized as one way of assessing the disease status of cattle (Mellau *et al.*, 2010a) and slaughter facilities played an important role in screening animal products with various abnormalities and diseases that are not fit for human consumption (Alton *et al.*, 2010). Major parasitic diseases such as fasciolosis, hydatid cyst, *Cysticercus bovis* and others causes like abscessation and cirrhosis cause a significant economic loss by lowering the productivity of cattle and condemnation of edible organs (Amene *et al.*, 2012; Hassan *et al.*, 2012). Several studies have been conducted through slaughter facility survey to determine the prevalence and economic loss resulting from organ condemnation in few slaughter facilities of Tanzania in big towns and Cities but not in small Town like Mahenge/Ulanga (Tembo and Nonga 20145; Mkupasi *et al.*, 2010). However, most of the studies were focusing only on specific diseases such as fasciolosis, hydatidosis and cysticercosis bovis. Furthermore, economic loss due various diseases/causes was estimated in some

slaughter facilities of the country (Tembo and Nonga 2015).

Hence, it would be essential to have information on occurrence of various diseases/causes and their economic loss from different parts of the country to establish appropriate strategy for prevention and controls. Currently, there is lack of information on occurrence of various diseases/causes and their economical loss due to organ condemnation in Ulanga. Therefore, the objectives of this study were to identify the major causes of organ condemnation and to estimate direct economic loss due to organ condemnation at Mahenge slaughter facility.

MATERIALS AND METHODS

Study area description

This study was conducted in Ulanga District, which is situated in the southwest of Morogoro region. The District headquarters is Mahenge located 312 km from Morogoro Municipality, 512 km from Dar es Salaam City. It borders with Kilombero District to the West and extends to the North, Liwale District to the East and Malinyi District to the South and West (Fig. 1). It is situated between latitude 9°00" South of equator and longitude 36°40" Eastern of Greenwich. According to the 2012 population and housing census, the district had human population of 265,203 with the growth rate of 2.9% per annum (PHCT, 2013). Administratively, Ulanga District is divided into four divisions namely; Vigoi, Mwaya, Ruaha and Lupiro with 21 wards, 59 villages and 222 hamlets. The economic activities in the district include mining, livestock and crop agriculture, fishing, and game hunting and lumbering. The study was done in Ulanga because it has never been conducted regardless of high levels of carcass/organ condemnations in the Mahenge slaughter facility.

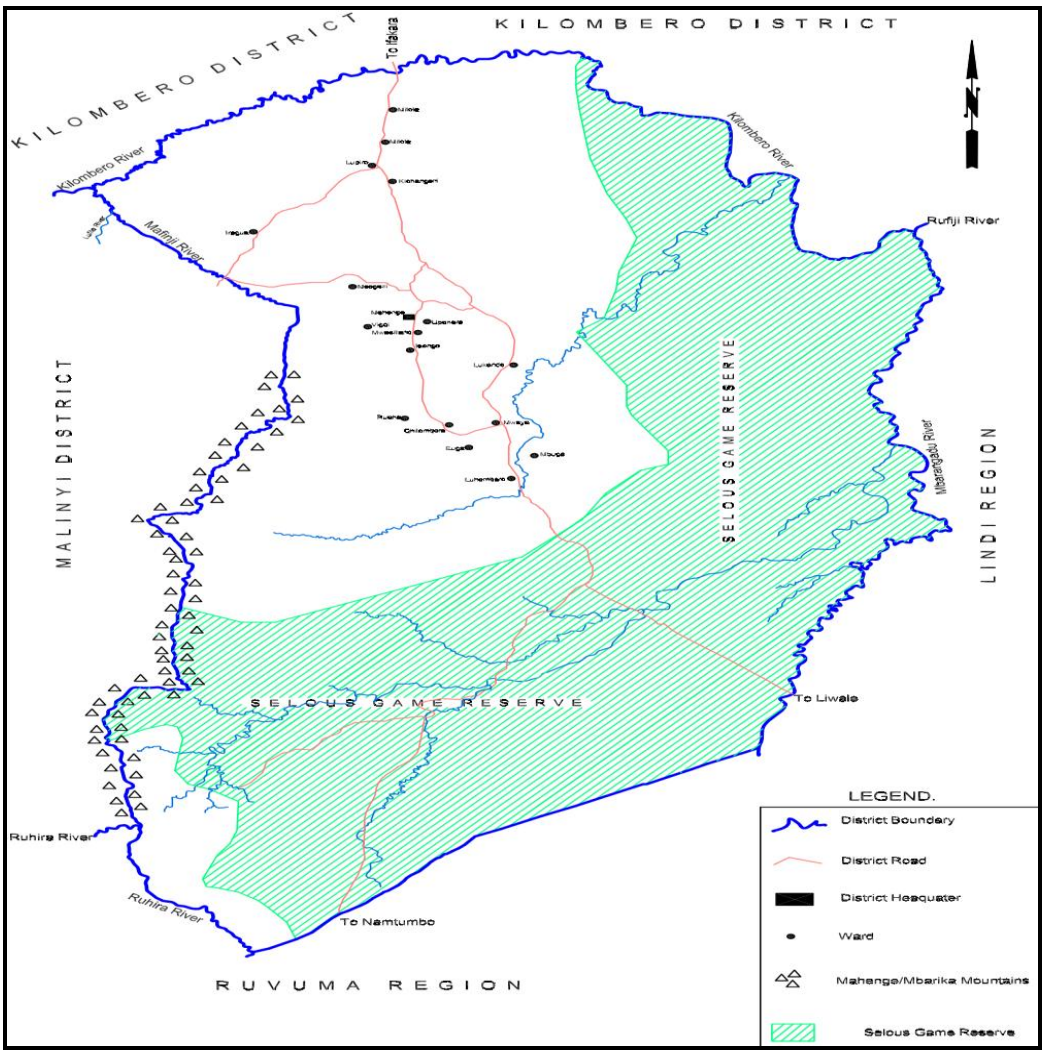

Figure 1. Map of Ulanga district showing boundaries, wards, district headquarters and Selous Game reserve.

Study animals

The study animals were all cattle brought to the District slaughter Facility for slaughter from different areas within Ulanga and Malinyi districts.

Study design

The study was conducted through two study designs involving both active slaughter facility survey and retrospective study.

Active slaughter facility survey

A cross sectional study was conducted from January to February 2017 to identify the major causes of organ condemnation and to estimate the direct economic loss due to organ condemnation in cattle slaughtered in Mahenge slaughter facility located at District Head quarter. A total of 150 cattle were examined by ante mortem and postmortem examinations using standard examination procedures given by FAO (1984). All animals were selected as sample units.

During the ante mortem examination each of study animal was identified based on the enumerate marks on their body marked before slaughter and their general behavior, signs of disease, nutritional status, cleanliness and any type of abnormalities were recorded (Gracey 1986). Judgment was also done based on the procedure given by FAO (FAO 1994). Postmortem examination was conducted through visualization inspection, palpation and systematic incision of each visceral organ particularly the liver, lung, heart and kidney for the presence of cysts, various adult parasites and other abnormalities (Getaw 2010).

Table 1. Abnormalities encountered during ante mortem examination at Mahenge slaughter facility

Abnormalities	No. of infected animal	Percentage
Hernia	2	0.78
Lacrimation	9	3.53
Lameness	5	1.96
Nasal discharge	4	1.57
Depression	2	0.78
Salivation	2	0.78
Local swelling	3	1.18
Pale mucus membrane	6	2.35
Blindness	2	0.78
Emaciation	6	2.35
Rough hair coat	3	1.18
Total	30	17.24

Retrospective slaughter facility study

A retrospective study was conducted using the postmortem meat inspection records of the slaughter facility from January 2012 to December 2016. Data was obtained by help of an experienced meat inspectors and information collected included number of cattle slaughtered, type and number of condemned carcass/organs and causes for each condemnation.

Assessment of direct economic loss

Total number of cattle slaughtered, average current local market price and number of each condemned organ were used to estimate the economic loss represented by the cause related condemnations over the study period. Average current local market price of each organ was obtained from the butcheries in Mahenge town, Ulanga.

Statistical analysis

Collected data were entered and stored in to Microsoft excel and analyzed by statistical methods using STATA 11 (Stata Corporation 2007). Descriptive statistics was used to determine the level of organ condemnation defined as the proportion of condemned organs to the total number of organs examined.

RESULTS

Active slaughter facility survey

Out of 155 cattle subjected to ante mortem examination, 30 (19.35%) were found with various types of abnormalities which includes; lacrimation, pale mucus membrane, nasal discharge, hernia, salivation, lameness, emaciation, depression, blindness, local swelling and rough hair coat (Table1).

Retrospective slaughter facility study

A retrospective data of 3967 cattle slaughtered was collected from January 2012 to February 2016 and it was observed that intestines (28.35 %) was the most condemned organ followed by liver, lung, kidney and spleen with condemnation rate of 21.29%, 13.34%, 9.74% and 4.53% respectively (Table-3). The major causes of organs condemnation were fasciolosis (13.95%), peritonitis (4.21%), calcified cysts (6.37%), pimply gut (7.15%), enteritis (4.1%) and hydronephrosis (3.9%), emphysema (10.0 %) respectively (Table 3).

Assessment of direct economic loss

The total direct economical loss incurred due to organs condemnation during active slaughter facility survey was estimated to be TSh. 3,567,000 (1,621.36USD) (1USD ~ 2,200 Tanzanian shillings by the time when the study was conducted). Higher economic losses were encountered mainly due to condemnation of whole carcass due to jaundice and bruises (TSh. 1,540,000.00) followed by pimply guts in the intestines (TSh. 812,000.00) and fasciolosis TSh. 724,500.00 respectively. From the retrospective study the overall economical loss during five consecutive years was estimated to be 26,477,000.00 Tanzanian shillings which is equivalent to 12,035 USD (Table 3).

Table 2. Financial loss during January and February 2017 prospective period at Mahenge slaughter facility, Ulanga District, Tanzania

Organ/Carcass	Total no. of organs/carcass	Ave. weight of organs/carcass (kg)	Ave. price/kg (Tsh)	Total loss (Tsh)
Carcass	1	220	7,000	1,540,000.00
Livers	23	3.5	9,000	724,500.00
Lungs	21	3.0	5,000	315,000.00
Intestines	29	7.0	4,000	812,000.00
Kidneys	22	0.3	9,000	49,500.00
Aorta	33	0.3	4,000	39,600.00
Spleen	12	0.8	9,000	86,400.00
Total				**3,567,000.00**

Table 3. Retrospective data on causes, percentage of organ condemnation and economic loss at Mahenge slaughter facility from January 2012 to December 2016

Condemned organ	Cause	No. (%) organ condemned	Loss money (TSh.)
Liver	Fasciolosis	554 (14.0)	4,986,000.00
	Calcified cysts	253 (6.4)	2,277,000.00
	Peritonitis	167 (4.2)	1,503,000.00
	Abscess	92 (2.3)	828,000.00
	Others (hepatomegaly, telangiactasis, hepatitis)	291 (7.3)	2,619,000.00
Lungs	Emphysema	397 (10.0)	1,985,000.00
	Haemorrhages	294 (7.4)	1,470,000.00
	Pleurisy	181 (4.6)	905,000.00
	Pneumonia	190 (4.8)	150,000.00
	Calcified cysts	64 (1.6)	320,000.00
	Abscess	6 (0.2)	30,000.00
	Congestion	3 (0.1)	15,000.00
Kidneys	Hydronephrosis	155 (3.9)	1,377,000.00
	Congenital cysts	103 (2.6)	927,000.00
	Others (nephritis, melanosis, necrosis)	129 (3.3)	1,161,000.00
Intestines	Pimply gut	284 (7.2)	1,136,000.00
	Enteritis	163 (4.1)	36,000.00
	Peritonitis	76 (1.9)	304,000.00
	Abscess	7 (0.2)	28,000.00
Spleen	Splenomegaly	174 (4.4)	1,566,000.00
	Peritonitis	2 (0.1)	18,000.00
	abscess	4 (0.1)	36,000.00
Total		3967	26,477,000.00

DISCUSSION

This study revealed that the conditions like fasciolosis, pneumonia, emphysema, hydro-nephrosis, cirrhosis, hepatitis, calcification, abscessation, peritonitis, congenital cysts and pimply guts were the major causes of organs condemnation in cattle slaughtered at Mahenge slaughter facility. This finding is similar with reports from different slaughterhouses in Tanzania (Mellau *et al*., 2010b; Tembo and Nonga 2015). Out of the total examined organs (34.1%) liver, (28.35%) lungs, (8.49%) kidneys, (13.34%) intestines, spleen (4.53%) and (9.72%) were condemned due to various causes.

In the present study, pimply gut was the leading cause of the intestinal condemnation with the prevalence of 18.71%. This was higher than that reported by Mellau *et al.,* (2010b) in Arusha and by Cadmus and Adesokan (2009) in Nigeria. The results indicated that the condition is mostly observed during the rainy than the dry season. Pimply gut caused by *Oephagostomum* helminthes, is prominent in rainy season due to the favourable conditions for increased helminthes activities in the endemic areas. By knowing this epidemiology of the parasites, it is therefore an important time to put forward the strategic effort of worm control so as to reduce loss of valuable meat from condemnation (Tembo and Nonga, 2015).

Liver condemnation due to fasciolosis was also revealed in this study with prevalence of (13.95%). This prevalence was greater than that reported by Tembo and Nonga (2015) in Dodoma slaughter facility which was 4.5%. Again, the study conducted in Arusha by Mwaibonimana *et al.*, (2009) found the prevalence of (6.7%) which was also lower than that of (8.6%) reported in the same area by Mellau et *al.,* (2010). A very high liver condemnation percentage as a result of fasciolosis (up to 30%) has been reported elsewhere by Kamwela *et al.* (2013) in Sumbawanga, and Nzalawahe and Komba (2013) in Kigoma, Tanzania. Studies conducted in Ethiopia and Nigeria (Mohammed *et al.,* 2012; Njoku-Tony 2011) have also revealed higher occurrence of fasciolosis. This shows that fasciolosis is a large burden in cattle in most African countries. Although fasciolosis rarely cause mortalities in cattle, its effects result in reduced production and condemnation of livers during meat inspection in slaughter facilities (Kambarage *et al.* 1995). Generally, in the prevalence was observed to high due to the nature of climatic condition of Ulanga of high annual rainfall and many water bodies which harbor the vectors for fasciolosis.

Kidneys accounted for (9.74%) of all organs and/or carcasses condemned. The main causes for kidney condemnation were congenital cysts and hydronephrosis. The results for hydronephrosis were two times greater than those reported by Mellau *et al.* (2011) in Arusha, who found a prevalence of 1.9%. Renal calculi, nephritis, necrosis and melanosis were other causes of kidney condemnation although at very low rates. Both hydronephrosis and renal calculi were observed more often in the dry seasons. This was probably as a result of scarcity of water for animals during dry as in Ulanga there are no water reservoirs which could assists animals in particular season.

Pimply gut (oesophagostomosis) was the leading cause of condemnations of cattle intestines in the current study. Incidence was lower than reported by Mellau *et al.* (2011) in Arusha and by Cadmus and Adesokan (2009) in Nigeria. In addition, the results indicated that pimply gut was observed more during the wet than the dry seasons, presumably as a result of favourable conditions for increased helminths activity in endemic areas. It is therefore imperative that control of helminths is stepped up during the wet season in order to reduce wastage of valuable meat due to condemnations. This study reported the rejection of intestines to be higher than the other organs although liver ranked the second.

Lungs are perhaps the organs most exposed to physical, chemical and biological injuries and this is because of their anatomical and histological characteristics (Tembo and Nonga, 2014). This is supported by the findings of the current study, which revealed that (28.5%) of all condemned organs were lungs. This was higher than the results obtained in Arusha by Mellau *et al.* (2011). Elsewhere, in Ismailia, Egypt, Ahmed *et al.* (2013) reported that lungs contributed up to 44.6% of all condemned organs. This is in agreement with findings by Mellau *et al.* (2010b), who reported that emphysema had contributed 13.1% to organ condemnations at Arusha slaughter facility in Tanzania. Ruminants, particularly cattle, have well-developed interlobular septa and lack of collateral ventilation, making them more susceptible to interstitial emphysema (Mellau *et al.,* 2010b). Pulmonary emphysema is associated with diseases such as East Coast Fever; it may also be caused by obstruction of airflow or by extensive gasping respiration during the slaughter process (FAO, 1994). Improper stunning, delayed slaughter after stunning and delayed hoisting after slaughter may

also have contributed to the high number of lungs with emphysema and haemorrhages (personal observation). Exposure of animals to stress factors like dust, overcrowding and exhaustion from long treks in search of pasture and water during the dry season may also contribute to respiratory conditions (Kusiluka and Kambarage, 1996).

Results from the present study revealed that the estimated financial loss resulting from organ and carcass condemnations in the prospective survey for two months (January and February 2017) was around TSh. 3,567,000 (1,621.36USD) (1USD ~ 2,200 Tanzanian shillings by the time when the study was conducted). Higher economic losses were encountered mainly due to condemnation of whole carcass resulted from jaundice and bruises (TSh. 1,540,000.00) followed by pimply guts in the intestines (TSh. 812,000.00) and fasciolosis in the liver which was TSh. 724,500.00 respectively. From the retrospective study the overall economical loss during five consecutive years was estimated to be 26,477,000.00 Tanzanian shillings which is equivalent to 12,035 USD. Similar study done by Tembo and Nonga (2015) has shown the condemnation of liver alone to be TSh.5, 953,200, although the greater different was due to sample size differences.

This liver condemnation due to fasciolosis is comparable with results obtained in Rukwa by Kamwela *et al.* (2013). Similarly, Mwabonimana *et al.* (2009) found financial loss as a result of fasciolosis to be $1169 in Arusha. The annual financial loss in the present study was comparable with results obtained from a study conducted in Nigeria (Cadmus and Adesokan, 2009), which indicated an annual financial loss of $110 968. This loss of revenue by farmers, traders and the livestock industry has serious financial implications and a negative impact on the socio-economic wellbeing of those involved in the livestock value chain. Therefore, disease control strategies should be implemented strictly.

The present study revealed that fasciolosis, pneumonia, emphysema, hydronephrosis, calcification, congenital cysts, peritonitis, onchocercosis, abscessation and pimply gut were the major causes of organs condemnation in cattle at Mahenge slaughter facility resulting in considerable economical loss in cattle production. Eradication of these diseases requires cooperation between the public health and official veterinary authorities. Public health education to avoid eating of raw meat, proper disposal of condemned organs, cattle management system, treatment of animals with anti-helminthes drugs and grazing management of animals during dry season to avoid access of the animals to the parasites eggs are important. In addition to this, proper and detail meat inspection at the slaughter facility are also recommended.

ACKNOWLEDGEMENTS

The authors extend their gratitude to the District Executive Director for kindly allowing this study to be conducted in the District. Furthermore, appreciations should reach the slaughter facility management, meat inspectors and entire staff of the Ulanga District Livestock Department for their valuable advice and assistance in the enhancement of this research.

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carcasses and organs in short-horn Zebu cattle

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Zulu, V., Mwanza, A.M., Banda, F.C., Yasuda, J. and Yoshida, M. Cattle reproductive wastage in Zambia: A case of Mongu slaughter facility', *Bull Facult Agricult, Kagoshima Univ* 63, 49-54, 2013.

Caudal mediastinal abscessation in an adult East African black headed ewe -A case report

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SUMMARY

A 4-year-old East African black headed ewe was presented to Sokoine University of Agriculture Animal Hospital with a history of loss of body condition and respiratory distress. Clinical examination revealed poor body condition, severe leukopenia with neutrophilia and lymphopenia. Radiographic examination of the thorax revealed an elongated caudal mediastinal mass with soft tissue opacity dorsal to caudal vena cava and caudal to the carina. The ewe was humanely euthanised due to deteriorated body condition. At post-mortem examination the mass contained caseous creamy material with early laminated appearance surrounded by a whitish fibrous capsule suggestive of chronic abscessation of caudal mediastinal lymph node. *Corynebacterium pseudotuberculosis* was isolated on bacterial culture of pus sample. A diagnosis of an abscessed caudal mediastinal lymph node as a result of *Corynebacterium pseudotuberculosis* infection was made. Thoracic radiography should be considered in sheep with chronic progressive emaciation and respiratory distress. Further, in sheep with caudal mediastinal mass, caseous lymphadenitis should be considered as one of the differential diagnoses.

Keywords: sheep, thorax, radiography, mediastinum, caseous lymphadenitis, *Corynebacterium pseudotuberculosis*

INTRODUCTION

The mediastinum is the space between the lungs. It extends from the thoracic inlet to the diaphragm (Thrall, 2007). The mediastinum may be divided into a cranial portion cranial to the heart, a middle portion at the level of and containing the heart, and a caudal portion caudal to the heart (Thrall, 2007). Further, it may also be divided into dorsal and ventral portions by a dorsal plane through the tracheal bifurcation (Thrall, 2007). Organs and structures within the mediastinum include; the oesophagus, trachea, thymus, nerves, lymph nodes, thoracic duct, heart and great vessels (Baines, 2008).

The most common sites for mediastinal masses are the cranial ventral and perihilar, however can occur anywhere within the mediastinum (Baines, 2008). There are various causes of mediastinal masses such as abscess, neoplasia, hernia and spirocerca lupi (Thrall, 2007). Mediastinal lymph node enlargement which is associated with various conditions is one of the most common causes of mediastinal mass (Thrall, 2007). Clinical signs such as dyspnea, coughing, exercise intolerance, and regurgitation may be observed in animals with mediastinal mass depending on the size and position (Baines, 2008).

Being one of the difficult areas to examine clinically, diagnostic imaging plays an important role in the detection and investigation of mediastinal abnormalities and for initial screening radiography is the imaging modality of choice (Baines, 2008). Two orthogonal views of the thorax i.e. the lateral and dorsovental (DV) or ventrodorsal (VD) views should be used to assess the mediastinum. The VD or DV view allows full assessment of the position of the mediastinum and is more sensitive for definitive diagnosis of mediastinal mass. The lateral view allows evaluation of many of the normal visible structures but does not allow assessment of mediastinal shift (Baines, 2008).

This case report on abscessation of the caudal mediastinal lymph node in East African black headed ewe as a result of *Corynebacterium pseudotuberculosis* infection.

Case presentation

A four-year-old, 15 kg East African black headed ewe was referred to Sokoine University of Agriculture Animal Hospital with a history of loss of body condition and respiratory distress of two months duration. The ewe was treated with procaine penicillin and dihydrostreptomycine sulphate (Pen & Strep®, Norbrook, Ireland) but failed to improve significantly.

At referral, clinical examination was performed and radiography of the thorax was done. Clinical examination revealed; poor body condition,

decreased rectal temperature (37.0°C, reference 39.1 ± 0.5), tachycardia (126 beats/min, reference 70-80 beats/min), and respiratory distress. Haematological parameters showed severe leukopenia (0.02, reference 4.0-12.0 x 10^9/L) with neutrophilia (93.8%, reference 10.0-50.0%) and lymphopenia (6.2%, reference 40.0-75.0%). Mild anaemia (RBC: 8.42, reference 9.0-15.0 g/L; PCV: 26.9%, reference 27.0-45.0; Hgb: 8.3 g/dl, reference 9-15 g/dl) was also observed. Thoracic radiographs revealed a single, well marginated, bilobed and elongated caudodorsal mediastinal mass dorsal to caudal vena cava and caudal to the carina (Figures 1 and 2). The mass had soft tissue opacity and measured 14.0 cm in length, 5.5 cm in height and 5.3 cm in width (Figures 1 and 2). Because of deteriorated body condition the ewe was humanely euthanised by intravenous injection of pentobarbitone (Euthapent®, Kyron laboratories [Pty] Ltd, South Africa).

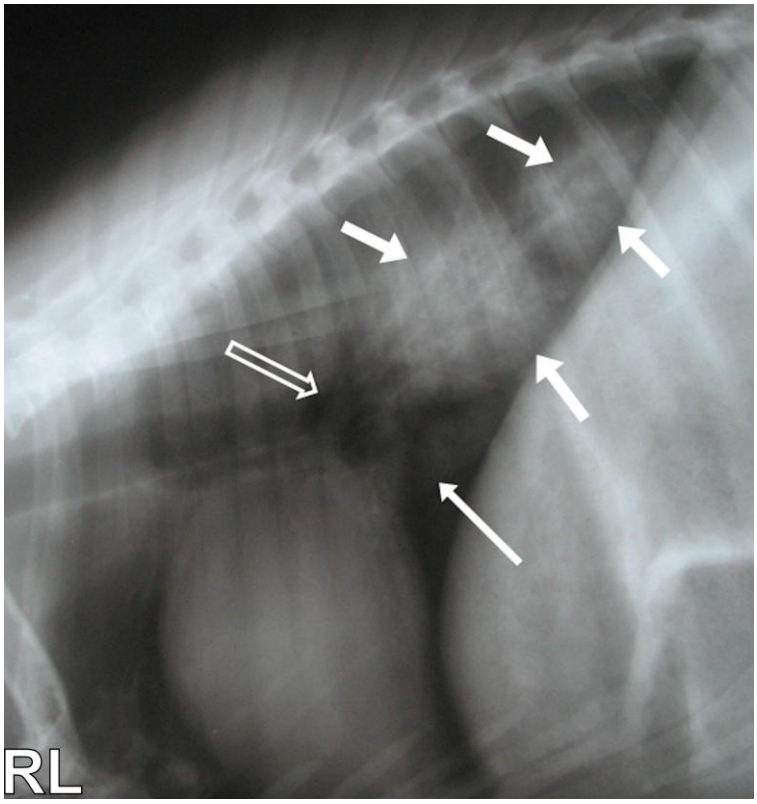

Figure 1. Right lateral thoracic radiograph of a four-year-old East African black headed ewe. Note the presence of an elongated bi-lobed mass with soft tissue opacity in the caudodorsal mediastinum (white arrows). The caudal vena cava and carina are indicated by a thin white arrow and an open white arrow, respectively. RL- right lateral.

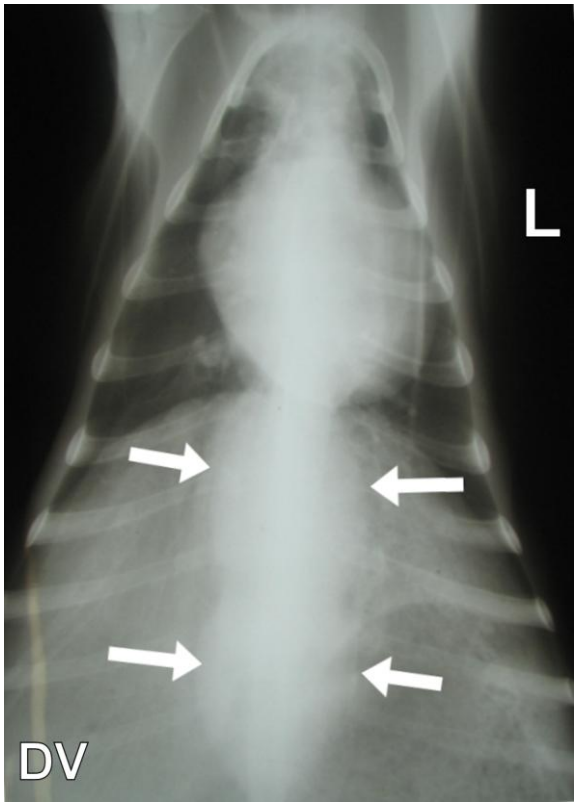

Figure 2. Dorsoventral thoracic radiograph of a four-year-old East African black headed ewe. The caudal mediastinal mass (white arrows) is superimposed with the spine and cranially is in contact with the caudal margin of the cardiac silhouette.

The necropsy confirmed the presence of an elongated bilobed mass in the caudal mediastinum ventral and to the right of the aorta between the caudal lung lobes (Figure 3). Adhesions were also observed between the mass and the lungs (Figure 3). The mass contained caseous (cheese-like) creamy material with early laminated appearance surrounded by a whitish fibrous capsule (Figure 4) and was interpreted to be an abscessed caudal mediastinal lymph node. Pus sample from the abscess was submitted for bacterial culture and *Corynebacterium pseudotuberculosis* was isolated.
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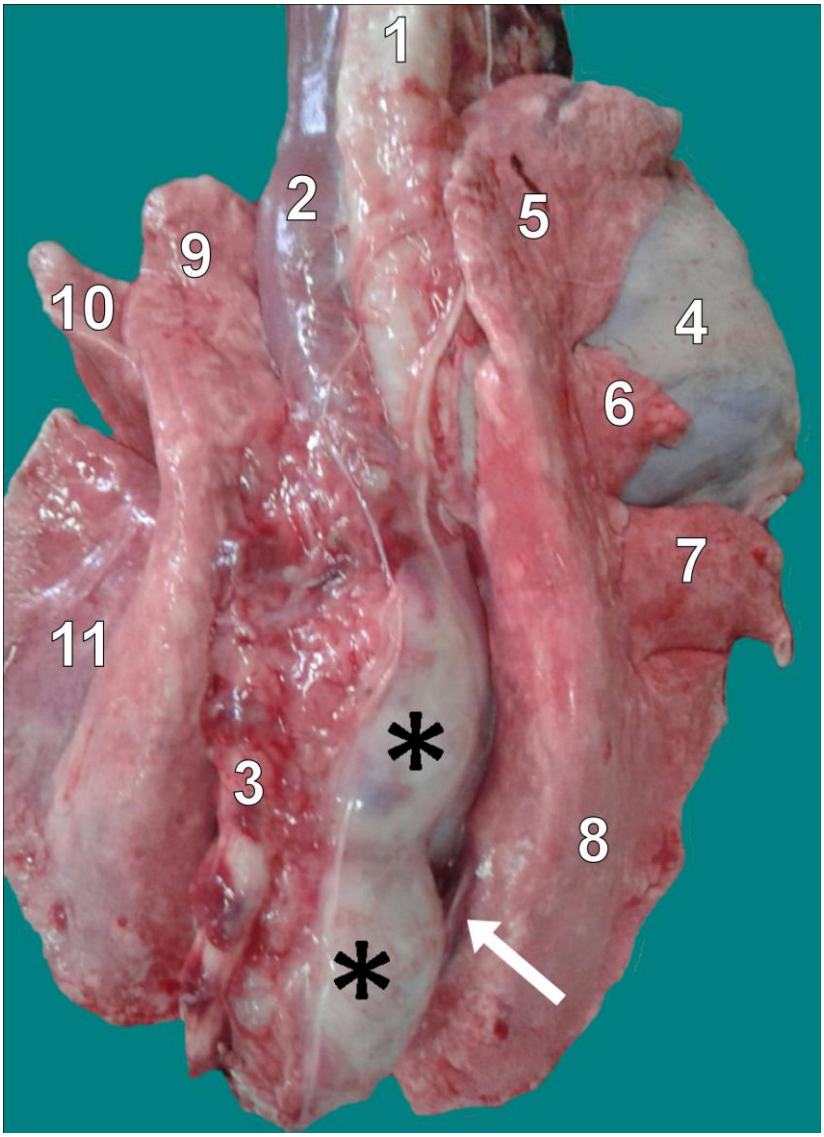

Figure 3. Dorsal photographic view of a gross specimen of lungs of a four-year-old East African black headed ewe. An elongated bi-lobed mass (asterisk) is seen between the medial surfaces of the caudal lung lobes (8, 11) and ventral to the right of the aorta (3). Note also the presence of adhesion (white arrow) between the mass and the right caudal lung lobe. 1, trachea; 2, oesophagus; 3, aorta; 4, heart; 5, cranial part of the cranial lobe of the right lung; 6, caudal part of the cranial lobe of the right lung; 7, middle lobe of the right lung; 8, caudal lobe of the right lung; 9, cranial part of the cranial lobe of the left lung; 10, caudal part of the cranial lobe of the left lung; 11, caudal lobe of the left lung.

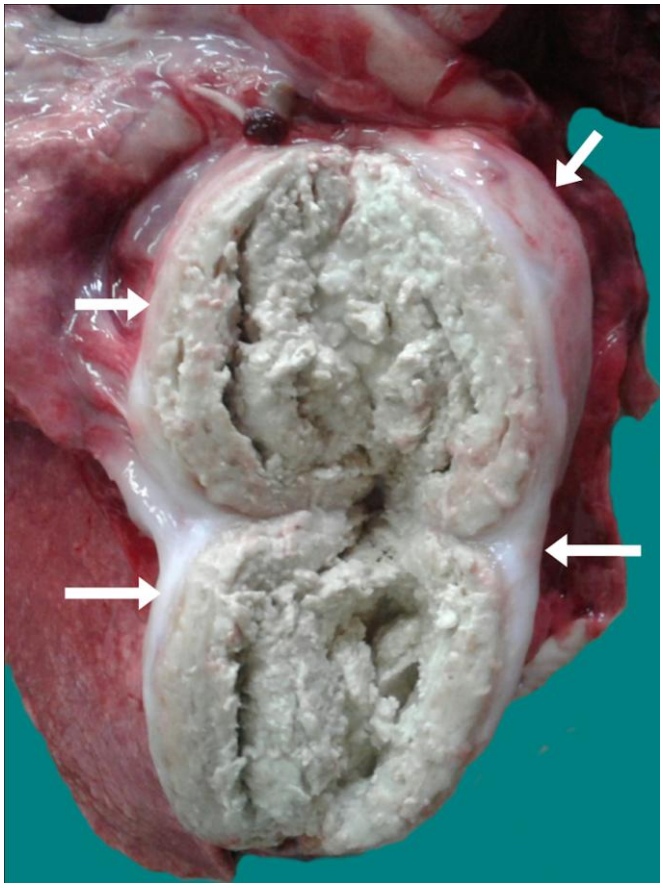

Figure 4. Dorsal photographic view of an opened elongated bi-lobed mass. Note the presence of caseous cream material with laminated appearance surrounded by a whitish fibrous capsule (white arrows).

DISCUSSION

Caseous lymphadenitis presents in two forms; namely external and internal (Baird and Fontaine, 2007; Fontaine and Baird, 2008; Serres*et al.*,2011; Williamson, 2001). The internal form, also known as visceral form is characterised by development of abscesses in internal lymph nodes (mediastinal, bronchial and lumbar) (Baird and Fontaine, 2007; Fontaine and Baird, 2008; Serres *et al.*, 2011; Williamson, 2001) as it was observed in this case. Abscesses may be also seen in other viscera such as the lung, liver, kidneys, spleen and uterus (Williamson, 2001; Baird and Fontaine, 2007; Fontaine and Baird, 2008; Serres *et al.*, 2011). It is more common in sheep (Williamson, 2001) and the principal location of the lesions is the lung parenchyma and mediastinal lymph nodes (Baird and Fontaine, 2007). In latter, abscesses may become so large and put pressure on the oesophagus, interfering with swallowing and rumination leading to chronic ill-thrift (Baird and Fontaine, 2007)

In sheep, the prevalence of caseous lymphadenitis has been reported to be significantly higher in ewe than in rams (Al-Gaabary*et al.*,2009) as it has been encountered in this case. Due to the chronic nature of the disease, it was postulated that the disease was commonly observed in ewe as a result of being kept for many years compared to rams, which are slaughtered at a young age (Al-Gaabary *et al.*, 2009). It is most likely that the abscessed caudal mediastinal lymph node interfered with respiration, which led to respiratory distress in this case. Furthermore, the pressure exerted by the abscess on the oesophagus interfered with rumination and swallowing, which lead to chronic ill-thrift. Chronic progressive emaciation and respiratory distress have been reported by several authors in sheep with visceral form of caseous lymphadenitis involving the thoracic cavity (Baird and Fontaine, 2007; Oreiby *et al.*, 2015). Leukopenia, neutrophilia and lymphopenia that have been observed in the present case are the result of chronic inflammation and a response to stress (Benjamin, 1978). Neutrophilia and lymphopenia have also been reported in an ewe with internal form of caseous lymphadenitis (Serres *et al.*, 2011).

There are few reports on the radiographic appearance of visceral form of caseous lymphadenitis involving the thoracic cavity in ewes (Williamson, 2001; Oreiby *et al.*, 2015). The visceral form of caseous lymphadenitis involving the thoracic cavity has various radiographic

appearances depending on whether there is a single or multiple abscesses. In one case with a single abscess; a radiopaque mass was seen over the heart base (Oreiby *et al.,* 2015). Williamson (2001) reported the presence of multiple pulmonary nodules in the dorsal lung field in an ewe with multiple abscesses.

The caseous creamy material with early laminated appearance, which was observed in an abscessed caudal mediastinal lymph node in this case, is almost similar to other reported cases of caseous lymphadenitis in small ruminants (Williamson, 2001; Baird and Fontaine, 2007; Leask*et al.,*2013).

Thoracic radiography should be considered in sheep with chronic progressive emaciation and respiratory distress. Further, in sheep with pulmonary nodules or mediastinal mass caseous lymphadenitis should be included in the differential diagnosis.

ACKNOWLEDGEMENTS

The authors would like to thank Mrs Sinao Kingongo and Mr Antony Mhando for their assistance during radiographic examination.

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Bacteriological assessment of chlorinated and non-chlorinated water in Morogoro Municipality, Tanzania

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SUMMARY

This cross sectional study was conducted to assess the bacterial contamination in chlorinated and non-chlorinated water in Morogoro Municipality from October 2013 – January 2014. Fifty two samples were collected from selected taps (chlorinated samples) and directly from the water sources (non -chlorinated samples). The total viable count (TVC) was performed on nutrient agar while the total coliform count (TCC) was done by Most Probable Number (MPN) using MacConkey broth. It was found that the TVC ranged between 530 CFU/100 mL and 600 CFU/100 mL during dry season and the same range during rainy season. The TVC ranged between 530 CFU/100 mL and 600 CFU/100 mL during dry season and 600 CFU/100 mL to 650 CFU/100 mL during rainy season. The results for TCC positive chlorinated samples during dry season ranged between 3.6/100 mL and 150/100 mL while during the rainy season it ranged between 15/100 mL and 150/100 mL. The highest TCC were found at Department of Animal Science and Production in both season for chlorinated water and 41% of non- chlorinated water during dry season while all the samples of non-chlorinated water during rainy season had high TCC count. Also the results further showed that TCC for positive non- chlorinated samples during dry season ranged between 210/100 mL and >1100/100 mL while it was >1100/100 mL during rainy season. In chlorinated water, significant difference (p=0.332) was observed when TVC during dry season was compared with that of rainy season. Based on the results of this study, it is concluded that chlorinated and non- chlorinated water show high number of TCC than that recommended by WHO and Tanzania Bureau of Standards (TBS). However, there was low TVC which was within recommended standards. The high TCC observed in treated water in this study may pose a risk of acquiring water-borne diseases to the Morogoro community.

Keywords: Bacteria, coliform, tap water, Morogoro Municipality.

INTRODUCTION

Water is vital to life and an important requirement to all kinds of living organism. Humans use water for drinking and other domestic uses, thus such use of water needs water which is clean and safe. Otherwise water may be the source of different health risks including biological, chemical and physical hazards. For example, cholera outbreak in Dar es Salaam has always been caused by contaminated water (WHO, 2011). Again several other effects like people with mottled teeth in Northern and Central Tanzania is caused by high level of fluorine in water (Yoder *et al.*, 1998). Such water contamination problems may be caused by natural causes or human related activities; the latter has a significant contribution to incidences of waterborne diseases.

In many developing countries, diarrheal diseases remain a major killer in children. Estimates by WHO and UNICEF (2004) indicate that 80% of all illnesses in developing countries is related to water and sanitation; and that 15% of all child deaths under the age of 5 years in developing countries are caused by diarrheal diseases (WHO and UNICEF, 2004; Thompson and Khan, 2003). In Tanzania for example, water-borne diseases contributed up to 10% of all diseases during 1985 (Jiwa *et al.*, 1991). A number of water-borne diseases including diarrhoea, dysentery, typhoid and cholera have been reported in Tanzania (Temu *et al.*, 2007; Penrose *et al.*, 2010; Mahende *et al.*, 2015; Chuma *et al.*, 2016).

Despite the Tanzania government's efforts to provide safe and adequate potable water to the majority of the population, water-borne diseases are still the problem. Diarrhoeal diseases and all forms of gastroenteritis average at 12% in Morogoro region (NBS, 2005). Interestingly, diarrhoea in children in Morogoro Municipality is reported to be up to 57.2% (Oketcho *et al.*, 2012). The most common problems of water-borne diseases are those

caused by infectious organisms like bacteria, protozoa, virus and helminthes. These microorganisms cause water-borne diarrheal diseases, including salmonellosis, campylobacteriosis, amoebiasis, shigellosis, cholera, or giardiasis are widespread in areas with contaminated water (Thompson and Khan, 2003; WHO and UNICEF, 2004; Grabow, 1996; Chuma *et al.*, 2016). Use of contaminated water, poor sanitation and poor hygiene causes up to 88% of diarrhoeal deaths around the world (Black *et al.*, 2013).

As means of control of the problems caused by contaminated water, authorities responsible with water supply have different means of water treatment. The most common method for water treatment in developing countries is chlorination (WHO, 1997). The method is effective, cheap and user friendly and is recommended by WHO as a solution to water-borne diseases in developing countries. This however disinfects water against bacteria, but chlorine is not effective against viruses, protozoa and helminthes (WHO, 1997). Other water treatment methods like flocculation and biological filtration may be useful but resources and infrastructure limitation in most developing countries has become obstacles to put these in use.

According to the census of 2012, Morogoro Municipality has the human population of 352,904 and demand of clean drinking water is 40,755m^3/day (MORUWASA, 2013). The community depends on piped water from Morogoro Urban Water Supply and Sanitation Authority (MORUWASA) which outsources the water mainly from Mindu dam and rivers like Morogoro river. Therefore 85% of all water provided to the Municipality comes from MORUWASA, 5% have own water sources, and the remaining 10% fetches water direct from streams, rivers and locally dug wells. Chlorination is the only means of water treatment practiced in Morogoro Municipality still there are several water-borne diseases. NBS (2005) and Oketcho *et al.* (2012) found that diarrhea cases are at high rate despite water treatment by chlorination this implies that water treatment methods used may not be effective. Yet there has been no study which tried to assess the effectiveness of water chlorination in Morogoro water treatment plants. This study was conducted to assess the bacterial load and coliform count in chlorinated water supplied in Morogoro Municipality.

MATERIAL AND METHODS

Study area

The study was conducted in Morogoro Municipality which covers 260 km^2 and has a population of 352,904 people (PHC, 2012). The Municipality is located in the Eastern part of Tanzania, 169 kilometres west of Dar es Salaam, the country's largest city and commercial centre, and 223 kilometres east of Dodoma, the country's capital city. The Municipality is supplied with water from different water sources such as Mindu dam, Morogoro river and independent Sokoine University of Agriculture (SUA) water supply. With this study the representative treated tap water from each source was used.

Water sample size and collection

Twenty four chlorinated water samples from the randomly selected taps supplied by three different sources (Mindu dam, Morogoro river and SUA independent source) were collected for analysis. Of these, 12 were collected during dry season and 12 during rainy season. Sample was collected from the representative tap at LITA, and Folkland (Morogoro river supply), Kichangani and Mafiga (Mindu dam source), Collegeof Veterinary Medicine and Biomedical Sciences and Department of Animal Science and Production (DASP) from SUA Independent water supply). Before water sample was collected from the tap, the tap openings were sterilized using flame and then water was allowed to run for 3 minutes. Then 500 mL of water sample was collected into sterile glass bottle and immediately the sample was placed in the cool box with ice packs.

To get the true picture on what is in the water sources, 28 un-chlorinated water samples from the selected sampling points at the three water sources (Mindu dam, Morogoro river and SUA independent source) were collected for analysis. The approach to sampling was done as described by WHO (1984). Briefly, the sterile sample bottle was opened and sunk at approximately six inches below the surface of the water. A total of 500 mL of water sample was collected leaving an air space of 2.5 cm close to the rid and immediately the sample was placed in the cool box with ice packs. Fourteen samples were collected during each of the dry and rainy seasons.

Therefore, a total, 52 water samples were collected for the whole study. Subsequently after the field work of each day, the water samples were being shipped to the University Microbiology laboratory for analysis within 24 hours after sampling.

Laboratory sample analysis

Total viable count (TVC)

TVC of water samples was determined as described by Harrigan and McCance (1976) and TZS (2007) in a such way that normal saline (9 mL) was added in ten test tubes arranged in a single row. Then 1 mL of water sample was added in each test tube above, followed by serial dilution where 1 mL was transferred from one test tube to another and the last 1 mL was discarded. The diluted samples were inoculated in the Petri dishes that contain nutrient agar and incubated at 37^{o}C overnight. The TVC on the plates was performed using a protocol described previously (ISO 721, 2007). Colony forming units (cfu) were counted on at least two critical dilution plates by the aid of colony counter. Two consecutive plates with 15 to 300 colonies were considered for record (ISO 4833:2003(E)). The countable colonies were converted into the mean colony forming units per millilitre (CFU/ml) using a formula: $N= \Sigma C/ (n_1+ 0.1n_2)d$ where N = the number of bacteria counted, C = sum of colony counted in two successful dilutions, n_1 = the number of dishes retained in the first dilution, n_2 = the number of dishes retained in the second dilution and d = dilution factor corresponding to the first dilution (ISO 4833:1991(E)).

Total Coliform Count (TCC)

Most Probable Number (MPN) method was adopted for determination of TCC. Nine test tubes with the Durham tubes in inverted position were arranged in three rows. The first row test tubes were added with 10 mL of double strength MacConkey broth media whereas the second and third rows were added with single strength media MacConkey broth. In the test tubes of the first row, 10 mL of the sample was inoculated where as in the second and third row a test tube 1 mL and 0.1 mL of the sample was inoculated respectively. The inoculated tubes were incubated at 37ºC over for 24 hours. Tubes with positive results were indicated by the change in colour of the media from pink to yellow and the formation of gas in the Durham tube. The MPN tables for 3 rows tubes were used to report the result of the MPN of coliform bacteria per millitre of water (WHO, 1984; Harrigan and McCance, 1976).

Data analysis

The data was analysed using Microsoft Excel Spread sheet 2010 to obtain Central tendency and One way Analysis of Variance (ANOVA) was used to compare the results of TCC and TVC in each season. Significance differences were observed at $P < 0.05$.

RESULTS

Results for assessment of bacteria load in chlorinated water by MPN and TVC methods are shown in Table 1. The results showed that TCC for positive chlorinated samples during dry season ranged between 3.6/100 mL and 150/100 mL with mean value of 47.2/100mL while it ranged between 15/100 mL and 150/100 mL with mean value of 40.5/100mL during rainy season. The highest coliform count was found at DASP in both seasons for chlorinated tap water. The results show no statistical significant when TCC during dry season was compared with TCC during rainy season (P=0.731). Also the results for TVC ranged between 530 CFU/100 mL and 600 CFU/100 mL during dry season and have the mean value of 561.6 CFU/100mL and the same range during rainy season with mean value of 572.5 CFU100/mL. Though the results show statistical significant when two seasons were compared (P=0.332).

Table 1. Bacteriological results of TCC and TVC in chlorinated water (tap water) during dry and rainy seasons

Water source	Tap water sampling sites	Dry season		Rainy season	
		TCC/mL	TVC/mL	TCC/mL	TVC/mL
Morogoro river	LITA 1	0.092	5.5	0.23	5.8
	LITA 2	0.036	5.3	0.20	5.6
	Folkland 1	0.15	5.6	0.43	5.9
	Folkland 2	1.5	6.0	0.20	5.4
Mindu dam	Kichangani 1	0.20	5.9	0.21	5.3
	Kichangani 2	0.23	5.3	0.23	6.0
	Mafiga 1	0.43	5.9	0.15	5.8
	Mafiga 2	0.21	5.8	0.93	5.6
SUA independent water supply	FVM 1	0.28	5.7	0.23	6.0
	FVM 2	0.11	5.0	0.20	5.7
	DASP 1	1.5	5.8	0.35	5.6
	DASP 2	0.93	5.6	1.5	6.0

Additionally, assessment of bacteria load in non-chlorinated water is shown in Table 2. The results showed that TCC for positive non-chlorinated samples during dry season ranged between 210/100 mL and >1100/100 mL while it was >1100/100 mL during rainy season. No statistical difference was observed when the TCC results of three sites were compared in each season. Also, it was established that the TVC ranged between 530 CFU/100 mL and 600 CFU/100 mL during dry season and 600 CFU/100 mL to 650 CFU/100 mL during rainy season. The TVC was also statistically insignificant between sites and season.

Table 2. Bacteriological results of TCC and TVC in non-chlorinated water during dry and rainy seasons

Water source	Sampling sites	Dry season		Rainy season	
		TCC/mL	TVC/mL	TCC/mL	TVC/mL
Morogoro river	Point 1	>11	6.3	>11	6.5
	Point 2	11	6.2	>11	6.3
	Point 3	>11	6.1	>11	6.1
	Point 4	2.1	6.0	>11	6.0
Mindu dam	Point 1	11	6.0	>11	6.2
	Point 2	11	5.9	>11	6.3
	Point 3	>11	6.2	>11	6.4
	Point 4	>11	6.3	>11	6.2
SUA independent water supply	At 0700h	>11	6.2	>11	6.2
	At 1000h	11	6.3	>11	6.3
	At 1300h	11	6.1	>11	6.1
	At 1600h	2.1	6.0	>11	6.4

DISCUSSION

The purpose of this study was to assess bacterial contamination in chlorinated tap water during both dry and rainy season. The sampling of water from the selected water taps was done from October 2013 to January 2014. It was found that all the water samples (chlorinated) contained coliform contrary to the standard by WHO (0 count per 100 mL) suggesting that water was likely contaminated with faeces. This predisposes the water consumers to dangers of waterborne pathogens. Also this call for thorough water treatment by the responsible authority and the water consumers should be educated to further treat the water at household level. The study found that during the dry and rainy seasons the TCC was high in chlorinated water which exceeded the standard according to WHO (0 count per 100 mL) and TBS which is 0 to 10 counts per 100 mL. This is an interesting finding since it was expected that chlorinated water would have no or very low counts of coliform bacteria. Similar findings was also noted by Shayo *et al.* (2007) in small community supplies in Kingolwira village in Morogoro, that faecal coliform ranging from 0.93 x 10^3 to 2.1 x 10^2/100 ml. The possible causes of high TCC in chlorinated water can be caused by several factors including presence of new contaminations that were likely due to leakages in the pipes in the pipeline distribution network.

In addition, inefficiency chlorination process may be another possibility for the high coliform count. It is suggested that for chlorination to be effective, water pH should be less than eight, turbidity of less than 5 Nephelometric Turbidity Unit (NTU) and 0.5 mg/L free chlorine residues for 30 minutes as contact time. Nevertheless, detection of bacteria in chlorinated water may be caused due to presence of suspended particles. Studies show that majority of the bacteria in water are attached to particles which act as a shield against chlorine. Microbes entrapped in particles or adsorbed onto surfaces are shielded from disinfection and are not inactivated by the process of chlorination. A study by Wolfe *et al*. (1985) reported presence of coliforms and faecal coliforms in chlorinated water. Again a study by Ridgway and Olson (1982) showed that the majority of viable bacteria in chlorinated water were attached to particles. All these are evidences that not always the chlorinated water is free from microbial contaminations.

Another finding of this study is the observed high TCC in non-chlorinated water. Under general situation contamination may occur due to natural and human related causes. The natural causes include nature of the water catchment area may predispose the water source to contamination. Natural water runoff that may cause floods and erosion enhances contamination from the upland areas. The other natural cause of water contamination is decomposing organic matter and faeces of wild animals. Meanwhile, the human related activities give effluent discharges which find their way to waterbodies and play a big role in water contamination. Such effluents may be municipal and industrial wastes, stormwater runoff and infiltration from waste disposal sites and animal wastes (Chapman, 1996). Mindu dam catchment areas and valleys of Morogoro river have a lot of human activities which are likely to be sources of water contamination which was observed in the current study (Mdegela *et al.,* 2010).

Furthermore, the results of TVC shows the highest count was 650 CFU/100 mL which is within the recommended standard by WHO (TVC should not exceed 1000 CFU/100 ml at 37°C). However, comparison of TCC and TVC for rainy and dry seasons showed no statistical significant ($P>0.05$) meaning that levels of contamination was almost the same during the two seasons. Although TCC and TVC recorded during the rainy season were slightly high. During the rainy season, there is a lot of surface run off which may carry all the contaminations from uplands to water bodies like dams and rivers. Nevertheless, the dry period is also associated with high use of water for irrigation and other human related activities which may expose the water bodies to contaminations. Also, during the dry season, there are low levels of water in the water bodies that may lead to concentration effects of the contaminants that even a minor contamination is detectable. All these account for a lack of differences in levels of bacterial contamination noticed during the rainy and dry seasons in the study areas.

Based on the results of this study, it is concluded that chlorinatedand non-chlorinated water show high number of TCC than that recommended by WHO and TBS. However, there was low TVC which was within recommended standards. High TCC poses a risk of acquiring water-borne diseases to the Morogoro community. Since high bacterial contamination was recorded in both chlorinated and non-chlorinated water, the community in Morogoro municipality should retreat the tap water. The water authority, MORUWASA should recheck the chlorination process they do to water and try to be doing routine water quality monitoring to ascertain the quality of water they supply to their clients.

ACKNOWLEDGEMENTS

The authors are thankful to the laboratory technicians Mr. N. Lukiko and Ms.S. Mgonja of the Department of Veterinary Medicine and Public Health (Sokoine University of Agriculture) for their technical assistance. Drs. J. Julius and I.B. Kashoma are thanked for kindly providing some laboratory materials support. This study was funded by Higher Education Student Loans Board (HESLB).

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Assessment of anticholinesterase contaminants in selected sites of Ruvu river in Tanzania using cholinesterase biomarker in African Sharptooth Catfish (*Claria gariepinus*)

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SUMMARY

Increase in human activities led to environmental contamination of physical, biological and chemical nature. Some of the contaminants can be determined using biomarkers such as Cholinesterases; Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) activities. A cross section study was performed to assess the anticholinesterase contaminants in Ruvu river using cholinesterase biomarker in African Sharptooth Catfish (*Clarias gariepinus*). A total of 40 fish was used to collectblood plasma and brain samples for AChE and BChE activities assessment. Ellman's methodand 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) chromophore were used during enzymes activities determination. The findings of AChE and BChE activities in blood plasma and BChE activities in brain showed no statistical significant difference ($p > 0.05$) among the fish from the various study sites and between fish from the study sites and the control. Also, no statistical significant difference in the brain AChE was observed among the fish from the study sites. However ($p < 0.05$) was observed in fish from Ruvu Darajani when its AChE activities were compared with that of control fish. The percentage of AChE inhibited was higher in brain than in blood plasma whereas the inhibition of BChE activities was higher in blood plasma than in the brain of fish from Ruvu Darajani.Fish samples from Ruvu darajani were observed to have highest inhibitions for both the brain AChE and blood plasma BChE activities (31.7% and 13.4%respectively).This indicates that Ruvu Darajani is highly contaminated with anticholinesterase compounds than other areas. Therefore, this study revealed that activities of AChE and BChE in *C. gariepinus*are useful biomarker for assessing aquatic environmental contaminants caused by anticholinesterases.

Keywords: Anticholinesterases, *Claria gariepinus*, Biomarker, Ruvu river, Tanzania

Agriculture plays a major role in Tanzania's economy. Its contribution accounts for more than 50% of the total Gross Domestic Product (GDP) (URT, 2015). The rapid human population growth in Tanzania like in any other developing countries in the world has led to an increase of food demand. As of 2002 census, the population of Tanzania was reported to be 37 million (URTNC, 2002) and currently is reported have increased to 45 million (URTNC, 2012). This increase in human population goes along with increase in crop production (Mati, 2005). Also, high occurrences of pests and diseases had led to increased indiscriminate use of pesticides and other agrochemicals in agricultureof which some has anticholinesterase properties (Ngowi *et al.*, 2007). Most pesticides target the family of enzymes (Cholinesterases; ChEs) formed by Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) (Nicolet *et al.*, 2003). ChEs are enzymes that hydrolyze the acetylcholine released at central and peripheral sites of the nervous system (Çokugras, 2003).

The AChE is synthesized in hematopoietic tissues and occurs in the brain, endplate of skeletal muscle and erythrocyte membrane, and its main function is to regulate neuronal communication by hydrolysing the ubiquitous neurotransmitter acetylcholine in synaptic cleft (Quinn 1987; Silman and Sussman, 2005).On the other hand, BChE is synthesized in liver and is highly present in blood plasma, smooth muscle, pancreas, adipocytes, skin and heart (Cokugras, 2003). Butyrylcholinesterase is pointed out as one of the main detoxifying enzymes able to hydrolyze or scavenge a broad range of xenobiotic compounds (Cokugras, 2003; Nicolet *et al.*, 2003).Furthermore, AChE enzymes can be inhibited by heavy metals, polycyclic aromatic hydrocarbons, detergents and other components of complex mixtures of contaminants as identified by Lionetto *et al.* (2004); Jebali *et al.* (2006); Vioque-Fern´andez*et al.* (2007). Also, the potential of some metallic ions, such as $Hg^{2+,}$ $Cd^{2+,}$ Cu^{2+}, and Pb^{2+}, to suppress the activity of AChE *in vitro* and/or *in vivo* conditions has been demonstrated in several studies in humans and animals (Frasco *et al.*,2005;

Ademuyiwa *et al.,* 2007). These contaminants cannot be easily detected by chemical analysis because of their relative short life in the environment, thus the use of biomarkers particular cholinesterase activitiesis an alternative method that provide links between the presence of chemicals in the environment and their biological effects in organisms (Chambers *et al.*, 2002; Reinecke, 2006).

Several animal species have been used to investigate presences of contaminants in the environment. Among the commonest used is *Clarias gariepinus* which is a benthic fish spp. with a long-life span of about 8 years and occurs naturally in contaminated and uncontaminated water bodies and entirely omnivorous. Considering its availability, ecological and feeding characteristics, makes it to be suitablefish speciesfor biomarker investigation in aquatic environments (Mdegela *et al.,* 2006a; Braathen *et al.,* 2008).

MATERIALS AND METHODS

Study area

This cross-sectional study was conducted at selected sites along the Ruvu river. The latter is one of the major East African Rivers that drain the Eastern Arc Mountains of Ulugulu, with a basin area of approximately 18,000 square kilometers. The basin is typically sub-divided into smaller catchments: the Mgeta, Ngerengere, Upper Ruvu in the Morogoro region, and the Middle and Lower Ruvu in the Coast Region. The main activities taking place at some parts of Ruvu river are fishing, local gold mining, livestock keeping, charcoal making and agriculture. This research targeted fish samples from Kidimla and Ruvu Darajani sites in Kilangalanga and Mlandizi ward respectively and Kidogozelo and Mtoni sites in Makurunge ward in Kibaha rural and Bagamoyo districts respectively. The sites were selected purposely based on high agricultural activities accompanied with usage of pesticides compounds also the location (distance) from one site to another in order to avoid sampling of the fish from the same area at different study site.

Fish and sample collection

*Clarias gariepinus*of both sexes were used for the study, whereas forty fish samples of either sex were used in the study. These samples were bought from fishermen from fourstudy sites which were regarded to have higher degree of contamination andMatombo as a site with minimal contamination thus was regarded as control site in Ruvu river. The control site was chosen based on low anthropogenic activity related to pesticide usage in agriculture and location (distance) from the study sites. Each site was sampled independently and eight fish samples from each sitewere transported while kept in a ventilated tank (100 L) to the laboratory for sample collection and analysis. In the laboratory fish was restrained manually and approximately two (2) ml of blood sample was collected directly from the caudal vein using a sterile hypodermic 21G needle, 5ml syringerinsed in EDTA. Blood sample was immediately transferred into EDTA vacutainer tubes. The collected blood was centrifuged at 3000XG, 4°C, for 15 minutes to obtain plasma. The later was separated and frozen at -21°C until analysis of the enzymes activities. Thereafter the fish was sacrificed by pithing and decapitating of the head. Fish heads were frozen at -21°C overnight. After overnight storage of the fish heads the whole brain were removed, weighed and immediatelyhomogenized using Potter-Elvehjem homogenizer, in ice- cold 0.1M phosphate buffer pH 8.0 (1:5w: v). Aliquot (3ml) of homogenates was stored at -21°C until analysis of the enzymes activities.

Determination of total protein concentration

The total protein concentration in blood plasma sample and aliquot of brain sample homogenates was determined using a total protein concentration determination kit (Micro Lowry, Peterson´s Modification) as described byLowry *et al.* (1951) and lyophilised bovine serum albumin was used as a standard.

Measurement of Cholinesterases activities

Measurement of AChE and PChE activities were performed using a spectrophotometric method described by Ellman *et al.* (1961).

Data analysis

Collected data were summarized, coded and verified before the analysis. SPSS version 16.0 and Microsoft Excel 2010 Office analytical tool pack computer software were employed in data analysis and the results were reported asmean± standard error of a mean (SEM). Testing of the means differences among the fish enzymes activities from the study sites and between the study sites and the control site were done by One Way Analysis of Variance (ANOVA) and T test with site as an independent variable. Also, Duncan´s Multiple Range Test (DMR) was used to compare the means of all four study sites. Significance differences were observed at$P<0.05$.

RESULTS

Following analysis of blood samples of fish from the study area, results showed that the percentage of the enzymes (AChE and BChE) inhibited were1.8, 5.4, 2.4, 5.4 for AChE and 8.5, 13.3, 10.5, 13.5 for BChE at Kidimla, Mtoni, Kidogozelo and Ruvu Darajani sites respectively. Figure 1 below shows the enzymes activities, these results showed no statistical significant differences (p>0.05) on both the levels of two enzymes activities in the blood plasma samples among thefish from the four sites and between fish from the four sites and the control.
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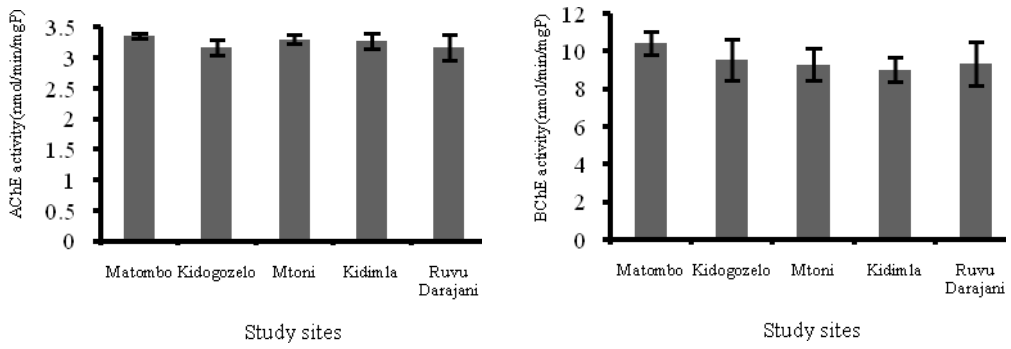

Figure 1. Acetylcholinesteraseand Butyrylcholinesteraseactivities in plasma

The activities of AChE and BChE in brain samples were presented and recorded as in plasma samples (Fig. 2). The percentage of the enzymes (AChE and BChE) inhibited were 7.4, 12.8, 19.1, 31.7 for AChE and 3.2, 2.6, 3.2, 9.0 for BChE at Kidimla, Mtoni, Kidogozelo and Ruvu Darajani sites respectively. In general AChE activities enzyme had no significant difference among the four study sites. A slightly significant difference (p = 0.06) of AChE activities was observed between Kidogozelo and Ruvu darajani. In comparison with the control site (Matombo), AChE activities were significantly higher (p = 0.032) at Ruvu Darajani. But for BChE activities no significant difference was observed among the study sites and between the study sites and control site.

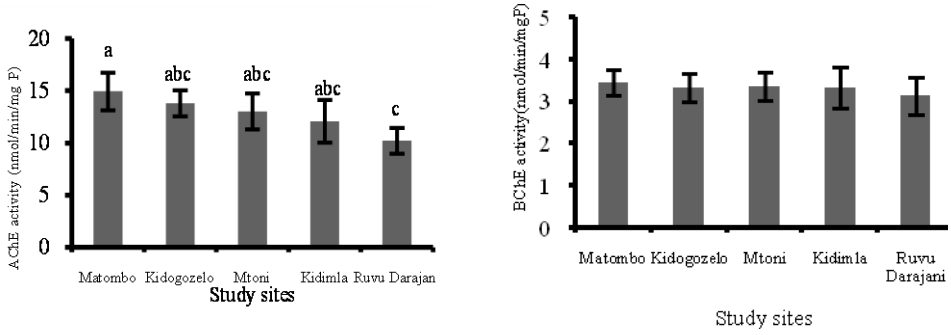

Figure 2. Acetylcholinesteraseand Butyrylcholinesterase activities in brain samples
** The error bars represent SEM at 95% confidence interval and a letter superscript different from control indicates significant difference (P < 0.05).

DISCUSSION

Cholinesterases (AChE and BChE) activities were performed in blood plasma and brain samples.Fish of either sex were used in the study since cholinesterase enzymes has no sex dependence as reported in the previous studiesby Mdegela *et al.* (2010) in *Clarias gariepinus* and also by Galgani and Bocquené, (1992) who found that no differences between male and female in *Limanda limanda*from the Atlantic Ocean when Acetylcholinesterase (AChE) enzymes were assessed in different sites.AChE is known to be highly found in nervous tissues while BChE is highly found in blood plasma. This statement was found to be true and concur with the findingsof the current workthat AChE activities

were higher in brain than in plasma and BChE activities were higher in plasma than in brain tissues. These findings also agree with the studies done Akman *et al.* (2009) and Frasco *et al.* (2010) who found the highest activities of AChE in the brain and muscle tissues of fish whereas BChE mostly predominates the liver and plasma. Furthermore, Jebali *et al.* (2013c) established tissue distribution rank of AChE activity in *Solea solea* fish and found highest activity in the brain than gills and kidney.

AChE and BChE activities in plasma and BChE activities in brain had no statistical significant difference ($p > 0.05$) among the fish from the study sites and between fish from the same study sites and the control. In the brain tissues, the AChE activities were also found to have no significant difference among the fish from the study sites. However, statistical significant difference ($p < 0.05$) was observed in fish from Ruvu Darajani when brain AChE activities of fish from the study site were compared with the control. Similar resultshave been reported by Bodin *et al.* (2004); Bocquene *et al.* (2004) that in the field, several species appeared to have baseline AChE activities of the same order of magnitude in different studies and or measurements. Also, it was noted that the differences between areas and seasons were obvious, e.g. with activity values in *Mytilus* species varying from 25 to 54 nmol min^{-1} mg $protein^{-1}$. Similar findings were also reported in *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea, where the mean values of AChE values vary two-fold depending on season, followingclosely changes in temperature (Leiniö and Lehtonen, 2005). Thus, to avoid such differences during Cholinesterase activities interpretation, samples used in this study were collected in the same period/season.

Also, the current work showed the highest inhibition of the AChE in the brain compared to the BChE. Theseresults are supported by Kamel and Hoppin, (2004) who pointed out that AChE inhibition is more sensitive than BChE in case of chronic exposure to organophosphate and reasoned out that AChE inhibition by OPs show a lower recovery rate compared to BChE and this produces cumulative inhibitory effect on the AChE activity. The same findings were reported by Cero´n *et al.* (1996); Sancho *et al.* (2000) that brain tissues are the sensitive tissues in assessing inhibitory responses resulting from anticholinesterases contaminants.

In addition, this study found highest inhibition of the AChE activities in brain (31.7%) observed in fish samples from Ruvu Darajani site whereas the lowest AChE activities inhibition (7.6%) were observed in fish from Kidogozelo site. Plasma samples had the highest inhibition (5.4%) of the AChE activities in fish from Ruvu Darajani and Mtoni sites with the inhibition of whereas the lowest enzyme inhibition was observed in samples from Kidogozelo (1.8%). Again, the inhibition of BChE activities was highest in fish from Ruvu Darajani site with the inhibition of 13.4% and 9.0% in plasma and brain respectively. These enzymes activities inhibitions can be linked with poor handling of the pesticides used in agriculture and other pesticides related activities in the area. These findings are comparable with the findings by Zinkl *et al*. (1987); Busby *et al*. (1989) who reported the 20% reduction in AChE activity in fish and invertebrates indicates exposure to neurotoxic compounds. Meanwhile, Wright and Welbourn, (2002) reported that reduction of brain AChE activity by 20% or more in birds, fish or invertebrates indicates exposure to OPs or carbamate pesticides, and a 50% or greater reduction is indicative of a life-threatening situation.

Furthermore, FAO (2007) reported that 20% inhibition of brain AChE activity is considered the endpoint to identify the no observed-adverse-effect-level (NOAEL) in organisms, while signs and symptoms appear when AChE is inhibited by 50% or more. Death occurs above 90% inhibition. However, in fish, the relationship between AChE inhibition and mortality is not clear because some species are able to survive with high percentages (90–95%) of brain enzyme inhibition (Ferrari *et al.*, 2004 and Ferrari *et al.,* 2007). On the other hand, some authors have established that 50% of AChE inhibition could indicate intoxication or poisoning (Dembélé *et al.,* 2000). Gruber and Munn, (1998) reported that cholinesterase inhibition of more than 70–90% at sub-lethal concentrations of organophosphates and carbamates has been observed in fish species such as Common carp.

Generally, the usage and mishandling of anticholinesterase compounds along the river in the study area may cause aquatic environmental contamination which can be linked with highest inhibition of brain AChE in fish from Ruvu Darajani site where multiple annual cropping accompanied with usage of pesticides arehighly practiced. This finding is supported by Ozmen *et al.* (2007) who observed the strong relationship between AChE inhibitions in the brain of C*yprinus carpio* collected from Sariyar Dam Lake (Turkey) and the organochlorines pesticides and their residues in water and sediments. In other field investigations using AChE as neurotoxicity biomarker, Lavado *et al.* (2006) reported that AChE was

strongly inhibited in the muscle of *C. carpio* sampled from some stations of Ebro River (Spain) whichhighly polluted by organophosphorus, carbamates, and heavy metals.

From the results of this study, it is concluded that mishandling of pesticides and other anticholinesterase compounds potentiallycontaminates terrestrial and aquatic environment. Therefore, integrated pest managements and farmers awareness on the adverse effects of these compounds is required. On the other hand, further studies are needed to assess the activities of the enzymes in other sites of the river and along its flow pattern throughout the year.

ACKNOWLEDGEMENTS

This study was kindly funded by Mwalimu Julius K. Nyerere University of Agriculture and technology (MJNUAT) and One Health for Central and Eastern Africa (OHCEA). We are grateful to the management and staff of Kibaha rural district and Bagamoyo district for granting permission to carry out the study in their area of management and for kind support during sampling. Also, we thank all staffs at the college of Veterinary Medicine and Biomedical Sciences of Sokoine University of Agriculture for their technical support and assistance.

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Possible involvement of *Dioscorea* species in human poisoning at Bwakila Juu in Morogoro Rural District, Tanzania

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SUMMARY

For many years' wild plants are known to be used as a source of food, medicines, poisons and for ornamental purposes. *Dioscorea* are reported to be poisonous but if well processed the local people in places where they are found eat them as food especially during hunger. The purpose of this study was to assess the toxicity of *Dioscorea* spp. after it was reported that the plant was involved in deaths of two humans in Bwakila Juu in Morogoro Rural District. Two types of *Dioscorea* bulbils were collected, one being aerial (D1) and the second was tuber (D2), they were sliced, dried under the sun and grounded to make coarse powder. Toxicity test of *Dioscorea* spp. was done using 4 weeks 60 cockerels which were allocated into 6 groups each consisting of 10 birds. Cockerels in groups I – III were fed with chick starter mash which was mixed with D1 *Dioscorea* bulbils coarse powder at a concentration of 0%, 10% and 20%; group IV - VI were fed D2 bulbils' coarse powder mixed with chick starter mash at a concentration of 0%, 10% and 20%. The feeding experiment was done for four days and any changes in clinical signs and deaths were observed for 7 days. In addition, blood samples were collected from all the birds on day 7 of the experiment to measure for aspartate aminotransferase enzyme (AST), albumin and total protein. All the cockerels in group III fed on 20% of D1 started developing clinical signs of inappetence, ruffled feathers, severe diarrhea, convulsion and torticollis. On day 1 of feeding, one bird died and the other two deaths were recorded on day 2. Postmortem results indicated generalized hemorrhages in the lungs and atria, hyperemia of the atria, congestion of kidneys, mucohaemorrhagic intestinal contents and generalized enteritis. The birds in the other groups appeared normal throughout the 7 days of observation but were all sacrificed for postmortem examination which again did not show significant lesions. The mean AST concentration in the plasma (16.9 ± 0.2 µg/l) of the treated birds was significantly higher ($p<0.05$) compared to the birds in the control groups which is an indication of the liver damage. The total protein and albumin in plasma of all the birds was within normal ranges. Almost six incidences of *Dioscorea* spp. poisoning in humans in which lead to death were reported by the local people in Bwakila Juu during different periods normally occurred during shortage of food. These preliminary results suggest that *Dioscorea* spp. may be involved in human poisoning. More studies are recommended before concluding with certainty the involvement of *Dioscorea* spp. in human poisoning.

Keywords: wild plants, *Dioscorea*, poisoning, cockerels

INTRODUCTION

For many years wild plants have been used as a source of food (Ruffo *et al.*, 2002), medicines (John, 1984; Sezik and Yesilada, 1992; De Foe and Senatore, 1993; Pamplona Roger, 2007), poisons (Thabet et al., 1999; Gidado *et al.*, 2007) and for ornamental purposes (Ruffo *et al.*, 2002). Plants belonging to the Genus *Dioscorea* are reported to be poisonous (Watt and Breyer-Brandwijk, 1962), but at the same time, if well processed, are used as food especially in times of food shortage. The genus contains about 600 species, which are distributed worldwide (Huber, 1998). In Tanzania, there are 14 *Dioscorea* species distributed in different regions /areas of the country; some of the species are found in the wild and some are cultivated as a food crop (Ruffo *et al.*, 2002). The plants produce underground as well as aerial tubers (bulbils) which contain large amounts of starch. As such the tubers are consumed particularly at times of food shortage, eaten fresh as roasted or cooked. Tubers are collected, peeled, cut into small pieces and soaked in water overnight to remove toxic substance before being cooked (Ruffo *et al.*, 2002). Alternatively, tubers have to be peeled then soaked in water for several days, washed, sliced into small pieces and dried under the sun. Dried slices may be pounded into flour and used for porridge and for stiff porridge (*Bondei* and *Zaramo* ethic groups) in Tanzania (Ruffo *et al.*, 2002).

Dioscorea species, both wild and some of the cultivated varieties, contain a toxic principle dioscorine, which may cause death when tubers are consumed fresh uncooked or partially cooked (Watts and Breyer-Brandwijk, 1962). Poisoning ability of *Dioscorea* species occurs after eating either aerial or underground tubers. It has further been reported that wild species are more poisonous than the cultivated ones (Watt and Breyer-Brandwijk, 1962). The genus *Dioscorea* consists

of plants which are twining dioecious pubescent (hairy) or glabrous (hairless) herbs with annual stems arising from tubers; leaves alternate or opposite, heart shaped, long and broad with prominent veins in some of the species, usually entire, occasionally compound with 3-7 leaflets. Aerial tubers of *Dioscorea* sometimes occur and arise in leaf-axils, and may be irregularly roundish. Inflorescence spicate, and may be pendulous. The capsules are rigid either deeply 3-lobed or triangular-ellipsoid, dehiscing into three valves while the seeds are variously winged or rarely wingless (Milne-Redhead, 1975).

In Tanzania, few cases of *Dioscorea* poisoning have been reported to occur, and are through hearsay. Nevertheless, *Dioscorea* spp are commonly eaten by the local community especially in areas where the plants are common. The purpose of this study was to assess the toxicity of *Dioscorea* spp. after the report that the plant was involved in deaths of two humans in Bwakila Juu in Morogoro Rural District. It appears that *Dioscorea* as a crop has not featured very much in recent years, notwithstanding its important role in times of food shortage.

MATERIALS AND METHODS

Study area and experimental area

The *Dioscorea* spp bulbils (Figure 1&2) were collected Bwakila juu village in Morogoro rural District and transported to Sokoine University of Agriculture (SUA) where the toxicity experiment was undertaken in cockerels. The brooding rooms in the Poultry Unit at SUA were cleaned thoroughly and well disinfected using glutaraldehyde/quaternary ammonium compound (V-RID®). Brooders were constructed in circular form using hard boards measuring 60 cm wide and 240 cm long. Electrical system was connected and fitted with several 200 watts bulbs, to generate light and heat (Figure 3).

Figure 1. *Dioscorea sansibarensi* aerial bulbis (regarded as more poisonous) Source: https://garden.org/plants/photo/347911/

Figure 2. *Dioscorea bulbifera* aerial bulbis (regarded as less poisonous types). Sources: https://garden.org/plants/photo/347911/

Figure 3. Brooder facility constructed of hard boards, showing cockerel chicks being provided light, heat, feed and water

Study design and animals

An experimental study design was conducted where by 250 day old cockerels were purchased from Ideal Chick Farm, Dar es Salaam, and reared for 4 weeks in the brooding facility within the Poultry Unit at Sokoine University of Agriculture (SUA) Farm (Figure 3). Brooder was constructed using two hard boards joined in a round form, attached together by means of bent nails. The smooth concrete floor was covered by spreading newspapers to protect chicks from cold. Three bulbs were used to generate heat and to supply light in the brooder. Water was provided using plastic water drinkers which were placed at the centre of the brooder. The chicks were fed chick starter mash put in plates, *adlibitum* and heat and light were provided for 24 hours during the first 4 weeks.

Chicks were vaccinated against Newcastle Disease on Day 3 and Day 21 of age, using live attenuated La-sota strain, orally in water. Vaccination against Infectious Bursal Disease (Gumboro) was done on the 13th day of age. The route of administration was oral via drinking water. Before administration of the vaccines cockerels were denied water for 2 hours to make them thirsty.

Processing of aerial tubers

Aerial tubers of two *Dioscorea* spp. collected from Bwakila Juu, and identified as D1 and D2, one of them being the poisonous type which was suspected to had caused death of the two people were separately sliced into very thin slices and dried in the sun for two days. The sun-dried slices were ground into coarse powder using an electric driven grinder. D1 and D2 bulbils were very similar in appearance although they belong to two different species, and on drying and grinding formed powders which differ in color, milky white and brown/chocolate, respectively.

Feeding trials and observations of changes

Cockerels in groups I – III were fed with chick starter mash mixed with D1 coarse powder at a concentration of 0%, 10% and 20%; group IV - VI were fed D2 bulbils' coarse powder mixed with chick starter mash at a concentration of 0%, 10% and 20%. The feeding experiment was done for four days and any changes in clinical signs and deaths were observed for 7 days. Blood samples were collected from all the birds on day 7 of the experiment to measure for aspartate aminotransferase enzyme (AST), albumin and total proteins.

Experimental birds were examined at interval of 2 hrs during the first 24 hours and subsequently at intervals of 6 hours and changes in behavior, clinical manifestations and any other abnormalities were recorded. Mortalities were recorded and a post-mortem examination was later conducted on all dead and sacrificed cockerels.

Blood samples collection, handling and processing

Blood samples were collected from the wing vein of cockerels using 23 gauge needles, and transferred to vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) as an in vitro anticoagulant. Blood was centrifuged at 3000 rpm for ten minutes

Data analysis

The toxicity test data from the experiment were calculated using Microsoft Excel and subjected to analysis of variance (ANOVA) and Chi-square tests at a critical probability of $P < 0.05$.

RESULTS

The feed intake in the cockerels in group I - III had poor feed intake as the consumption rates was lower compared to the control group which were being fed on D2. All the 10 cockerels in group III fed on 20% of D1 developed clinical signs of inappetence, ruffled feathers, severe diarrhea, convulsion and torticollis (Figure 4). On day 1 of feeding, one bird in group III fed on 20% of D1 died and the other two deaths were recorded on day 2. Postmortem examination indicated generalized hemorrhages in the lungs and atria, congestion of kidneys, mucohaemorrhagic intestinal contents and generalized enteritis. The mean AST concentration in the plasma (16.9 ± 0.2 µg/l) of the treated birds was significantly higher ($p<0.05$) compared to the birds in the control groups which is an indication of the liver damage (Table 1). However, the total protein and albumin in plasma of all the birds was within normal ranges.

The birds in the other groups appeared normal throughout the 7 days of observation but were all sacrificed for postmortem examination which again did not show significant lesions.

to isolate plasma which was used to determine concentrations of AST enzyme, albumin and total protein. The absorbance of the standard and test samples was read at wavelength 630 nm in spectrophotometer.

The formula that was used in calculation of concentration of AST enzyme, albumin and total protein was:

$$\text{Concentration of test sample} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of standard}} \times \text{Concentration standard}$$

Figure 4. Dead cockerel with chalky faecal materials on the floor

Table 1. The mean plasma concentration of albumin, total protein and AST enzyme in cockerels

Groups	Albumin µg/l	Total protein µg/l	AST µg/l
i - D1 0%	16.69 ± 2.05	7.37 ± 0.65	7.76 ± 1.34
ii - D1 10%	16.24 ± 0.96	7.41 ± 0.38	10.7 ± 1.62
iii - D1 20%	16.71 ± 0.18	7.45 ± 0.48	16.85 ± 0.21
iv - D2 0%	16.71 ± 0.18	7.41 ± 0.66	7.62 ± 1.24
v - D2 10%	16.67 ± 0.19	7.36 ± 0.59	7.66 ± 1.15
vi - D2 20%	16.69 ± 2.01	7.41 ± 0.71	7.69 ± 1.51

DISCUSSION

The purpose of the current study was to assess the toxicity of *Dioscorea* spp. after it was reported that the plant was involved in deaths of two humans in Bwakila Juu in Morogoro Rural District. The results indicated that all the cockerels in fed on 20% of D1 were affected by the *Dioscorea* spp. since all the birds showed some clinical signs suggestive of poisoning and some deaths were experienced in the group. The mean AST concentration in the plasma was high (16.9 ± 0.2 µg/l) further giving evidences of liver damage. Postmortem results indicated generalized hemorrhages in different visceral organs

and generalized enteritis which further supported possibilities for poisoning. With the results observed in toxicity trials in cockerels, it is imperartive to state that *Dioscorea* spp. may have been involved with the poisoning of humans in Bwakila Juu in Morogoro Rural District. Nevertheless, studies show that if the *Dioscorea* bulbils are well processed by peeling, soaking in water for several days, sliced, dried under and pounded into flour can be used as food with minimal or no poisoning (Watt and Breyer-Brandwijk, 1962; Ruffo *et al.*, 2002).

The current study established that *D. sansibarensis* (*Dendego*) was given in-feed to cockerels and caused deaths in two days of feeding indicating that the species is poisonous. The other species *D. Bulbifera* (*Libika*) given in-feed caused no harm to cockerels, an indication that they are harmless. The distribution of *D. sansibarensis* in Tanzania is wide; it has some features which resemble the less poisonous species. The people who consume the tubers of *D. sansibarensis* were posioned as compared to those who took *D. Bulbifera.* Dioscorea are known to contain a toxic principle called dioscorine which causes various toxic effects in mammals that are related to liver damages. To date, almost six incidences of *Dioscorea* spp. poisoning in humans has been reported and two individuals in Bwakila Juu after they consumed the plant during shortage of food. These preliminary results suggest that *Dioscorea* spp. may be involved in human poisoning.

The clinical signs of severe diarrhoea, torticolis, and narcosis that were observed in cockerels after being fed on bulbils of *D. sansibarensis* have also been previously reported by other scholars (Broadbent and Schnieden, 1958; Bhandari and Kawabata, 2005; Azanza and Patricia, 2006). The active ingredient *Dioscorin* is a neurotoxin that acts by blocking the nicotinic acetylcholine receptor. It has an LD50 of 60 mg/kg in mice through an intra peritoneal route of administration. Saponin has a wide range of effects; it cause haemolysis of red blood cells, vacuolization of the cytoplasm of hepatocytes, canalicular cholestasis and cause liver necrosis (Bhandari and Kawabata, 2005; Azanza and Patricia, 2006). The post-mortem lesions of generalized haemorrhages in the lungs and atria, congestion of kidneys, mucohaemorrhagic intestinal contents and generalized enteritis suggests an acute poisoning. This further show that *D. sansibarensis* if not well prepared has a potential of toxicity effects to whoever consuming the plant.

Experimental cockerels in the present study had low feed intake and did not readily accept the feed containing *Dioscorea* powder. This again may be due to presence of bitter alkaloids dioscorine, furanoid norditerpenes and saponins in *Dioscorea* plant (Broadbent and Schnieden 1958; Bhandari and Kawabata, 2005).

The analysis of the total protein and albumin in plasma of the control group and the experimental cockerels showed no variations between the groups. The difference was not observed probably because of the short duration of the experiment, therefore there was no much damage to the liver. Damage to the liver usually interferes with protein synthesis which results into decreased plasma protein concentration (Maxine, 1979). The high mean (16.85 ± 0.21µg/l) AST concentrations in the plasma of the treated birds than that measured in the control groups of birds suggestive of some extent of liver damage, specifically liver cells (hepatocytes) (Maxine, 1979).

From the results obtained from the toxiciy study of *D. sansibarensis* cockerels show that the plant is poisonous. It caused severe illness to the exposed cockerels and may be responsible for deaths in humans in Morogoro at Bwakila Juu. Therefore, more studies are recommended before concluding with certainty the involvement of *Dioscorea* spp. in human poisoning. These preliminary results should build the foundation to further explore the toxicity of the plant and possibly identify the toxic ingredients of *Dioscorea* plants of Tanzania. Local people in the locality can be used as the starting point towards bioprospecting of the importance of *Dioscorea* plants. Nevertheless, education to the people in localities where the *Dioscorea* are found should be educated on poisonous and non poisonous species and where they are in acute shortage of food, better methods of *Dioscorea* preparations should be practiced.

ACKNOWLEDGEMENTS

The authors are are thankful to the local people in Bwakila Juu.

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Awareness on mycotoxins among commercial poultry feed handlers in Morogoro

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SUMMARY

Successful control of the mycotoxins contamination in animal feeds needs to integrate variety of techniques which involve different stakeholders along the livestock management and feed handling chain. A study was conducted in three districts in Morogoro region, Tanzania, to assess the degree of awareness among poultry feed handlers including feed processors and retailers. A structured questionnaire was delivered to 186 respondents including 36 feed miller owners or managers, 54 feed miller workers and 96 feed retailers. The respondents were randomly sampled in Morogoro municipality, Morogoro rural, and Kilosa districts. It was found that 85% of the respondents in the three districts had basic knowledge on mycotoxins. Among the respondents with the basic knowledge on mycotoxins 52% had very unsatisfactory knowledge, 44% had unsatisfactory knowledge and 4% had satisfactory knowledge. The respondents from Morogoro municipality (urban area) had significantly higher awareness on mycotoxins as compared to the ones from Morogoro rural and Kilosa ($p< 0.05$). Managers and/or owners of feed millers were more aware on mycotoxins as compared to other miller workers. Feed miller workers and retailers conducted 82% and 72% respectively of activities potential for mycotoxins control during feed handling processes while managers conducted 56% of the activities. The findings of this study display the potential roles of commercial poultry feed handlers on occurrence and control of mycotoxin accumulation in feeds and subsequent exposure to poultry. Utilization of the obtained information on implementation of mycotoxin control programs will facilitate better allocation of the limited resources, by understanding what inputs are required by different groups of role players.

Keywords; feed handlers, Morogoro, mycotoxins, poultry feeds

INTRODUCTION

Mycotoxins are toxic secondary metabolites of some which grow in soils and plant materials. A number of the fungi are known to colonize food crops and produce the toxins. *Fusarium* spp and *Aspergillus* spp are the major fungi which grow in food and feed and produce mycotoxins (Sweeney and Dobson, 1998). The toxins cause health effects to exposed humans and animals and also significant economic effects along food chain (Darwish *et al.,* 2014). Among the health effects include cancers, retarded growth, immunological suppression and reproductive disorders (Zain 2011).

Mycotoxin occurrences in foods and feeds have been reported worldwide (Binder *et al.,* 2007, Rodrigues and Naehrer 2012). While different zone of the world are affected at different levels by different types of mycotoxins, tropical countries are affected more by aflatoxins and fumonisins (Lee and Ryu 2017). The occurrence of the toxins in the tropical developing countries is higher as compared to other parts of the world. Aflatoxin B_1 which is the most toxic and carcinogenic mycotoxin is reported in most countries in the region. Contamination of foods and animal feeds has been widely reported in Tanzania (Geary *et al.,* 2016, Kajuna *et al.,* 2013). Exposure of humans and animals to mycotoxins in the country has been documented at different levels. Whereas aflatoxins and fumonisins are the mostly encountered mycotoxins in Tanzania, fatal cases associated with acute aflatoxicosis in humans have been reported for years (NEWS-DESK 2017, TFDA 2012).

Occurrence of mycotoxins in foods is associated with climate condition of a given area, agronomical practices and crops grown (Bernhoft *et al.,* 2012, Diao *et al.,* 2015, Mutegi *et al.,* 2012). Aflatoxins are known to occur more in high temperature, low rainfall areas while fusarium toxins are more experienced in cold and wet regions (Bernhoft *et al.,* 2012). In low income countries high rate of mycotoxin occurrence in foods is precipitated by low input agriculture practised (Mutegi *et al.,* 2009). There has been an importance to control mycotoxins in feeds in order to reduce the burden of diseases and improve productivity in livestock. Controlling mycotoxins has always been achieved by integrating different methods as there is no single bullet method that has indicated to have total control of the problem. Using different methods require efforts from different stakeholders involved in different ways of production, trading, regulation and consumption of agricultural products (Milicevic *et*

al., 2015). It is therefore of high importance for every part along the feed chain to play appropriate role in achieving that.

Among the factors that have been mentioned to be important for facilitating different stakeholders do their part well is having proper and adequate knowledge of the mycotoxin problem and how to handle it (Bhat *et al.,* 2010). Studies, especially in developing countries have indicated low level of awareness among common people, health and livestock officers on mycotoxins (Adekoya *et al.,* 2017, Strosnider *et al.,* 2006). Insufficient knowledge among different key players in the food chain is also thought to contribute to continued occurrence of the toxins in foods and feeds (Strosnider *et al.,* 2006). While different efforts on awareness building have been taken in different areas, maximum achievement of the efforts requires strategic planning. This should involve, among other things, considering who does what and hence what information is required by each stakeholder. One of the important areas for consideration is on the production and trading of commercial livestock feeds.

The mycotoxin contamination status in commercial livestock, especially poultry feeds has been widely studied. In most of the studies conducted on poultry feeds in Tanzania, more than half of the feeds are indicated to have mycotoxin contamination above the accepted levels (Kajuna *et al.,* 2013). The source of contamination is assumed to be both in field and postharvest. Therefore, controlling the toxins in the feeds should focus both on using clean raw materials as well as practicing proper handling and storage to reduce postharvest contamination. In order to achieve that, feed handlers along compounding and trading part of the feed chain should have a proper conduct depending on their different parts they play.

The important roles played by the handlers along the feed chain parts they are involved with include selecting raw feed materials, transportation, compounding and storage. Traders are also involved with transportation and storage of the feeds before they are delivered to farmers. These parts are very important in managing mycotoxins contamination in the feeds. Selecting suitable raw materials, even by visual analysis can have a good impact in avoiding contaminated products. Rotten grains including maize and peanuts are more often contaminated with mycotoxins (Karlovsky *et al.,* 2016). Feed processors also have to buy adequately dry products as high moistures facilitate fungal multiplication and formation of mycotoxins during handling of the products. Ensuring suitably clean materials goes hand in hand with avoiding building conditions which facilitate mycotoxins production. Processors have therefore to ensure milling and mixing the feeds avoid further contamination with fungi and leave the feeds dry. Proper packaging is another important area. Tight packaging of fully dry materials, which ensure very limited air supply, will greatly discourage mycotoxin accumulation during storage (Lane and Woloshuk, 2017). Traders also need to ensure they buy feeds which are visually sound and with minimal signs of going bad. The feeds have to be kept in proper environment in the stores as well as ensuring minimum storage time before they are delivered to the farmers. In order for all these to be conducted successfully, feed processors and traders have to have proper knowledge on mycotoxins and how to control them. Planning education programs for them have to consider their average level of awareness they currently have in order to know the recipe to provide.

While most of the studies on awareness on mycotoxins have been focused on farmers and health and livestock officers, less is known about the same for persons who handle the feeds during processing and trading. Therefore, this study aimed at assessing the awareness among the group on mycotoxins, their effects and how to control them. The information provides an insight of how the levels of knowledge the people have influence the presence and accumulation of the toxins in poultry commercial feeds.

MATERIALS AND METHODS

Study area and selection of respondents

The study was conducted in Morogoro region. The region of located along the east-southern part of Tanzania, and characterised with diverse climatic conditions, with dry savanna areas on the north eastern parts and high rainfall areas in west-southern parts. The region is divided into seven districts and for this study three districts were involved; Morogoro urban, Morogoro rural and Kilosa. Respondents for the study were feed processors and traders, and were purposively selected to get representation from feed miller managers, workers and sellers.

In conducting the study, it appeared that sometimes owners are the activity overseers hence qualified also as managers. List of individuals who run

poultry feed processing and selling was obtained and poultry farmers. Identified respondents were approached, briefed on the study and its intention and requested to willingly consent. A total of 186 respondents from the three districts consented to participate in the study (Table 1). The biographic and educational characteristics of the respondents are also summarized in Table 2.

Table 1. Number of respondents indicating owners/managers, miller workers and retailers and their distribution in the three districts studied

	Owners / managers	Miller workers	Retailers
Morogoro Urban	21	22	41
Morogoro Rural	7	15	22
Kilosa	8	17	33

Table 2. Biographic and educational characteristics of the respondents

	Primary education	Secondary education	Post-secondary education
Morogoro urban			
Male	29	19	4
Female	11	14	7
Morogoro rural			
Male	17	15	2
Female	6	4	0
Kilosa			
Male	27	18	3
Female	5	4	1

Study design

The study was conducted in a cross sectional design where, a structured questionnaire was delivered in form of interview to the respondents. The questionnaire was developed to capture information on general awareness of a respondent on mycotoxins, awareness on effects of the mycotoxins and awareness on actions required during handling of feeds to reduce contamination and avoid further accumulation of the toxins. Questions were also included to capture the activities a given respondent is directly involved and assess tendency of sharing skills among people who work together. The first version of the developed questionnaire was tested to five respondents in Mzumbe ward, Mvomero district, in Morogoro. The questionnaire was found possible and understandable to respondents and with the help of district and ward livestock officers average questionnaire delivery time was found to be 15 minutes. The questionnaire was developed in English language, then translated to Kiswahili and delivered in the same language.

Assessing the general knowledge of the respondents

The first part of the questionnaire was designed to capture the respondents' general awareness on mycotoxins. Gradual interviewing on what he/she knows about feeds going bad, growth of moulds was done. The respondent was asked if he/she is aware on effects of moulds that grow on feeds, about toxins that may be present in mouldy feeds and then if he/she knows the name of the toxins. Asking if the respondent knows about mycotoxins (yes/no) was avoided, as chances are most people respond "yes", even when they are not sure what is being asked about. Being aware about growth of moulds in grains was considered as basic awareness and was gave a 10 mark score. The respondents were also asked about being aware of toxic substances produced by the moulds, different types of moulds (on appearance), name of the toxins (in Kiswahili), and crops that are commonly affected.

Assessing specific knowledge on effects and control of mycotoxins

The respondents with basic knowledge were taken to the second part of the questionnaire which queried on awareness on effects and management activities required to control the problem. The target was effects and control of mycotoxins in animals, more specific on poultry, but answers focusing on humans were also taken positive. This was because in some circumstances, respondents have information on mycotoxins in foods and their control in human, but not in livestock.

Involvement on activities significant for mycotoxin management

In a part of the questionnaire the respondents were asked to indicate different activities they are directly involved with, from sourcing raw feed materials to packaging and storage. The section was separated to customize activities for feed miller operators and retailers.

Assessing the tendency of sharing information among respondents

A question was set to ask on the source of information on mycotoxins, and if one was used to share any similar information with colleagues in the working place.

Data analysis

The answers from the questionnaires were coded and recorded in Microsoft Excel computer program and analysed for descriptive and inferential statistics using the same program.

RESULTS

Mycotoxin general knowledge awareness

Among the respondents involved, 158 (85%) had at least basic knowledge on growth of moulds in some crops and could indicate that the moulds can be harmful to consumers. The remaining 28(15%) of the respondents were not aware on the harmful growth of moulds in grains and were considered lacking basic knowledge on mycotoxins (Figure 1). Among the 158 respondents with basic knowledge, 76 (48%) could at least indicate that the harmfulness of the fungi was associated to production of toxic substances in the grains. The respondents in this group could also specify effects of the toxins or ways to control them, but not both. Whereas this was still considered unsatisfactory information on mycotoxins, the other 82 participants (52%) with basic knowledge were not aware of the toxins and this was taken as very unsatisfactory knowledge. Six respondents, who were almost 4% of the respondents with basic knowledge, had what was considered as satisfactory knowledge on mycotoxins. These respondents could at least indicate one health effect of the toxins and one measure for control.

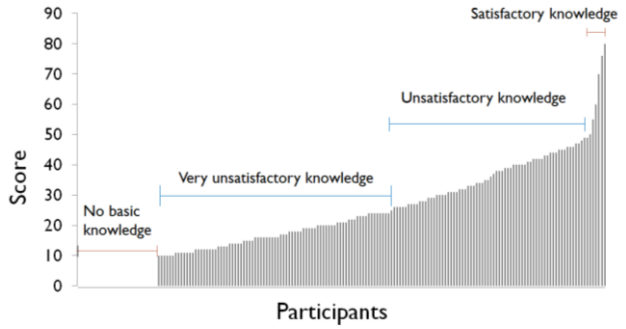

Figure 1. Distributions of the respondents along different knowledge categories depending on their scores

The level of knowledge was compared between the respondents on basis of geographical origin and specific roles of the individuals. More respondents from Morogoro urban (95%) had basic knowledge on mycotoxins as compared to Morogoro rural (73%) and Kilosa (79%) and the difference was significant ($p < 0.05$) when tested by Chi-square at 95% confidence interval (**Figure 2**).

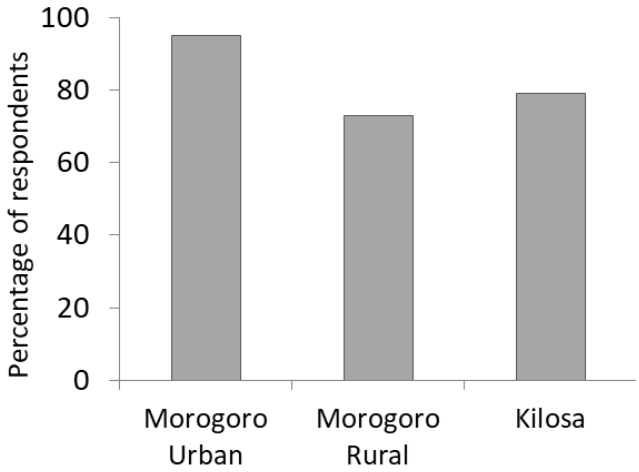

Figure 2. Percentage of respondents from the three districts with basic knowledge on mycotoxins

In general, more miller managers and owners were aware at different aspects on the mycotoxins as compared to other miller workers and feed retailers (Figure 3).

Figure 3. Percentage of managers and owners, workers and retailers with basic knowledge, knowledge on effects and knowledge on control of mycotoxins

Roles and information sharing among respondents

It was found that in average miller workers and retailers conducted 80% and 72% respectively of activities directly related to control of mycotoxins. The rates were significantly higher as compared to managers who were directly involved with 56% of the activities. Activities considered include inspecting quality of feed raw materials before being brought to the miller, packaging of the materials for storage and ensuring adequate drying of the materials. Others include packing materials in the storage facilities, and monitoring clean, dry and sound storage environment.

Five percent of the respondents indicated that they make discussions with fellow workers to share information on matters related to feed (and raw materials) safety. The low sharing of information among respondents was reflected by poor correlation between managers the levels of knowledge of managers/owners and workers (Figure 4).

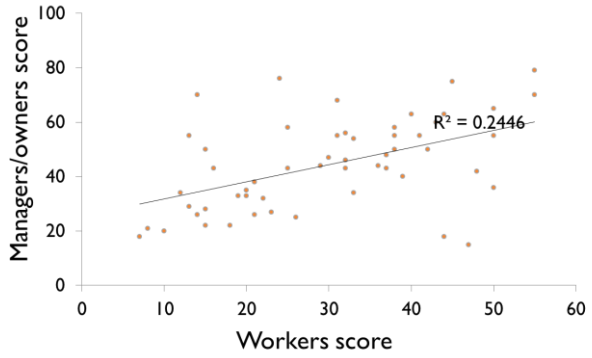

Figure 4. Scatter diagram indicating relation between scores for managers and owners against scores for other workers. The line of fit and regression coefficient is provided.

DISCUSSION

The findings of this study have once again indicated poor awareness and knowledge among key stakeholders of mycotoxin control in Tanzania. It has previously been indicated in another study that awareness on mycotoxins among heads of households in Kilosa Tanzania is low (Magembe *et al.*, 2016). Human and animal populations in Africa suffer adverse effects from mycotoxin exposure. Effective control of the problem is a subject to full participation of all key players in the food and feed value chain. Infections of most crops by mycotoxin producing fungi start in the field (Oliveira *et al.*, 2014) and continues during harvesting and postharvest times (Neme and Mohammed, 2017). That means inadequate awareness in farmers, processors, traders and consumers has a significant role in propagating mycotoxins and their exposures.

The low awareness on mycotoxins among public member in the country is contributed to different factors including socio-economic and psychological factors (Udomkun *et al.*, 2017). Naturally, most health effects due to mycotoxins are manifested chronically and come over time. Consequently, many people miss the direct link between observed health implications and mycotoxin exposure, and as a result little attention is drawn. Current advances in mycotoxin research have generated timely information that has awakened the efforts for strategies to reduce problem (Lee and Ryu, 2015).

The current study indicates variation on level of awareness between geographical locations; being higher for participants in the urban area (Morogoro urban) as compared to the ones in the rural areas (Morogoro rural and Kilosa). This, among other factors may have been due to the higher accessibility of information tools in urban communities (Temba *et al.*, 2016). Better infrastructure and advanced information and communication technology have resulted into shifting trend of the sources of extension from hard written materials to digital sources (Mtega, 2012).

The results also indicate higher knowledge among owners and managers as compared to normal workers in animal feed value chain. The fact that awareness of people on mycotoxins is influenced by different factors including socio-economic factors (Udomkun *et al.*,2017) can partially justify the findings. Occurrences of mycotoxins in feeds decline market acceptability and hence impair business flow (Mitchell *et al.*, 2016). Owners and managers play the first role in propagating their business and therefore may have more interest of learning issues like quality of what they produce. The study however indicate low tendency of information sharing among owners, managers, and other staff working together.

The findings from this study demonstrate the importance of strengthening awareness building initiative in efforts to fight the mycotoxin contamination in foods and feeds. Although farmers were not involved in this particular study, they are the first part of poultry feed chain who need to be educated on mycotoxins and how to avoid. Operators of grain storage points, grain mills and feed mills paly important part in dealing with postharvest safety measures of the feeds. Traders and poultry keepers also need to know about techniques of safe handling of the compounded and

raw feeds in their points to protect poultry against mycotoxin exposure.

ACKNOWLEDGEMENTS

The author thanks the Australian Government through anAustralian Awards scholarship for sponsoring the study, the district directors in Morogoro urban, Morogoro rural and Kilosa district for granting permits to carry on the study and assistant given by the district agriculture and livestock officers in the respective villages. All the respondents are appreciated for their cooperation.

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Evaluation of stress hormone (cortisol) levels and some biochemical parameters of pigs kept under intensive management systems in Morogoro, Tanzania

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SUMMARY

The worldwide increase in demand for animal products in recent decades has necessitated raising of food animals under intensive systems that have been demonstrated to cause stress to animals. A cross sectional study was carried conducted to evaluate the welfare of pigs kept under intensive system using serum cortisol levels and some biochemical parameters as indicators of stress. A total of 302 pigs, aged three months to two years, from urban and peri-urban areas of Morogoro township were purposively involved in the study which assessed farm management aspects (housing and feeding) and blood levels of cortisol and some biochemical parameters (glucose, total protein and cholesterol). Results showed that serum cortisol levels were significantly higher ($p < 0.001$) in pigs from the peri-urban farms than those dwelling in the urban settings. Weaner pigs and boars had the highest serum cortisol levels compared to other groups. Significantly high levels of plasma cholesterol ($p < 0.05$) were measured in boars followed by gilts and weaners all kept in the urban areas whereas no significant differences were observed as far as plasma glucose and total proteins were concerned. Result on farm management assessment showed that urban piggery housing was of better quality than in the peri-urban and that feeding was largely influenced by local availability of the major feed ingredients such as maize bran and vegetable residues. About 80% of urban pigs were maintained on high concentrate feed due to availability of swills (restaurant leftovers) and maize bran whereas low concentrate feed with mainly vegetable residues dominated in the peri-urban settings. It is concluded that, serum cortisol levels observed in the current study were a reflection of stress to different groups of pigs kept under the intensive system and that feeds had some direct effect on biochemical parameters such as plasma cholesterol levels.

Key words: Stress factors, biochemical parameters, cortisol, pigs, urban, peri-urban

INTRODUCTION

Pig farming is an important component of the livestock sector in Tanzania. Pig keeping is practiced by small scale farmers due to its relatively low cost of establishment and its potential role as a contributor to increased rural household incomes and to improved food security in households (Wilson and Swai, 2014). In recent years there has been a growing concern about animal welfare due to the undesirable consequences on general productivity performance (Miró *et al.,* 2016). Increased demand for quality animal products has led to intensification of production that compromises with animal welfare (Barnet *et al.,* 2001; Cadiani *et al.,* 2008). Intensive management has been reported to cause stress in animals particularly pigs. Investigations of stress in pigs have shown that restricted movements and freedom to feed causes stress in pigs (Verdon *et al.,* 2015). Social interactions and aggressive behaviour has been shown to increases as the space allowance decreased in group-housed sows (Weng *et al.,* 1998; Verdon *et al.,* 2015). Other factors include environmental stress such as temperature, humidity, light, concentration of dust and gases, ammonia levels and sound intensity. Metabolic stress results from food and/or water restriction or deprivation (Pearce *et al.,* 2013; Sanz *et al.,* 2015). Subjecting pigs to a stressful environment has lead to increased level of blood serum cortisol and also interfere with biochemical parameters such as blood glucose, cholesterol and total protein.

Cortisol, a steroid hormone in the glucocorticoid class of hormones, is normally released into the blood stream in response to stress and low blood-glucose concentration in most mammals (Aronson, 2005; Maduka *et al.,* 2015). In most diurnal species, cortisol is secreted with a circadian variation, which is high concentrations at late night and early morning and with low concentrations in the afternoon and early night (Sjaastad, 2003). Blood glucose, cholesterol and total protein are biochemical parameters which have also received considerable attention as indicators in assessing

animal welfare (Doyle *et al.,* 2015). Recently, animal welfare report has highlighted the need for improving animal welfare for good production. There is paucity of information on studies to assess the influence of management aspects on serum cortisol levels and some blood parameters in pigs in the country. The purpose of this study was therefore to evaluate the welfare of pigs kept under intensive system using serum cortisol levels and some biochemical profiles as indicators of stress.

MATERIALS AND METHODS

Study area and animal descriptions

The study was conducted between April and June in urban (Morogoro town, 500 - 600 m asl, 25 - 30^{0}C) and peri-urban areas (Mgeta, 1500 - 1700 m asl, 16 - 20^{0}C) in Morogoro region, Tanzania. These areas were chosen because they were known to have substantial number of small scale pig farms having least 10 animals and above per household. A total of 302 pigs of both sexes ranging from 3 months to two years of age were involved in the study. 140 pigs were from peri urban whereas 162 were from urban Morogoro. Distribution of sampled areas, stage and sex of pigs is shown in Table 1

Table 1. Number of pigs by sex and growth stage

Growth stage	Total number	Urban	Peri urban	Female	Male
Boars	14	7	7	NA	14
Sows	92	69	23	92	NA
Gilts	34	5	29	34	NA
Growers	70	23	47	23	47
Weaners	92	34	58	44	48
Total	302	138	164	193	109

Study design and assessment of farm management

A cross-sectional study was employed; it involved purposive sampling of units with at least 10 animals and above. At the farm level, all pigs above three months of age were involved in the study. A well-designed checklist was used to assess farm management aspects (housing and feeding). Pig house were classified as 1= ideal (with concrete floor, division of pens and roof with corrugated iron sheets; 2 = moderate (i.e. concreate floor, divisions of pens and thatch roof; 3 = poor (i.e., sandy floor, no divisions of pens and roof with thatch/no roof). Feeding was categorised into three levels based on energy value: 1 = high concentrate diet (> 80% depends on food remains from restaurants i.e., swills and 20% maize bran), 2 = moderate concentrate (50% mixture of food remains and 50% maize bran) and 3 = low concentrate diet (70% depend on horticultural crop residues and 30% maize bran).

Blood sample collection and handling

Blood samples were collected early in the morning between 8 and 10 am to avoid a circadian variation of cortisol and heat stress. Animals were restrained using pig snare; to minimize stress to animals only two operators were allowed in a pen at a time. Blood samples (about 5 mL) were collected directly from the anterior vena cava using plain and heparinized vacutainer for cortisol and biochemical analyses respectively. Samples were transported to a laboratory at the CVMS using cool boxes. In the laboratory, the samples were centrifuged at 3,000 rpm to obtain serum and plasma, which were refrigerated at -4 ^{0}C pending analysis.

Determination of cortisol and biochemical parameters

Serum cortisol levels were determined using micro plate enzyme immunoassay (Accu Bind ELISA Microwell). This is a competitive immunoassay specifically designed and validated for the quantitative measurement of cortisol in serum. Biochemical parameters such as plasma glucose, total protein and cholesterol were analysed because they are good indicators for nutritional status of animals and their levels are also affected by the stress hormones. Determinations of the blood glucose and cholesterol levels were done using standard methods as explained by Trinders (1969). Total protein was determined by Biuret method using Erba ® test kit as described by WHO (2006).

Data analysis

The results were analyzed using SPSS statistical package version 20 (2011). Data were subjected to univariate analyses using two-way ANOVA and t-test to compare mean values among different variables. Tests were conducted at 95% confidence

interval and significance level of 5% was considered significant.

RESULTS

In the current study, pigs from the peri-urban farms had significantly higher serum cortisol levels (4.96 ± 0.19 µg/dL; $p < 0.001$) than their urban counterparts (3.11± 0.15 µg/dL). Moreover, weaner pigs and boars had significantly higher levels ($p < 0.001$) compared to the other tested groups. The diet offered had significant influence ($p < 0.05$) on serum cortisol level in that pigs fed on low concentrate diet had significantly higher serum cortisol levels than those maintained on moderate or high concentrate diets (Table 2).

Plasma cholesterol levels were significantly higher ($p < 0.05$) in male pigs than in their female counterparts particularly in boars from the urban farms. It was further observed that pigs maintained on level 1 feed (> 80% depends on food remains from restaurants i.e., swills and < 20% maize bran) had high cholesterol concentration than those on low concentrate diet. Plasma concentrations of glucose were not significantly different ($p > 0.05$) among different groups although boars had higher levels in comparison to other groups in both study areas. Also, pigs maintained on moderate concentrate diet had higher plasma glucose levels than those receiving high concentrate diets. Plasma total protein did not differ significantly ($p > 0.05$) among pig groups of location and the levels were within normal range i.e.6.1 - 6.9 mg/dL.

Table 2. Serum cortisol levels in different groups of pigs

		Cortisol (µg/dL)*		
		General	Urban	Peri urban
Sex	Female	3.67 ± 0.17	3.08 ± 0.18	4.85 ± 0.29
	Male	3.81 ± 0.24	3.39 ± 0.37	4.21 ± 0.25
Location	NA	NA	3.11 ± 0.15	4.94 ± 0.22
Stage	Boar	4.96 ± 0.19***	4.11 ± 0.13	5.66 ± 1.79***
	Gilt	3.12 ± 0.20	2.87 ± 0.59	4.39 ± 0.39
	Grower	3.21 ± 0.88	4.31 ± 0.28	5.19 ± 0.56***
	Sow	3.39 ± 0.72	3.13 ± 0.28	4.98 ± 0.56**
	Weaner	3.77 ± 0.28	4.57 ± 0.18	5.71 ± 0.52***
Diet	High	NA	3.01 ± 0.27	NA
	Moderate	NA	3.23 ± 0.28	NA
	Low	NA	NA	4.96 ± 0.19

Normal values of serum cortisol in pigs: 2.75 – 3.18 µg/dL [Radostitis et al 2000]

Table 3. Plasma biochemical parameters of pigs from selected urban and peri-urban areas of Morogoro

		Glucose (mg/dL)			Total protein (g/dL)			Cholesterol (mg/dL)		
		Overall	Urban	Peri urban	Overall	Urban	Peri urban	Overall	Urban	Peri urban
Sex	Female	80.2 ± 1.9	82.1 ± 3.1	78.4 ± 2.3	6.5 ± 0.9	6.4 ± 0.1	6.5 ± 0.1	78.4 ± 2.8	98.9 ± 4.3	57.9 ± 2.4
	Male	86.5 ± 2.5	87.7 ± 4.1	85.3 ± 3.2	6.2 ± 0.1	6.2 ± 0.1	6.2 ± 02	84.2 ± 3.8	107.4 ± 8.0	61.0 ± 2.6
Stage	Boar	90.7 ± 2.2	104.1 ± 10.1	94.6 ± 10.3	6.3 ± 0.1	5.8 ± 0.2	7.0 ± 0.6	59.5 ± 3.4	131.9 ± 31.8*	44.6 ± 5.4
	Gilt	83.0 ± 2.2	81.2 ± 4.6	77.8 ± 10.0	6.3 ± 0.1	6.4 ± 0.5	6.2 ± 0.2	103.2 ± 3.3	110.8 ± 40.2	66.3 ± 3.8
	Grower	98.0 ± 9.0	88.4 ± 4.6	80.2 ± 3.7	6.2 ± 0.5	6.4 ± 0.5	6.2 ± 0.2	81.9 ± 10.8	110.9 ± 40.2*	66.3 ± 3.8
	Sow	76.8 ± 7.3	88.8 ± 5.5	82.0 ± 3.1	6.3 ± 0.4	6.6 ± 1.6	6.9 ± 0.3	106.3 ± 8.8	95.7 ± 5.5	52.4 ± 4.2
	Weaner	84.1 ± 2.8	78.7 ± 3.6	78.9 ± 4.9	6.4 ± 0.1	6.1 ± 0.2	6.2 ± 0.1	86.9 ± 3.4	102.1 ± 8.6	59.6 ± 3.0
Diet	High	84.2 ± 2.5	68.4 ± 2.6*	NA	6.7 ± 0.1	6.2 ± 0.2	NA	79.1 ± 3.0	150.5 ± 5.1	NA
	Moderate	77.8 ± 2.9	96.5 ± 2.8*	NA	6.1 ± 0.1	6.4 ± 0.3	NA	89.6 ± 3.5	56.7 ± 3.1	NA
	Low	68.4 ± 2.6	NA	83.5 ± 2.0	6.1 ± 0.2	NA	6.5 ± 0.1	NA	NA	59.2 ± 6.9

Normal values: [Plasma glucose 85–150 mg/dL; Total protein 3.5–6 g/dL; Total cholesterol 28–48 mg/dL adapted from Radostits et al 2000]

Result on farm management assessment showed that intensive system, whereby pigs are housed in a flock of 5 to over 40 animals and partitioned into different pens depending on the growth stage and physiological status was the most commonly practiced pig farming system in the study area. About 53% of piggery housing were categorized as class 1 and 2 and were all found in the urban area whereas the remaining percent was class 3 and were all in the peri-urban settings. The nature/type of feeds used in the two study areas was largely influenced by local availability of the major feed ingredients such as maize bran and vegetable residues. About 80% of urban pigs were maintained on high concentrate feed due to availability of swills (restaurant leftovers) and maize bran whereas low concentrate feed with mainly vegetable residues dominated in the peri-urban settings.

DISCUSSION

The current study evaluated the welfare of pigs kept under intensive system using serum cortisol levels and some biochemical profiles as indicators of stress. The high levels of serum cortisol in peri urban pigs could be a result of some stress factors which were observed to associate management, i.e., housing and feeding. For instance, poor housing without proper roofing and partitioning was a characteristic feature of peri-urban farms involved in this study; which may have subjected the animals to environmental and social stress. Feeding of low concentrate diet to peri-urban might have also contributed to higher serum cortisol as a compensatory mechanism to low energy intake. As a regulator of metabolizable energy in the body, serum cortisol increases hepatic gluconeogenesis and the peripheral release of substrates, primarily from muscle, required for gluconeogenesis (Velazco *et al.*, 2013; Maduka *et al.*, 2015).

Findings on plasma biochemical parameters revealed a remarkable association with the type of feed offered to the animals. That is, the high levels of plasma cholesterol in urban pigs were due to the fact that the animals were maintained on high concentrate feed with large proportion of swills known to contain large quantities of fats and carbohydrate. A similar trend was observed in case of the plasma glucose although the difference was not statistically significant. Undoubtedly, the nature of feeds used in the study area was mainly affected by availability and affordability of feed components such as maize, wheat, sorghum, soybean and sunflower which increases competition for basal feed with human and poultry for feed resources, thus increasing cost of production. Also, recurrence of droughts due to global warming are exacerbating the situation thus resulted into increased cost of production in the pig industry (Kanengoni, 2016).

The current study also revealed that intensive system, whereby pigs are permanently housed in a flock of 5 to over 40 animals, is the most practised system in Morogoro. Similar findings were reported by Wilson and Swai (2014) who observed that in rural areas of Tanzania, pigs are totally confined in rudimentary housing of local materials and with little consideration for hygiene or general welfare. In this study, it was further noted that majority (80%) of pigs kept were breeding sows, growers, weaners and fewer males. This showed that most farmers get rid of male pigs through selling, culling or exchanging them with males from a distant pig keeper to avoid inbreeding; this is an interesting observation implying that most small-scale farmers had knowledge on good piggery management. This observation differs from findings by Karimuribo *et al.* (2011) who noted that the majority of small-scale farms in Iringa Region had more growers, followed by adult pigs and relatively small proportion of piglets.

It is concluded that serum cortisol was a reliable indicator to assess stress in pigs and this was affected by some observed stressors such as housing and type of diet.

Acknowledgements

This study was funded by DANIDA - Urban and Peri Urban Livestock Farming and Environment and Public Health Challenges project in Tanzania and we are grateful for the financial support. We also acknowledge the assistance provided by the extension officers and also sincerely thank farmers for participating in this study.

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Assessment of health status, handling and management of working animals in Tanzania: A case study of Donkeys in Kilosa district

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SUMMARY

The use of donkeys as drought animal has been a tradition in various parts of Tanzania. Donkeys transport goods to markets, children to schools, sick people, women in labor and water and supplies to remote communities. Despite the importance of donkeys in the community, little is known on the common diseases affecting donkeys in Tanzania. In addition, there is no structured management systems, handling, reproduction and/or disease control programmes. According to a report by ministry of Livestock development and Fisheries, the number of donkeys is decreasing. Several measures have been undertaken to save these animals including stopping of the slaughter operations which were carried out in Dodoma municipality. However, to fulfill the demand, capacity building on proper management system, handling, disease surveillance and control systems is important. This study therefore assessed the health status of donkeys, handling and management system practiced in Kilosa. Field visits, interviews, structured questionnaire and focus group discussion were used to gather information. Clinical examination was undertaken and samples collected for disease diagnosis. Sick animals were treated. The observation showed that donkeys suffer several ailments including poor management systems (housing, feeding, and manure disposal), infectious diseases, overworking, injuries and inbreeding. Community training, empowerment and veterinary service provision is recommended to improve the health and survival of donkeys in Kilosa.

Keywords: donkey, health, handling, Kilosa

INTRODUCTION

The human history shows that equine species (horses and donkeys) has been used as working animals for many years. In Tanzania, donkeys are the major working animals reared for various purposes. They play an important role for the survival of poor people in rural areas. They are traditionally used as means of transport, carrying water, firewood, harvests, commercial items and sick family members to hospital. Observations show that donkeys are the only means of transport in remote areas where road infrastructure is poor. According to a report by Ministry of Livestock development and Fisheries (2016), the number of donkey in Tanzania is decreasing. The decrease in donkey population is caused by deaths, poor fertility as well as slaughtering for skin and hides. Several measures have been undertaken to save the decrease in donkey population. One of the intervention was stopping of the slaughter operations which were carried out in Dodoma municipality. However, based on the importance of donkeys for rural livelihood, protecting these animals is important. The present project based on outreach programme undertaken at Kilosa District under support of SPANA. The goal of the project was to improve health of donkeys through community training and demonstration of good management practices. The project assessed the knowledge, attitude, behaviour and training needs of farmers; handling and management of donkeys in the area; social economic value of donkeys; and carried out diagnosis and treatment of diseases encountered.

MATERIALS AND METHODS

The present study based on the outreach project which was conducted at Kilosa District. A total of 37 households were visited in 9 wards. The households were selected randomly. Structured questionnaires were used to assess farmers' knowledge on health, handling and management aspects. In addition, field visits and on-site observation method were used to assess the housing status and manure disposal. Through group discussion, the behavior and perception of community towards animal welfare was examined. Clinical examinations were done on site and sick animals were treated. Faecal samples were collected from rectum and evaluated for endoparasites by floatation method following standard procedure.

RESULTS AND DISCUSSION

Knowledge

It was observed that peoples' knowledge on recognizing sick animal was above average. Sixty percent (60%) of visited farmers in Kilosa could recognize a sick animal. Farmers recognized a sick animal by clinical signs such as dullness, reluctant to carry the load, rough hair coat and swelling under the brisket. However, there was weakness in relating the observed clinical signs and the disease versus malnutrition/exhaustion. All visited farmers showed quest of knowledge about donkey diseases, management of donkey diseases and access to veterinary services.

Handling

The donkey owners showed high attachment to their animals. Donkeys were used as source of income for the household i.e. used to transport water for domestic and commercial purposes, carry goods to the market, carry harvested crops from farms to the house for the owners family and other families on payment basis, as well as used as means of transport to take sick family members especially during rainy season. Despite all the benefits, it was observed that there was no saddle used to protect the animals. Ropes were used to hang the loads on animals back (Fig 1a). This act caused bruises and large wound on the back of the animal and under the tail (Figure 1b&c).

Figure. 1a. Photograph of donkey carrying luggage. Note type of saddle and ropes holding the luggage

Figure 1b&c. Photographs of donkeys after removal of the luggage. Note presence of wounds on the back caused by local saddle.

Management systems and Housing

In wards visited, donkeys were reared under free range grazing system. A few farmers were using maize bran as supplement in some occasions. Water was observed to be a problem in some villages. In these villages, animals rely on salty well and rain water. More than 90% of visited farmers had no proper house for donkeys. Three types of houses were encountered. Type one was an open roof boma made up of thorny shrubs (Figure 2a).

Figure 2a. Type 1: Ope roof Boma

Type two was open or closed roof boma made up of timber and pieces of wood with gravel/cemented floor (Figure 2b). Type three was timber house covered by pieces of iron sheet (Figure 2c). In this district type two was most common whereas type three was rarely seen. In addition, a few farmers were mixing donkeys and cattle in the same house. Some families tired their animals under the tree outside the house. There was neither proper cleaning of the houses nor manure disposal.

Type 2b. Open roof wood Boma: Ilonga

Figure 2c. Type 3. Timber house covered by pieces of iron sheet

Veterinary services

There was no veterinary service provision specifically for donkeys. Farmers claimed not to have ever been visited by state veterinarian specifically for treating a donkey. Animals were left to recover themselves or die when there is health problem. There was no history of vaccination, deworming or control of ectoparasites.

Social economic value of donkeys

At Kilosa all visited farmers showed high dependency of donkeys as a means of transport and income generation. In addition, the live animals were sold locally to other farmers. The cost of live animal range between Tsh. 70,000/= and 200,000/=. For commercial purposes, the cost of transporting luggage depends on the distance. For example, at Mowe village to the market place, they charged between 10,000/= and 12,000/= (Tsh.) per trip for a load weighing 100kg. The observed challenge was low price of live animal and difficulties in accessing the national market in Dodoma.

Disease surveillance and/or diagnosis and treatment

Clinical examination was carried out in all animals attended. A total of 172 animals were examined. During clinical examination faecal samples were collected and stored pending analysis at parasitology laboratory in the College of Veterinary medicine and Biomedical Sciences (Figure 3a).

Figure 3a. Faecal sample collection from rectum

Based on clinical signs, all animals which had health problems were treated on site. Analysis of faecal samples revealed worm infestation (EPG range 200-900). The observed species were *Strongyles* and *Parascaris* spp (Figure 3b&c).

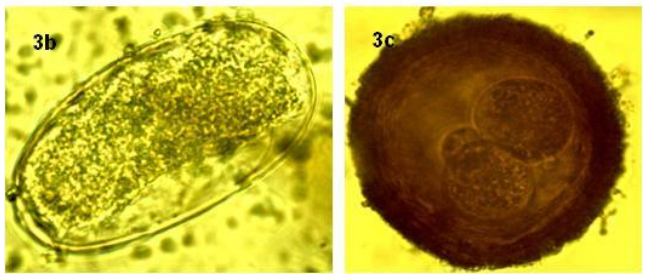

Figure 3. Photomicrographs showing (a) *Strongyle* egg and (b) *Parascaris* egg observed in infested donkeys

Other health problems found included lameness (fractures, foot rot, overgrown hooves skin lesions, wounds (Figure 4), worm infestation, ectoparasite burden, tick and fly bites. A few animals in had history of respiratory problems and nervous signs. Infested animals were dewormed by ivermectin.

Figure 4. Photograph of a donkey at kilosa district. Note overgrown hoof which caused lameness in this animal

Training

Training of farmers on good management practices and overview of donkey diseases were carried out in three wards in Kilosa (i.e. Msowero, Mkwatani and Ilonga). The training covered issues of management (housing, feeds and feeding), deworming and control of ectoparasites, access to veterinary services and market of donkeys.

Based on the findings of this study it is concluded that Donkeys are valuable animals and important for the survival of rural people living in remote areas (economic and social value of the animal). Capacity building on animal welfare (handling, management practices) and disease control strategies is needed to improve health of donkeys and livelihood of rural communities living in poverty.

ACKNOWLEDGMENTS

This project was funded by SPANA. We acknowledge cooperation and smooth coordination offered by the office of District Veterinary officer at Kilosa (Dr Yuda and his co workers.). We thank farmers for their participation and providing information for implementation of the project. Technical assistance provided by Mr Msalilwa (Parasitology technician) is greatly acknowledged.

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Welfare and contribution of draft animals to the transformation of the agricultural sector

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SUMMARY

Draft animals were in common use in Mesopotamia before 3000 BC for farm work and for pulling wheeled vehicles. Their use spread to the rest of the world over the following 2,500 years. Despite the enormous technological upheavals of the 20th century, both in agriculture and in many other sectors of the global economy, animal draft has remained important in many countries, even though is very old. The draft animals enable people especially in some of the poorest communities to increase their incomes and productivity significantly in the agricultural sector (plowing, sowing, weeding and transportation). These animals simplify the circulation, distribution and commercialization of agricultural products but this benefit is often compromised by their poor welfare i.e. how they cope with the conditions in which they live. An animal is in a good state of welfare if as indicated by scientific evidence it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress. The common problems are poor animal health, nutrition, overwork and poor harness materials. Studies show that simple interventions mainly community engagement in humane education, awareness and policy enforcement can improve draft animal's welfare thus bringing huge transformations in the agricultural sector both for the animals and people.

Keywords: draft animals, global economy, agricultural sector, income, animal welfare, interventions, transformations

INTRODUCTION

Animal welfare is defined as how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare if (as indicated by scientific evidence) it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress. Good animal welfare requires disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling and humane slaughter/killing. Animal welfare refers to the state of the animal; the treatment that an animal receives is covered by other terms such as animal care, animal husbandry, and humane treatment (OIE).

The work of the draft animals in agricultural sector include draft animals exist in all regions of the world; they assist in eliminating poverty, reducing drudgery and creation of wealth. Also animal traction is particularly important for food security in smallholder farming systems, draft animals can assist directly with crop production ploughing, planting, and weeding). Food production, distribution and rural trade are also assisted through animal-powered transport (on farm, marketing, riding, pack transport). Other works are animals save household (women and children) time and effort by carrying water and fuel wood. Animal power can also be used for water-lifting, milling, logging and land excavation and road construction. Working Animals although are often overlooked, they are the foundation of economy and productions for millions of people in both rural and urban settings (The Donkey Sanctuary).

General Benefits of draft animals in Agricultural transformation

There are many benefits associated with **draft animals in agricultural transformation and these include** the draft animals themselves contribute to food production through milk, meat, manure and offspring. Pack animals and carts facilitate the marketing of produce, stimulating local trade and production. Animals can be very important for carrying domestic water and fuel, reducing drudgery (particularly for women) and releasing time that can be used in other productive or socially important tasks, normally available and affordable to people in rural areas and fragile environment Animal power requires little or no foreign exchange. Money spent on motors and machinery is exported from rural areas. Money invested in animal power circulates within rural areas, helping to revitalize rural economies.

Women, men and children all benefit from animal power

Provided access to animal power is widespread, it can benefit all members of society. Access may come from ownership, borrowing or hiring which

provides timeliness and so spreading the costs and benefits.

Historically, men have tended to control animal power technologies, including ploughing and transport. In many countries, women are having increased access to work animals. In much of the world women, as major carriers using shoulder and head for water, fire wood, food grains and agricultural products can benefit particularly from draft animals. "A woman without a donkey is a donkey herself

Draft animals need care as they are dependable power sources

Suitable animals must be able to thrive under local conditions and be strong enough for the work required. They must be affordable and locally available. Donkeys are becoming increasingly important in agricultural production and rural transport systems because of their low cost and longevity e.g. can live up to 50 years. Good animal health is a prerequisite for the success of animal traction. Many health problems can be avoided by a combination of indigenous knowledge and modern prevention systems (vaccination/prophylactics) and good management e.g. grooming, feeding, watering and attention to harnessing.

Draft animals are complementary to other power sources

Manual, animal and engine power sources are all important in agricultural development. Animals and engine powered machines help to reduce human drudgery and allow people to achieve more with their time. Engine power, where available and affordable, can achieve the highest savings in time and labour. Many smallholder farmers would like to benefit from tractor power, but such aspirations are often unrealistic. Engine power tends to be appropriate for large-scale farming and long-distance transport. Animals are often more affordable and appropriate for small-scale farming and local transport.

Challenges of draft animal power

Low or no animal welfare awareness to stakeholders as result animals are suffering while working. Animal power is always not incorporated within development strategies and programmes. No favourable policy environment to be sustained by the private sector. Draft Animal Power (DAP) is forgotten- the media have tended to exaggerate the decline and the outdated aspect of animal power and disregarded. This results in a lack of general knowledge among decision-makers and scientists, and leads to policies that further marginalize rural communities. Lack of data on working animals including on their economic contributions and roles. Global fast growing Donkey Skin and Meat Trade

Possible solutions

A number of possible solutions to challenges can be forwarded that include: (i) Humane Education to all animal power stakeholders e.g. farmers, professionals, leaders and young generation; (ii) Including animal powers issues within development strategies and programmers -In recent years, it has become common to include environmental and gender impact statements in development strategy documents. In a similar way, animal power options in plans relating to food security, rural infrastructure and services and transport should be considered; (iii) Favourable policies to private sector-The aim of governments and development agencies should be to ensure a suitable policy environment to enable private sector support services to be maintained or to develop; (iv) Legislation or development processes should not isolate animal power users or support services, either directly or indirectly e.g. subsidies, exclusion of animal-powered transport and legislation more favourable to factories than village blacksmiths

Making Draft Animal Power (DAP) data available:

The draft animal power data can be made available through: (i) The qualitative benefits of working animal use are mostly well recognized by the animal owners and their families. There is very little evidence of the monetary value of DAP versus human labour, thus farmers and other groups that use working animals may not be aware of the impact of those animals on their income e.g. massive selling of donkeys for skin and meat trade; (ii) Both the qualitative and quantitative especially financial impacts of DAP will provide a compelling case for greater recognition of the importance of working animals to human livelihoods by national and international decision and policy makers, NGOs and educational institutions. There are very few well-designed and large-scale studies of those impacts.

Conclusions

Based on this paper, it is concluded that

(i) Mainstreaming the welfare of draft animals in the Agricultural Sector is highly important for transformation in the sector.

(ii) Despite the years of neglect, animal traction is still a major farm power component by farmers, animal traction still offers the best option and opportunities for increase in farm sizes, reducing drudgery in farm work, reducing labour costs, raising yields and farm production in general.

(iii) However, these opportunities can only be utilized if farmers are assisted to acquire working animals and accessories at reasonable prices.

(iv) The availability of quality spare parts to ensure sustainability in the use of implements is also a condition for success.

(v) Finally, the need to research into the design and development of comfortable harnesses and more appropriate and durable implements for planting, weeding and harvesting should be supported and promoted.

ACKOWLEDGEMENTS

The author acknowledges the support from Tanzania Animal Welfare Society (TAWESO). All donkeys stakeholders are thanked for their contribution towards accomplishment of this keynote TVA paper.

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An Assessment of donkeys' welfare using physical and emotional parameters: a Case of Mkwinda EPA, Bunda Area, Lilongwe, Malawi

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SUMMARY

A donkey (*Equus africanus asinus*) is commonly used for traction by smallholder farmers in Lilongwe, the capital city of Malawi, where most of the donkey population is concentrated. Though donkeys' behaviour is often mistakenly interpreted as aggressive, they arestoic and generally considered weaker animals compared to oxen. This in turn raises concern over their treatment and general wellbeing as they are being used to support livelihoods of smallholder farmers. A study was done in Lilongwe, Mkwinda EPA, using the hands-on donkey welfare toolin form of a semi structured questionnaire, on 48 donkeys, to examine physical parameters which were body condition, lameness, wound availability, other signs of injury and disease and the emotional parameter which looks at the donkey's behavior which were scored 'best' to 'worst' numerated by values ranging from one to five respectively. Analysis was done using descriptive statistics and Spearman's rank order correlation. The results indicate most of the sampled donkeys fall under the ideal BCS of 3 while other parameters scored differently, giving "worst" scores of 5 for lameness and behaviour, "worse" scores of 4 for wounds and other signs of injury and disease parameter. There is a positive correlation between wounds and Lameness score, significant at 0.362 ($P< 0.05$). This insinuates that prolonged standing in grazing wetlands causes softening of the skin in the hoof regions which results in wound prevalence hence the difficulty in the gait. The study specifically revealed that the majority of the sampled donkeys were affected by poor water and feed intake, poor housing, lack of donkey veterinary clinics, unhygienic grazing areas, inappropriate handling of donkeys and lack of knowledge on the need for good donkey welfare. Furthermore, most of the donkeys, after reaching the end of their production cycle, were used for light loading, left on free range, or sold to others. Therefore, there is an intensive need to enhance donkey welfare.

Key words: hands-on donkey welfare, semi structured questionnaire, physical and emotional parameters, descriptive statistics, Spearman's rank order correlation

INTRODUCTION

A donkey (*Equus africanus asinus*) is a domesticated member of the horse family, Equidae (Rossel and Marshall, 2008). The wild ancestor of the donkey is the African wild ass, *Equus africanus*. The donkey has been used as a working animal for at least 5000 years (Rossel and Marshall, 2008). However, there are more than 40 million donkeys in the world, mostly in underdeveloped countries, where they are used principally as draught power (Starkey and Starkey, n.d.). Working donkeys are often associated with those living below subsistence levels where a small numbers of donkeys are kept for breeding especially in developed countries.

Overview of donkey farming in Malawi

Donkeys were introduced in Malawi in the late 1950's from neighbouring countries. Since the introduction of these donkeys into the country, there has been a high rate of inbreeding which lowers breeding values (Kumwendan.d.). There is very little knowledge in managerial practices in donkeys in areas where they are kept. Some of management practices which smallholder farmers fail to accomplish are: good housing, feed requirements and formulation, grooming and disease control (Kumwenda n. d.).

Approximately, there is a population of 15,000 donkeys in Malawi were a large number of these animals are mostly concentrated around Lilongwe where there is a high demand for carting (Mayers, 2015). Donkeys play an important and developing role in the whole agricultural system of Malawi, mainly through traction (Mayers, 2016; Kumwenda, n.d.). Donkeys easily adapt to environmental conditions such as drought, flooding and are hardworking as they support smallholder farmers. Significantly, donkeys are not meant for meat production in comparison with cattle and goats which are susceptible to theft, hence making the use of donkeys cost sound effective (Kumwenda, n.d).

As donkeys alleviate some of the productivity constrains, their welfare incur a lot of problems at farm level. Some of the welfare problems that donkeys face in Malawi are use of carts that are designed for oxen which cause wounds and sores on the shoulders and neck (Mayers, 2016). This results

in behaviours which are misread as aggression or stubbornness.

Most common welfare problems that donkeys experience, in Malawi, range from the usage of carts, more especially those designed for oxen (Mayers, 2016) since the kinds of harnesses donkeys used are not cost sound effective. The anatomy of a donkey is different from an ox which makes it unsuitable for donkeys to use the type of harnessing that is designed for oxen which causes wounds and sores on the neck and shoulders of donkey. As a result, the donkeys try to express their pain and discomfort through aggression, which is misinterpreted by viewers (Mayers, 2016) and has a negative impact on the welfare (Dibbits, 1997). Other problems include foot rot, skin lesions, malnutrition and dehydration which are common in areas where donkeys are raised (The Donkey Sanctuary, 2015).

In 2015, Malawi experienced floods in the Lower Shire which led to the Donkey Sanctuary Team to propose the need of a data census which will aim at improving donkeys' welfare (The Donkey Sanctuary, 2015). Currently, the initiative is facilitated by Lilongwe Society for the Protection and Care of Animals (LSPCA).

The Hands-on or 'the Hand' donkey welfare assessment

The Donkey Sanctuary introduced the approach where the Hands-on Donkey Welfare or 'the Hand' toolwhichexamines the physical parameters namely: body condition, lameness, wound availability, other signs of injury and disease and the emotional parameter which looks at the behaviour of the donkey as shown in Figure 1.

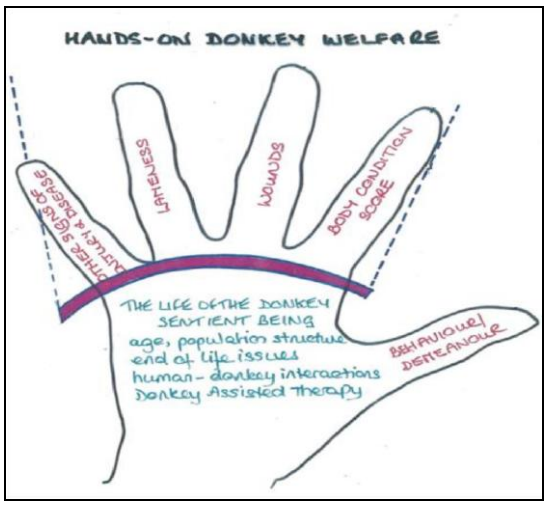

Figure 1. The Hands-on Donkey Welfare (Zedlacher, 2014)

Each parameter gives scores from a scale of one to five where one being the "best" and five the "worst". Furthermore, 'the Hand' relates to the 'five freedoms' which is a significant approach of checking whether a donkey is 'free from' hunger, thirst, distress, pain, and if the donkey is 'free to' express its natural behaviour (Mayers, 2015).

The main objective of this study was to assess the status of the welfare of donkeys around Bunda area using physical and emotional parameters and to specifically, identify factors which affect the welfare of donkeys and the use of donkeys after reaching the end of their production cycle.

METHODOLOGY

The study was conducted at Mkwinda Extension Planning Area (EPA) specifically Bunda area. A sample of forty-eight donkeys out of an approximate population of 1500 (Mathala, personal communication) was randomly selected from the households and a specific age of two and above was a benchmark.

The sampled donkeys were monitored using the 'Hands-on' approach in form of a semi structured questionnaire, asseen in Figure 1 in the Appendices. Furthermore, details of the donkey owner included the name, gender; problems associated with donkey husbandry were collected. Data were analysed using Statistical Package and Service Solutions (SPSS) where descriptive statistics and Spearman's rank order correlation was used to assess the score and below is the Spearman's Rank Order Correlation formula which was used.

(Geiger and Hovorka, 2016)

$$r = 1 - \frac{6\sum d^2}{n^3 - n}$$

Where: "*r*" is the Spearman Correlation Coefficient; "*d*"is the difference in rank between values of each variable; "*n*"is the total number of donkeys used for sampling.

Furthermore, Microsoft Excel was used to make further analysis on descriptive statistics after Spearman's rank order correlation.

RESULTS

The sampled donkeys encountered were left to graze or used for cart pulling.

BCS in the donkey sample population

Majority of the donkeys fall under the idealBCS of

3 (46%) followed by 2 (18.8%), 3.5 (16.7%) then 2.5 (14.6%) and lastly 2.75 (4.2%) as indicated in Figure

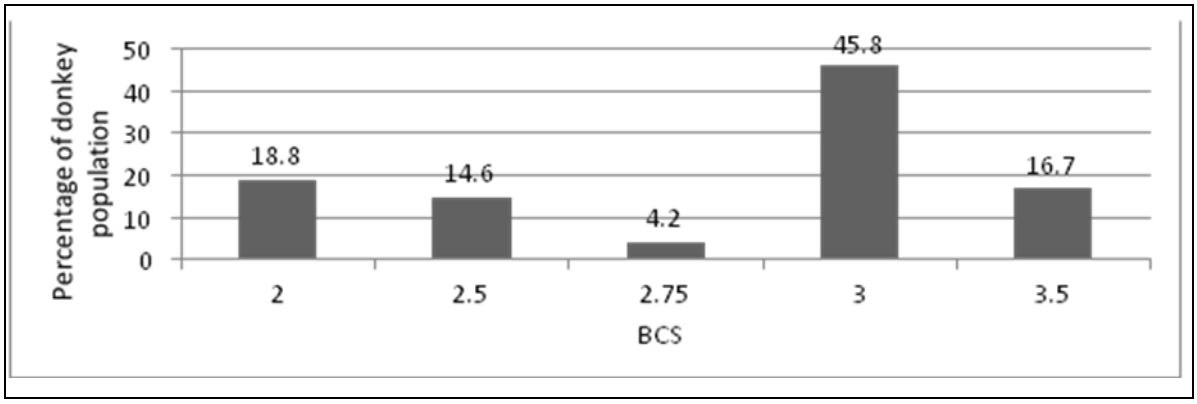

Figure 2. BCS in the donkey sample population

Wounds' score in the sample of donkey population

Figure 3 shows that majority of the sampled donkeys fall under the "worse" score of 4 (39.6%) followed by 5(35.4%) being the "worst", then 3(18.8%) and lastly with 2 (6.4%).

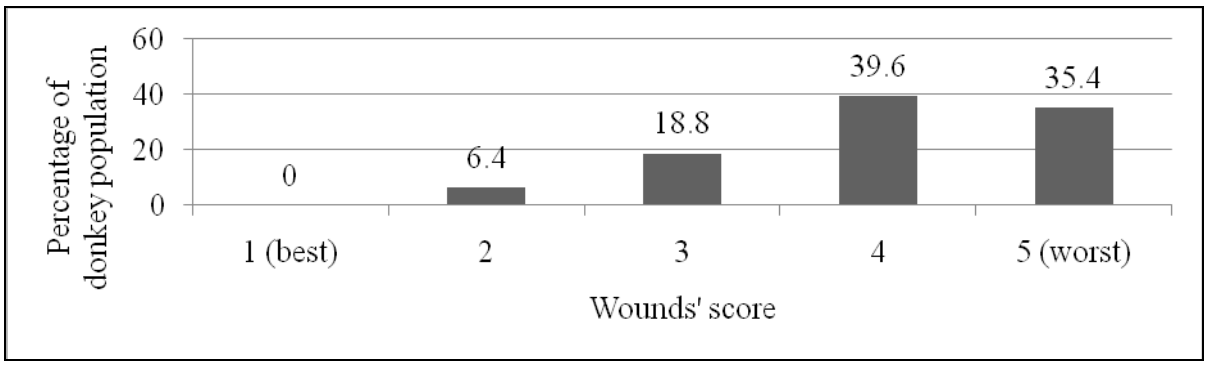

Figure 3. Wounds score in the sample of donkey population

Lameness score in the sampled donkeys

Figure 4 shows that majority of the sampled population fall under the "worst" score of 5 (75%) then the "worse" score of 4 (16.7%), and then followed by the "better" score of 2 (8.3%).

Other signs of injury and disease score 'Other signs' of in the sampled donkey population

Figure 5 indicates that most of the donkeys had a "worse" score of 4 (58.3%) on the other signs of injury and disease ('Other signs'). However, many of donkeys registered the "worst" score of 5 (37.5%), then, lastly by the "normal" score of 3 (4.2%).

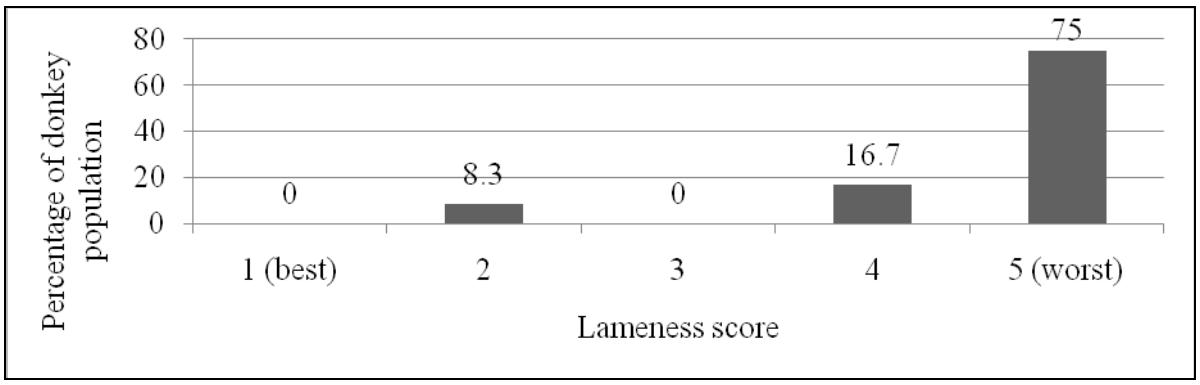

Figure 4. Lameness score in the donkey sample population

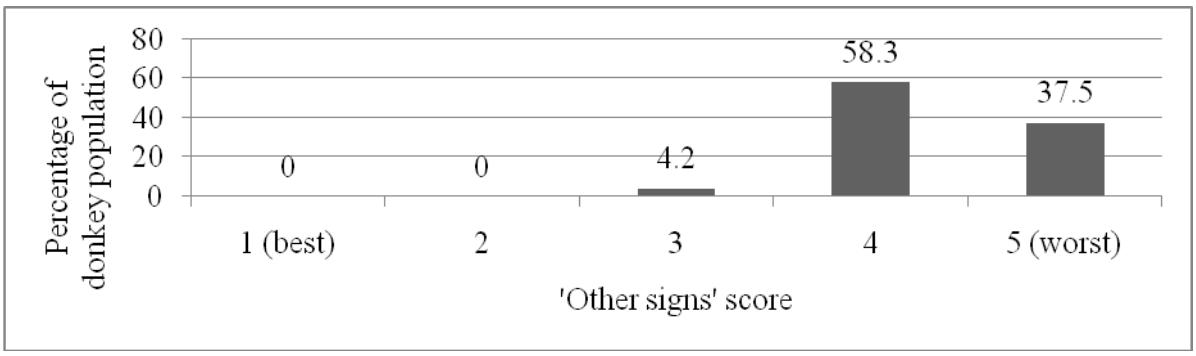

Figure 5. Other signs of injury or disease ('Other signs') score in donkey sample population

Behaviour score in the sampled donkey population

Figure 5 shows that there was prevalence in all behaviour scores having the "worst" score of 5 (35.4%) with the highest, followed by "normal" score of 3 (33.3%), then, by "worse" score of 4 (20.8%), "better" score of 2 (6.3%), then the "best" score of 1(4.2%) consecutively.

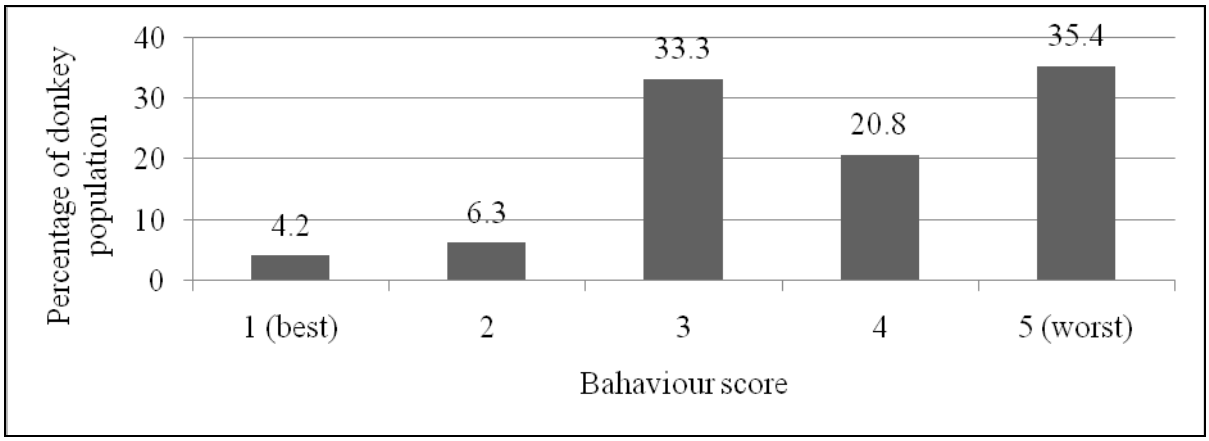

Figure 5. Behaviour score in the sampled donkey population

Correlation between emotional and physical parameters of the donkey

The physical and emotional parameters of the donkeys indicate that either some are positively or negatively significant correlated ($P< 0.05$, $P< 0.01$) as shown in Table 2. However, a number of parameters shows significant difference where correlations found to be significant for ($0.00\leq P\leq$ 0.19) being "very weak", ($0.20 \leq P\leq 0.39$) "being weak", ($P=0.40 \leq P\leq 0.59$) for "moderate", ($P=0.60 \leq P\leq 0.79$) for "strong" or ($P=0.80 \leq P\leq 1.00$) for "very strong", thus signifying varying strengths of correlation.

Table 8: Correlation between emotional and physical parameters of the donkey

	BCS	WO	LM	OS	PSL	NOG	FS	RTR	DOI	DRE	HCC	SOA
BC	-0.392**	0.440**	0.101	0.415**	-0.341*	-0.078	-0.174	0.886**	0.243	0.721**	0.357*	-0.369**
BCS		-0.603*	-0.570*	-0.503**	0.629**	0.441**	0.311*	-0.384**	-0.032	-0.403**	-0.369*	0.660**
WO			0.362*	0.473**	-0.778*	-0.240	-0.360*	0.399**	-0.136	0.461**	0.353*	-0.723**
LM				0.407**	-0.398**	-0.778**	-0.318*	0.055	-0.042	0.116	0.353*	-0.360*
OS					-0.344*	-0.368**	-0.400*	.424**	.028	0.220	0.887**	-0.097
PSL						0.354*	0.217	-0.272	-0.035	-0.374**	-0.226	0.583**
NOG							0.187	-0.111	0.179	-0.028	-0.346*	0.282
FS								-0.102	-0.092	-0.181	-0.162	0.236
RTR									-0.061	0.609**	0.389**	-0.391**
DOI										0.070	0.005	-0.017
DRE											0.188	-0.272
HCC												-0.510**
	BCS	WO	LM	OS	PSL	NOG	FS	RTR	DOI	DRE	HCC	SOA

Key: WO= Woundsscore, LM= Lameness score, OS= Other signs of injury or disease, PSL= Presence of skin lesions, NOG= nature of gait, FS= Faecal soiling, HCC= hair coat condition, PLA= presence of limb abnormalities, PHP=presence of health problems, STS= Signs of thermal stress, POS= Presence of swelling, PDW= presence of deep wounds, SOA=Signs of alopecia, BC= Behaviour score, RTR= reaction to researcher, DOI= Donkey owner interaction, DRE=Donkey's reaction to environment.

DISCUSSION

Most of the donkeys fall under the BCS score of 3 and 3.5 which indicates good BCS and optimum weight. This may be likely due to the time of data collection which was in the rainy season where plenty pastures were readily available for consumption and as some were pregnant mares hence the good BCS.

Many donkeys fall under a "worse" score of 4 for the Wounds' score due to owners mistreating their donkeys through whipping and providing poor harnessing. In addition, the usage of yokes causes wounds and sores on the neck and shoulders which cause skin lesions (Dibbits, 1997).

A large number of donkeys fell under "worst" score for Lameness which was attributed since the donkeys had overgrown hooves which results in lameness as well (Usman*et al*., 2015). Donkeys were overworked for over eight hours in a day without adequate feed or health care due to the high demand for transportation of goodsin Mkwinda EPA, Bunda area, hence making donkeys' necessitated animals (Kumwenda, n.d.). In addition, most smallholder farmers lackequid harnesses hence donkeys are forced to use ox-carts which carry heavyweight loads. The carts are often unbalanced hence this kind of load distribution affects the conformation of the donkeywhich causes swelling in the joints hence affecting the gait of the donkey (Kumwenda, n.d.; Animal Welfare Indicators, 2015).

Approximately half of the sampled donkey population falls under the "worse" score for the 'Other signs of injury and disease'. This implies that there was insufficient supply of balanced rations for working donkeys which resulted in poor hair coat condition. This can also be attributed by parasite infestation, poor housing conditions such as accumulation of dung and presence of tsetse flies and mosquitoes; worms from unclean temporary water points or pastures from contaminated wetlands which contribute susceptibility to diseases (Farmers, verbal communication). Furthermore, the donkeys are associated with grazing in wetland areas where the unhygienic water seeps through the skin of the donkey and causes irritation which results into scabbing and alopecia (The Sathya Sai Santuary, 2010).

Donkeys are naturally social animals as shown on the Behaviour score where many donkeys fall under the score range of 3, the ideal, to 5 which is the "worst". Furthermore, donkeys are stoical such that they do not live gregariously hence they have adapted and express stoicism as a defense mechanism. Additionally, most of the donkeys are mishandled by the owners hence they express a typical overprotective reaction towards strangers (Mayers, 2016).

Wounds and Lameness score are positively correlated and significant at 0.362 ($P< 0.05$) level of confidence as shown Table 1. This indicates that donkeys were overworked using improper harnesses which were also heavy which led to the change in body balance and gait (Kumwenda n.d.).

Reaction to the environment and researcher are positively correlated and significant at 0.609 ($P< 0.01$) level of confidence as shown in Table 1. Mishandling of donkeys by the owners is an indicator which fuelled the avoidance and fright of donkeys towards the researcher hence the researcher approaching them in their natural environment altered the calm behaviour of donkeys in the grazing areas or kraals which is contrary since donkeys are considered social animals (The Donkey Sanctuary, 2014).

Reaction towards researcher and Wounds score are positively correlated and significant at 0.399 ($P< 0.05$) which implies that the harsh treatment of owners toward donkeys resulted in availability of wounds on the donkeys hence frightful expression toward the researcher.

In conclusion, the study has revealed that majority of the sampled donkey population in Mkwinda EPA undergo the following factors: poor nutrition, poor housing, and lack of donkey veterinary clinics, unhygienic grazing areas, lack of knowledge and inappropriate handling of donkeys. These factors were assessed using the Hands-on donkey welfare tool which relates to the five freedoms of animal welfare. Henceforth, the study has revealed that most of the sampled donkeys fall under the ideal BCS of 3 while other parameters scored differently, giving "worst" scores of 5 for lameness and behaviour, "worse" scores of 4 for Wounds and Other signs of injury and disease parameter. Secondly, majority of the donkeys after reaching the end of their production cycle were left on free range and some were sold whereas the rest, were used for light loading. Organisations like Lilongwe Society for the Protection and Care of Animals (LSPCA) should keep on working on improving the welfare of donkeys and various community groups should be educated on treating donkeys equally as with other livestock species that have a high social importance to farmers.

ACKNOWLEDGEMENTS

The authors acknowledge the Department of Animal Science at Lilongwe University of Agriculture and Natural Resources (LUANAR) for sponsoring this study and would like to give heartfelt thanks to my supervisor, Dr. O. Bakili and co- supervisor, Mr. J. Tanganyika. I am grateful for their academic mentorship that has shaped this project. I would also like to thank the Agricultural Extension Development Coordinator, Mr. Lorent Mwamutafya; the scout, Mr. Isaac Mathala, and all smallholder farmer participants of Mkwinda Extension Planning Area for facilitating the process of me getting the relevant information for the project. Lastly, I would like to give thanks to my parents and LUANAR and all the various people who gave me an opportunity to have the right conducive learning environment. Thank you very much all.

Table 9. Prevalence of physical and emotional parameters in the sampled donkeys

	Donkey category			
	Male	Female	Gelding	Pregnant
Presence of skin lesions	11(61.1)	12 (48)	0 (0)	0 (0)
Presence of deep wounds	2 (11.1)	2 (8)	0	0
Alopecia	12 (66.7)	9 (36)	0 (0)	0 (0)
Body condition score				
2	6 (33.3)	3(0)	0 (0)	0 (0)
2.5	3 (16.7)	4 (16)	0 (0)	0 (0)
2.75	1 (5.6)	1(4)	0 (0)	0 (0)
3	7 (38.9)	12 (48)	1 (50)	2 (66.7)
3.5	1(5.6)	5(20)	1(50)	1(33.3)
Wound score				
1 (best)	0 (0)	0 (0)	0 (0)	0 (0)
2	2 (11.1)	1(4)	0 (0)	0 (0)
3	5(27.8)	4 (16)	0 (0)	0 (0)
4	6 (33.3)	12 (48)	1(50)	0 (0)
5 (worst)	5 (27.8)	8(32)	1(50)	3 (100)
Lameness score				
1 (best)	0 (0)	0 (0)	0 (0)	0 (0)
2	3 (16.7)	1(4)	0 (0)	0 (0)
3	0 (0)	0 (0)	0 (0)	0 (0)
4	5 (27.5)	2 (8)	0 (0)	1(33.3)
5 (worst)	10 (55.6)	22 (88)	2 (100)	2 (66.7)
Nature of stride				
Natural stride	13 (72.2)	23 (92)	2 (100)	1 (33.3)
Delayed stride	5 (27.8)	2 (8)	0 (0)	2 (66.7)
Other signs of injury and disease score				
1 (best)	0 (0)	0 (0)	0 (0)	0 (0)
2	0 (0)	0 (0)	0 (0)	0 (0)
3	1 (5.4)	1 (4)	0 (0)	0 (0)
4	13 (72.2)	12 (48)	0 (0)	3 (100)
5 (worst)	4 (22.2)	12 (48)	2 (100)	0 (0)
Heat stress	9 (50)	9 (36)	1 (50)	2 (66.7)
Presence of faecalsoiling	1 (5.6)	1 (4)	0 (0)	0 (0)
Limb abnormalities	5 (27.8)	2 (8.3)	0 (0)	0 (0)
Poor condition coat	14 (77.4)	13 (52)	0 (0)	3 (100)
Behaviour score				
1	8 (44.4)	7 (28)	1 (50)	1 (33.3)
2	3 (16.7)	6 (24)	0 (0)	1 (33.3)
3	5 (27.8)	9 (36)	1 (50)	1 (33.3)
4	1 (5.6)	2 (8)	0 (0)	0 (0)
5	1 (5.6)	1 (4)	0 (0)	0 (0)
Reaction to researcher				
Apathetic	2 (11.1)	1(4.2)	0 (0)	0 (0)
Avoidance	4 (16)	10 (40)	1 (50)	1 (33.3)
Avoidance and fright	4 (22.2)	10 (40)	1 (50)	1 (33.3)
Curious	7 (38.9)	6 (25)	1 (50)	0 (0)
Interested	4 (22.2)	6 (24)	0 (0)	1 (33.3)
Indifferent	1 (5.6)	2 (8)	0 (0)	1(33.3)
Donkey owner interaction				
Calm and gentle	5 (27.8)	9 (36)	2 (100)	1 (33.3)
Rough and jerky	11 (61.1)	13 (52)	0 (0)	1 (33.3)
Angry and scolding	5 (27.8)	9 (36)	2 (100)	1 (33.3)
Donkey's response to environment				
Alert and inquisitive	12 (66.7)	13 (52)	1(50)	3 (100)
Startled	3 (16.7)	7 (28)	1 (50)	0 (0)
Startled and frightened	2 (11.1)	5 (20)	0 (0)	0 (0)
Fearful	0 (0)	0 (0)	0 (0)	0 (0)
Indifferent	1 (5.6)	0 (0)	0 (0)	0 (0)

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Husbandry practices, disease management and production profiles among smallholder layer chicken farms in Morogoro Municipality, Tanzania

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SUMMARY

Husbandry practices, disease management and production profiles were examined among 46 smallholder layer chicken farms in Morogoro, Tanzania; using a structured questionnaire and direct observations. Farmers kept their chickens in deep litter system or in cages. The average flock size was 350 birds (97-8000). Chickens were stocked at day old or at 12 to 14 weeks of age. For day old chickens farmers used antimicrobials in the first seven days, combined with multivitamins. Farmers vaccinated chickens against Newcastle disease, gumboro and fowlpox. Beak trimming was performed at 12 to 16 weeks. Most farms had a foot bath at the entrance to poultry houses. Chicken house cleaning was regular for cage system. Some farmers sent dead birds to veterinary centres for necropsy and others disposed or fed them to dogs. Some farmers used commercial feeds for their chickens whereas others made their own. The average age at point-of-lay was 20 weeks (16-22) and peak lay was attained two to three months later. Laying percentage varied greatly between flocks (55-90; mean 76); and fluctuated within flocks. Farmers attributed drop in egg production to diseases, feed quality, stresses and use of sulphur drugs. Viral, bacterial, parasitic and nutritional diseases were reported to be common in the flocks. The culling age was 18 to 24 months. The study revealed inadequacies in layer chicken husbandry and flock health management. Eventually the production was poor with irregularities. Improvements in husbandry and disease management would increase and sustain production.

Keywords: Bio-security, Husbandry practices, Layer chickens, Morogoro, Production profiles

INTRODUCTION

Chicken production constitutes one of the major agricultural activities in Tanzania, with 94% of the total chicken population kept in villages and in peri-urban areas under the traditional free range system, in most cases owned by women (MAFC, 2008). The traditional poultry system is the largest, supplying more than 90% of poultry meat and eggs consumed in rural areas, and 20% of the same consumed in urban areas. A decade ago, improvements in husbandry practices and adoption of a thermostable vaccine (strain I_2) to control Newcastle disease, resulted into an increase in egg production from 790 million in 2002 to1.8 billion in 2006 (MLD, 2008; Msami, 2008). Eventually, the per capita consumption of eggs rose from 23 eggs in 2002 to 50 eggs in 2008 per person per year. Despite an increase in supply of eggs the demand is still high, and the per capita consumption of eggs in Tanzania is quite low, compared with 106 eggs per person per year for Africa and 190 for high income countries (Gueye, 2004). The rising demand for eggs calls for more investments in the intensive layer chicken production and the poultry industry as a whole.

Layer chickens are among the most adaptable domesticated animals and more people are directly involved in layer chicken production throughout the world than in any other single agricultural enterprise (Bishop, 1995). Commercial poultry farming in Tanzania was introduced during the 1980s, and overtime visible growth in the production of layers has been observed to supplement egg supplies particularly in urban areas. The production of layer chickens is a better source of earning cash because it offers higher net returns (Paul *et al.*, 1990); as compared to the production of local chickens which however predominate in the country. Small and medium enterprises have increased the numbers of layers from 27 million in 2001 to 38 million in 2008 while the commercial stock increased from 20 million to 25 million. On average, 5.5 million hatching eggs and one million day old chicks are imported annually to produce a total of 25 million day old chicks for commercial purposes (MLD, 2008). This figure is low compared to the actual requirement of 60 million day old chicks per year.

The production of layer chickens is gaining popularity in Tanzania with people's engagements at different scales. The small scale backyard production predominates, mostly engaging women either as a primary or secondary source of income at household level. This study aimed at evaluating husbandry practices, disease management and production profiles of layer chicken kept by smallholder farmers in Morogoro Municipality. The information created will provide basis, for different players in the livestock sector with bias on the poultry industry, to identify entry points for interventions aimed at making the industry a

profitable venture.

MATERIALS AND METHODS

Study area

The study was conducted in Morogoro Municipality, Eastern Tanzania. Geographically the study area is located within the tropical Savannah woodland at the foot of the Uluguru Mountains at the altitude of 500-600 m above sea level; and the latitude 5.7 N and 10^0S and longitude 35.6^0W and 39.5^0E. The average annual rainfall is between 600-1000mm with peaks usually in December and April. Among the farming activities in the Municipality are commercial (broiler and layer) and free ranging chicken production, mainly as backyard activities involving family members. Layer chickens produce eggs which are sold directly to consumers or to retailers and whole sellers. At the end of the laying period layer chickens are sold as spent layers for human consumption.

Study design, sample size determination and sampling procedure

This was cross-sectional study conducted from April to July 2017 with the aim of determining husbandry practices, disease management and production profiles of layer chicken kept by smallholder farmers in Morogoro Municipality. In total 46 farmers from different locations in the Municipality were involved based on availability and willingness to provide information and allowing the researcher to access poultry units for onsite observation of some attributes. A list of layer chicken keepers in the Municipality was made by the aid of chicks suppliers, input suppliers and veterinary practitioners involved in provision of services to the farmers. A snowball sampling technique was also adopted as farmers tend to know fellows in the industry for different reasons.

Data collection

During collection of data, questionnaire survey (face to face interviews) and direct observation methods were used. At each household, general information of the respondent and poultry farming history were collected first. Collection of information on husbandry practices focused on chicken housing, flock management, feeding, daily routines and animal waste handling. Disease management information targeted areas of prevention and control. Production profiles were assessed based on collection of information in the following areas: the average age at point-of-lay, age at peak lay, the laying percentage, production trends, the culling age and factors affecting egg production.

RESULTS

Respondents socio-demographic profiles and poultry farming history

A total of 46 layer chicken keepers were interviewed in this study reached in different wards i.e. Boma (4.4%), Kichangani (8.7%), Kihonda (8.7%), Kihonda Maghorofan (17.4%), Kilakala (4.4%), Lukobe (13.04%), Mafisa (4.4%), Magadu (4.4%), Mazimbu (21.7%), Misufini (4.4%), Mlimani (4.4%) and Mkambalani (4.4%). Their socio-demographic attributes are summarized in Table 1. There were different experience levels in chicken keeping among the 46 farmers interviewed in this study. Twenty farmers (43.5%) had an experience of up to 5 years, 16 farmers (34.8%) had an experience of between 6-10 years, 10 farmers (21.7%) had an experience of 11 or more years the longest being 28. On entering the poultry industry majority of the interviewed farmers (52.2%) started with layer chicken keeping, 16 farmers (34.8%) started with broiler keeping, four farmers (8.7%) started with local chicken keeping and two farmers (4.4%) started with both broilers and layers. Sixteen famers (34.8%) among those interviewed had attended seminars on chicken husbandry and health. Most of the farmers attended the seminars in Morogoro Municipality (95.6%), while a few (4.4%) attended the seminars in Dar es Salaam. Organizers of the different training events were NGO's (21.7%), feed manufacturing companies (4.4%), drug companies (60.8%), chick supply companies (8.7%) and veterinarians in private practice (4.4%). The sources of information for different aspects of poultry production among the respondents were: Agro- vet shops staff (42 respondents; 84%), fellows (25 respondents; 54.3%) and animal health professionals (40 respondents; 87.0%).

Table 1: Socio-demographic profiles of visited small holder layer chicken keepers in Morogoro Municipality

Attribute	Level	Number of responses	Proportion (%)
Sex	Males	14	30.4
	Females	32	69.6
Age distribution	20-30 years,	6	13.04
	31-45 years	18	39.1
	46-70 years.	22	47.8
Marital status	Married,	40	86.9
	Single	4	8.7
	Widowed	2	4.4
Level of education	Post-secondary	24	52.2
	Secondary level	16	34.8
	Primary	6	13.04
Household size	1-5 people,	30	65.2
	6-10 people	14	30.4
	11-15 people	2	4.4
Primary occupation	Chicken keeping	22	47.8
	Wage employees	12	26.1
	Business	10	21.7
	Retirees	2	4.4

Layer chicken housing and flock characteristics

Majority of the interviewed farmers (78.3%) used deep litter system of rearing their layer chickens; the rest (21.7%) used battery cages. On assessment of the house condition 34 farmers (73.9%) had their poultry houses in good condition and 12 farmers (26.1%) had the houses in a poor condition. Spots of wet litter were common for the deep litter system particularly around drinkers. Ventilation problems were among the deficiencies seen in chicken houses in poor condition. Of the farmers involved in this study, 43.5% kept layer chickens together with other types of birds in the same compound; whereas 26 farmers (56.5%) kept only layers. More than half (56.5%) of the farmers had single flocks at the time of visits during this study. Some of those with multiple flocks had bird flocks that were below the egg production age. The average flock age as found during the data collection period was 46 weeks (23-67). The average flock size was 350 birds (97-8000). Most of the farmers (84.8%) stocked chickens at day old, while a few stocked at 12 to 14 weeks of age. For multi-flock farms there was no clear physical separation between flocks; and most of the farms (60.9%) allowed movement of attendants between the flocks whereas the remaining few farms (39.1%) restricted such movement. Most of the farmers (86.9%) had brooding system and six farmers (13.04%) had no that facility. Of the interviewed farmers 20 (43.5%) had one attendant while the rest had more than one attendant.

Figure 1. Layer chickens kept in deep litter system in Morogoro, Tanzania

Figure 2. Layer chickens kept in battery cages in Morogoro, Tanzania

Feeding practices and flock management

Fourteen farmers (30.4%) used commercial feeds only, sixteen farmers (34.8%) made their own feed at commercially run poultry feed mills and 16 farmers (34.8%) used both commercial and own made feeds interchangeably. The feeding of chick starter, growers mash and layers mash to respective age groups differed in regimes among farms. Thirty seven farmers (80.4%) used starter from day one up to two months, whereas nine farmers (19.6%) used chick starter from day one to three months. Thirty five farmers (76.1%) fed their chicken with growers mash from two to four months and 11 farmers (23.9%) fed their chicken with growers mash from three to five months. In cases of delayed age at point-of-lay most farmers kept their grower chickens on "growers' marsh" until when egg laying started before switching over to layers' mash. Almost two thirds of the interviewed farmers (65.2%) switched over from one feed category to the other gradually over a week or two by mixing the two types until

they had them on the subsequent; the rest (34.8%) did the switch over abruptly. The majority of the farmers (8.7%) once per day and two farmers (4.3%) three times per day. Five brands of commercial feeds were commonly used by interviewed farmers, with majority of the farmers (69.6%) sticking to only a single brand. Layers were fed between 125 g and 130 g a day. The drinker/feeder: chicken ratio varied considerably both between and within farms for the deep litter system, ranging from 1 to10 for 100 birds. Chickens in most of the farms (69.6%) received drinking water from the Municipal supply, whereas the remaining (30.4%) used underground water. Treatment of water prior to supply to birds was practiced by very few (15.2%) of the interviewed farmers

Other routine management practices

Apart from feeding, watering and cleanliness; other routine management practices among the layer farms included manure collection and disposal, eggs collection, beak trimming and observation of sick birds. Some farmers associated some of these practices with stress to birds and thus resulting in drop in eggs production (34.8%) and sometimes disease occurrence (23.9%).

Disease management

Access to veterinary services

Forty out of the 46 interviewed farmers (87.0%) make use of animal health professionals in disease management while 6 farmers (13.0%) don't. Of those who make use of animal health professionals, fourteen of them (35.0%) use degree holders, 14 farmers (35.0%) use diploma holders and 12 farmers (30.0%) use animal health professionals whose qualifications they don't know. Some farmers (42.5%) use a specific animal health professional but others (57.5%) use any conveniently available. The animal health professionals providing services to farmers come from a Livestock Training Agency (15.0%), private facilities (65.0%), Sokoine University of Agriculture (15.0%) and from Non Governmental Organizations (5.0%). Among others, services delivered by the animal health professionals include disease treatment, disease prevention, input supplies, extension services; and market search for eggs and spent layers.

farmers (87%) fed their chicken twice per day, four

Disease prevention by vaccination and biosecurity

Farmers vaccinated their birds against Newcastle disease (100.0%), Infectious bursal disease (100.0%) and Fowl pox (47.8%). Vaccines were sourced from agro-vet shops and were handled on ice packs (43.5%) or in the fridge (52.2%) prior to use. Two farmers didn't use any cold chain in handling the vaccine before use claiming instant use. Two types of Newcastle disease vaccines are used in the study area i.e. LaSota® strain administered in drinking water and the thermo-tolerant I_2 strain vaccine administered as eye drops. For vaccines administered in drinking water, some farmers (19.6%) used underground water and rain water for reconstitution claiming to avoid chlorine in tap water. Following reconstitution farmers allowed the birds to drink vaccine water for an average of 1.5 hours (30 minutes to 3 hours). The fowl pox vaccine is administered through the wing web. Bio-security measures adopted by farmers in disease prevention included isolation of sick birds, disposal of dead birds by burying, traffic control and use of footbaths with disinfectants on their farm entrances (91.3%). Some farmers (15.2%) treated drinking water for birds using recommended levels of disinfectants.

Disease treatment and prophylaxis

A large proportion of the farmers (91.3%) uses prophylaxis for various disease conditions particularly coccidiosis (52.2%), avitaminosis (60.9%), worms (26.9%) and egg peritonitis (10.9%). Some of the prophylactic treatment products are included in feeds as seen for some commercial brands that indicated inclusion of coccidiostats in their feeds. All the farmers who stocked day old chicks supplied them with sub-therapeutic doses of antimicrobial agents in the first seven days, combined with multivitamins. Farmers in the study area used mainly water soluble powders for treatment of different disease conditions (Table 2). They obtained the drugs from veterinary centres, prescribed by animal health professionals following diagnosis based on necropsy or clinical picture as described by farmers or seen by a professional on a farm visit. Forty farmers (87.0%) were aware about the drug withdrawal period following bird treatment, while the rest (13.04%) were not.

Table 2: Common diseases among layer chicken flocks in Morogoro Municipality (diagnosis based on clinical picture and necropsy)

Category	Diseases	Drug used for treatment
Viral	Newcastle	None; supportive therapy provided
	Gumboro	None; supportive therapy provided
	Fowl pox	None; supportive therapy provided
	Avian leucosis	None; supportive therapy provided
	Marek's	None; supportive therapy provided
Bacterial	Fowl typhoid	Oxytetracycline, Sulphur drugs
	Omphalitis	Oxytetracycline, Doxycycline
	Fowl cholera	Sulphur drugs, Norfloxacin
	Necrotic enteritis	Norfloxacin, Enrofloxacin
	Infectious coryza	Chlortetracyclines, sulphur drugs
Parasitic	Coccidia	Sulphur drugs, amprolium
	Helminths	Ivermectin, piperazine citrate, albendazole
	Mites	Carbamates, pyrethroids, ivermectin
	Lice	Carbamates, pyrethroids
Nutritional diseases	Avitaminosis A	Vitamin supplements
	Hypocalcemia	In-feed mineral supplements
	Vitamin E deficiency	Vitamin supplements

DISCUSSION

Production profiles of layer chickens in the study area

It was revealed from farmers' responses that the average age at point-of-lay was 20 weeks (16-22) and peak lay was attained two to three months later. The laying percentage varied greatly between flocks (55-90; mean 76); the highest recorded for caged layers. Within flocks fluctuations in laying percentages were reported by all farmers and many of them attributed these to changes in feed quality, weather, disease, stress and vices. The average production longevity of the layer chicken stocks as captured from interviewed farmers was 19 months (18 to 24) after which the birds were culled. Some farmers had an opinion that the layer chickens they are keeping had a potential to produce eggs for a longer period, but the period is shortened due to several challenges including disease and poor feed quality. Apart from lower percentage rates, other production problems reported by layer chicken keepers included thin shelled eggs, relatively small eggs and occasionally extremely large eggs impacting the vent.

Challenges in layer chicken production

Main challenges mentioned by layer chicken keepers in relation to layer chicken production were diseases, extreme weather, predation, cannibalism, vaccine failures, housing, poor chick quality, fluctuation in poultry feed price and quality, and poor market structure for eggs. Furthermore, farmers mentioned the following problems specific to battery cage housing system: breakages in battery cages, wet floor (17.4%), injury to chickens caused

by wire mesh (8.7%), egg perking (8.7%). Problems linked to deep litter housing system included; easy spread of diseases (30.4%), stress (30.4%), dusting and ammonia as a result of poor ventilation.

DISCUSSION

Poultry production is one of the rapidly growing food industries throughout the world (Abdul-Cader *et al.,* 2017). The current study involved 46 layer chicken keepers of which about two thirds were females (Table 1). About half of the participants (47.8%) mentioned chicken keeping as their primary activity in terms of income generation for the family. Other farmers identified it as a secondary activity. This underscores the role of the industry to peoples' livelihoods by contributing to their economic affairs. Some farmers have sustained in the industry for up to 28 years suggesting dependence on the activity for a living. Limited supply of eggs from the industry in the country, as a result of engagement of few farmers and/or small scale of production, provides opportunities for more people to venture in.

Battery cage system of layer chicken rearing is one of the most common methods used in many countries. Relatively few farmers (21.7%) in this study adopted the system in rearing their chickens. Majority of them adopted the deep litter system due to low investment capital needed compared to that for battery cage production system. Studies in Sweden (Fossum *et al.,* 2009) and in Bulgaria (Gerzilov *et al.,* 2012) have attributed housing system to mortality and low production in chicken farming. Both studies have reported higher mortality rates in litter-based system as opposed to battery-cages due the easy with which diseases can be controlled in the later. Similarly, farmers in the current study mentioned this as one of the advantages of battery cages. Other benefits of the battery-cage system include: easy collection of eggs, cleaner eggs, little space requirement; and control of internal and external parasite. On the other hand, bird injury caused by wires and hen's inability to exercise are among the main welfare concerns associated with the battery cage housing system. According to Webster (2004) lack of exercise can easily result in skeletal damage at peak production as a result of bone weakness. The lack of exercise also subjects birds to frustration and boredom and their behaviors may change which affect their production. The deep litter system which is practiced by majority of the farmers in the study area has an advantage of allowing bird mobility but is associated with problems of dust and accumulation of ammonia gas.

Regarding feeding, some respondents reported to use commercial feed made by different companies for their chickens; whereas some opted to make the feed at home or at commercial feed mills offering feed compounding services. One of the major concerns for those buying different brands of commercial feeds was inconsistencies in feed quality as reflected in the performance of their chickens. They associated poor feed quality with increased price of maize the main poultry feed ingredient at certain time of the year of low supply. The speculation is that, for manufacturers not to increase the price of feed proportional to the increase in price of maize, they tend to reduce other components which are not very conspicuous in the feed to take care for the elevated maize cost. Most of them mentioned limited amounts of premixes, lysine and methionine; which are among the most expensive ingredients in feed formulation.

Sufficient amount of feed and fresh water are essential during all stages of layer chicken production. According to Duguies *et al.* (2016), layer chicken feeding must be on a continuous basis; therefore feed should be available to the birds for 24-hours. Farmers in this survey supplied water at adlib where as the amount of feed supplied ranged from 120 g to 130 g per bird per day. They claimed that the supplied feed sufficed their chickens' feed requirements for 24 hours. Most of the farmers fed their chickens twice a day portioning the amount of required feed into two. Others fed their layer chickens once a day preferably in the afternoon. A few portioned the amount of required feed into three and fed their birds three times a day. Those who fed their chickens once a day filled feeders to the brim leading to loss of a significant amount of feed due to bird's "pecking" behavior while eating (Duguies et al., 2016). Extremely full feeders also tend to promote spoilage of feed at the bottom of the feeder which can affect a bird's health and eventually egg-laying performance (Duguies *et al.,* 2016). Providing adequate amount of feed twice a day that is once in the morning and again in the afternoon, is a better and highly recommended practice (Duguies et al., 2016).

Insufficient feeder and drinker space was a feature in most of the farms that adopted deep litter system. The deficiency may lead to competition among chickens resulting in exclusion of low-ranking hens from the feed and water (Thogerson et al., 2009). As hens prefer to feed synchronously, competition at the feeder during times of intense feeding may disrupt feeding and induce expression of agonistic behaviour (Hughes, 1971; Webster and Hurnik,

1994). Lower ranking birds may be prevented from access to feed and hence suffer adverse effects under conditions of competitive feeding (Hughes, 1983). This eventually leads to poor welfare, reduced productivity, and sometimes mortality. Conversely, increased feeder space could improve well-being by reducing the negative effects of aggressive behaviors or social dominance during feeding (Thogerson et al., 2009). The main problem observed in cages related to water supply to birds was blockage of drinker cups such that birds are prevented from accessing water.

Manure collection and disposal, eggs collection, beak trimming and observation of sick birds were mentioned by farmers as other routine management practices among the layer farms, apart from feeding and cleanliness. Some farmers associated these practices with stress to birds and thus resulting in drop in eggs production and sometimes disease occurrence. Beak trimming involves the removal of two thirds of the upper and one third of the lower mandibles (Van Liere, 1995; Gentle et al., 1995; Sandilands and Savory, 2002). The procedure is mainly aimed at preventing feather pecking, egg pecking and cannibalism; which are common vices in growing and laying flocks resulting to production losses (Allen and Perry, 1975). Though aimed at controlling these vices, beak trimming can cause acute or even chronic pain; and may be associated with undesirable effects on birds' well being (Fahey et al., 2007). According to Lee and Craig (1991), the relative benefits of beak trimming vary among genetic stocks. In the current study the layer chicken keepers mentioned to conduct the procedure to the chickens when they are 12 to 16 weeks old. Early trimming is usually in response to early occurrence of feather pecking and cannibalism. Elsewhere beak trimming is performed at the hatchery where it is considered to be convenient and relatively cheap before delivery of the chicks. Previously, however, reported that beak trimming done to day old chick increases the risk of mortality (Wells, 1983).

Several diseases were mentioned by farmers in this study to negatively impact on production and cause mortality to layer chickens. They included viral, bacterial, parasitic and nutritional diseases (Table 2). The frequently mentioned diseases have been linked to production losses among layer chickens in previous studies (Lambert and Kabar, 1994; Anjum *et al.,* 1993; Savic, 1999; Demir, 1992; Farooq *et al.,* 2001; Singh *et al.,* 1994; Amin et al., 1995; Bains, 1979; Nicholls, 1984; Reece *et al.,* 1986; Sorensen, 1992; Qu *et al.,* 1997; Sandoval et al., 1999; Taylor et al., 1999; Zanella et al., 2000; Mukhopadhyay *et al.,* 2000; Dhillon *et al.,* 2004). Occurrence of most of these diseases in the small holder farms is associated with poor bio-security and lack of sound disease control programs. This could partly be addressed by involving qualified animal health professionals in this farming activity, an element which lacks in some visited farms.

Viruses constitute a group of pathogens causing diseases with severe impacts in poultry production. The control of diseases caused by these agents solely relies on bio-security measures and vaccination practices (Aichi, 1998; Capua and Marangon, 2006 a&b). All the respondents in the study area vaccinated their chickens against Newcastle disease, gumboro and fowlpox. Most of them use lentogenic LaSota vaccine which has been used by farmers in the country for prevention of Newcastle disease for long time. However, complaints on occurrence of ND in vaccinated flocks have continuously been raised by many farmers in different parts of the country. The same concern was raised by farmers involved in the present study. Some of the mentioned possible causes of vaccine failure have included use of expired vaccine, mismatch between vaccine strain and the circulating strain, poor vaccine handling, vaccinating incubating birds and use of chlorinated water for reconstituting vaccine. This observation of vaccine failure is an area which requires an intensive, thorough investigation to identify the actual causes; and eventually plan and institute mitigation strategies aimed at stemming ND associated losses among chicken keepers.

Failure to institute bio-security at farm level is associated with many disease occurrences in the layer chicken industry as disease pathogens and vectors can gain access to farm premises (Sylejmani *et al.,* 2016). Bio-security measures which include traffic control, sanitation and isolation thus contribute significantly to the poultry industry profitability (Trampel *et al.,* 2014). Most farmers however do not realize the importance of bio-security such that the industry is exposed to different hazards. Evidence of presence and use of a clean foot bath containing disinfectant at the entrance to poultry houses and isolation of sick birds from others in most of the visited farms reflect some degree of bio-security. Lack of regular cleaning of chicken houses was however observed in the farms adopting the deep litter system increasing possibilities for persistence of disease causing agents in the houses. Further, lack of separation between chicken flocks of different ages seen in most of the multi-flock farms in this study facilitates disease transmission between them, more so from older flocks to younger, susceptible flocks.

It is always recommended to progress from younger to older and from healthy to sick flocks during bird care. Other practices that increase farm bio-security include maintaining litter in good condition, adjusting ventilation to avoid moisture and ammonia build-up in the house, strict control of human and equipment movement into the farm, quick and proper disposal of dead birds, limiting workers to a single house and prevention of vermin (e.g. wild birds, insects and rodents) exposure into the houses. Rodents, for instance, are known to be carriers of many poultry diseases and the most common reason for re-contamination of a cleaned and disinfected poultry facility. They are also responsible for house-to-house spread of disease on a farm.

among egg laying birds. According to Fulton (2017) many of the natural causes of mortalities among layers are associated with making an egg. Fulton (2017) further insists that performing necropsies on a regular basis helps to detect deaths due to unforeseen causes in a way providing for timely intervention to allow for the continued health and welfare of the birds (Fulton, 2017). Identified natural causes of mortalities among layers in the current study include; egg yolk peritonitis, cannibalism (pick out), fatty liver syndrome and prolapsed vent which also were reported elsewhere (Fulton, 2017).. Necropsy findings in the study area were frequently supplemented with clinical presentations of different diseases for which most of the farmers had basic knowledge obtained from animal health professionals.

Several factors are known to influence egg production; and these include chicken strain, feeding, mortality, culling, health and management practices, age at point-of-lay, and peak lay and persistency of lay (Farooq et al., 2002). In the present study the average age at point-of-lay was 20 weeks and peak lay was attained two to three months later. An observation on the average age at point-of-lay is more or less similar to what was reported in previous studies (Singh and Belsare, 1994; Petek (1999). Other earlier researchers reported a lower age (Tolimir and Masic. 2000; Farooq *et al.,* 2002). Farooq *et al.* (2002) however, reported a more or less similar age at peak-of-lay to the present findings. A higher age at peak-of-lay than what is reported in this study has been reported by Lai and Kan (2000). According to Bell and Weaver (2002), however, the recommended age at point-of-lay is 112 and the age of flock at peak hen-day egg production is 182 days. The results obtained in the current study for these parameters therefore indicate

It was revealed in this study that most farmers send dead birds to veterinary centres for necropsy; though some few disposed or fed them to dogs. Through necropsy veterinarians were able to establish common disease conditions responsible for morbidities and mortalities in the flocks. Sending dead birds for necropsy underscores a recent argument that determining the cause of death in egg laying bird flocks by routine necropsy of daily mortality is useful in helping farmers and the examiner to establish what is normal for a particular flock (Fulton, 2017). Routine autopsy examination of dead hens is a source of disease information (Grimes, 1975); including "background" disease which causes continuous low-level morbidity and mortality. Such a practice is also useful in providing information on the natural causes of mortalities delays in start of production and attaining peak production among the flocks in the study area.

The current study observed variable egg production performance for different flocks. The study also revealed irregularities in egg laying performance within flocks. The average laying percentage was 76 (55-90). The highest laying percentage was recorded for caged layers. Comparable levels of hen-day egg production performance have been reported by previous authors (Akyildiz *et al.,* 1993; Tanaka, 1993; Kristensen and Sillebak-Kristensen, 1996; North, 1984). Factors responsible for varied performance among egg laying flocks include chicken strain differences (North, 1984; Petek, 1999; Tolimir and Masic, 2000; Lai and Kan, 2000), stocking rate (Adams and Craig, 1985; Lee and Moss, 1995), persistency of lay (Akyildiz et al., 1993; Tanaka, 1993; Kristensen and Silleb-Kristensen; 1996) and housing system. Regarding housing, authors found better egg production performance of layers reared in cages than on deep litter (North, 1984; Horne-Van and Van-Horne, 1994; Moorthy *et al.,* 2000). According to farmers, irregularities in production within their flocks were caused by diseases, fluctuations in feed quality, stress and use of sulphur drugs. The influence of the different factors on egg production performance between and within flocks was however not investigated during this survey.

Commercial egg laying hens normally reduce production after 70-72 weeks of age (Petek, 1999). Hens that are in production for almost two years are considered "spent" layers. Although layers can lay eggs longer than two years, the quality and quantity of the eggs produced are generally considered poor and uneconomical (Duguies *et al.,* 2016). After the chickens' laying capacity decreases, producers cull and sell them as spent layers for slaughter purposes.

Farmers involved in this study performed culling of their layer chickens at the age of 18 to 24 months. Some of them opted to continue keeping hens that have dropped production as a way of maintaining their customers for produced eggs while waiting for replacement flock to begin laying eggs. At culling the customers include individuals for home consumption, owners of bars for making soup and businessmen who buy at wholesale price from producers and sell them on retail basis at the market. The entry of spent layer chickens into the food chain is however with some limitations, which include low meat yield and tough meat (Loetscher *et al.*, 2015). Consumers however tend to equate the spent layer chicken meat to meat from free ranging indigenous chickens which are the mostly preferred in the country.

Layer chickens production in the tropics is featured by severe losses attributable to the harsh climate, high disease incidents, stress, poor biosecurity, poor disease management, poor husbandry, poor chick quality, feed-associated causes, and unintended accidents (Sorensen, 1992; Farooq et al., 2002; Shittu et al., 2014). They are at higher risk due to long term exposures (≥72 weeks) to these factors on farms (Sorensen, 1992). Most of these limitations were raised by the layer chicken keepers during this survey. According to literature (Shittu et al., 2014), hot-dry weather is linked to heat stress, waning immunity and inefficient feed usage and increase probability of death with reduced egg production. The authors point out that younger birds (19–38 weeks) are at higher risk of death due to stress of coming into production, management changes and diseases; and speculate that older chickens' better protection from death could be associated with many prophylactic and metaphylactic regimen of medications/vaccination. FAO has demonstrated that at a temperature above 28°C, egg production will significantly wane both in quantity and quality. It should be noted that an environmental temperature between 25-40°C will cause the bird to pant and may lead to heat stroke and eventual death. Farmers in the study area also mentioned low temperatures to be a cause of drop in egg production.

Conclusions and recommendations

The current study highlights on different important aspects of layer chickens production in Morogoro, Tanzania. The study revealed inadequacies in layer chicken husbandry and flock health management. The deficiencies in the two aspects were reflected in poor chicken performance both in health and productivity, eventually limiting profitability of the industry. Improvements in husbandry and health management (including biosecurity) are highly recommended so as to increase and sustain production. Future research should include attempts to devise intervention strategies to address factors attributable to poor productivity in the layer chicken industry. Regulatory agencies should play their part in ensuring the supply of genuine vaccines and quality chicken feeds with all essential nutrients and supplements to support production performance.

ACKNOWLEDGEMENTS

The author acknowledges the willingness of farmers to participate in this study.

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Prevalence of claw lesions in free range short horn cattle (zebu) in Kwimba district, Tanzania

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SUMMARY

Claw lesions are the problems which affect the hoofed animals and may or may not result into lameness. In cattle, this is a disease of economical importance as it affects the animal wellbeing as well as the economy of the farmer. The disease has been shown to affect animals kept in different production systems, although much information is available from the intensive dairy units and beef in feedlot systems. Limited studies have investigated the problems of claw lesions in cattle under pastoral and agro-pastoral systems in Tanzania. The present study was carried out to determine the prevalence and characteristics of claw lesions in the free range cattle in Nyambiti Village Kwimba district in Mwanza. The study was carried out during dry and rainy season between 2014 and 2015. A total of 19 households were selected for study whereby 367 cattle were examined. A total of 206 cattle were examined during dry season and 161 during rainy season. The overall prevalence of the claw lesions was 7.6%. During the dry season, 13 (6.3%) cattle had claw lesions while during rainy season 15 (9.3%) cattle had claw lesions. The observed lesions traumatic injury, claw abscess, hoof overgrowth, inward and outward curved claw, laminitis, hardened groove and swelling of the coronary area, foot rot, interdigital dermatitis, heel erosion and double sole. Most of the lesions were realized in cows which accounted for 71.4% of all lesions. Hind legs had most of the claw lesions (82.1%). It is concluded that claw lesion is a problem in free range short horn zebu hence the farmers should be given proper information and advices on the claw problems as to how it occurs and the associated effects as well as management on animals.

Keywords: Claws, Lesions, Free range, Zebu, Kwimba district

INTRODUCTION

Claw lesions are the problems which affect the claws in the legs of animals (cattle). The lesions can result in lameness although in some animals the sign of lameness is not obviously presented (Manske, 2002; Erin, 2004). Cattle shows the sign of lameness when there is a problem in any part of the limb but the mostly affected are the claws and the lesions which are normally reported on the claws are; sole erosion, interdigital dermatitis, claw/hoof overgrowth (Mpanduji *et al* 2012), flattened claw, sole ulcer, sole hemorrhage, white line disease, vertical fissure, axial fissure, corkscrew claw, toe ulcer or foreign body penetration. These lesions can be observed when the hooves are examined and more clearly after hoof trimming (Eric, 2004). The claw lesions have variable appearances and can be found at a range of sites including the classic site on the skin around the heel, under the heel horn, on the coronary band, on the skin between the claws, under the due claws as well as the pastern skin. Claw lesions and Bovine lameness are the function of multi factorial effects (Mpanduji *et al.,* 2012) in most of cattle in Tanzania as well as other countries. The lesions are of greatest importance in animal production as it become the great insult in productivity while most of farmers take negligence on it. These conditions appear in cattle following one or in combination of the factors such as, congenital anomalies, hereditary, nutritional factor, moisture, poor hygiene, infections as well as poor animal management. Claw lesions and lameness represents the major health problem in cattle and where the incidence is high it accounts for tremendous economic loss (Shear *et al.*, 2000). This condition affect the rate of production such as: milk production, meat production, growth rate, reduced farm activities, reduced fertility. Also it can cause prolonging in calving interval, weight loss, loss of animal (death of animal), cost of extra labor as well as veterinary expenses (Erin, 2004).

Kwimba district is among the areas with high number of small scale cattle farmers in Tanzania. Most of the cattle kept in Kwimba are the Tanzanian short horned zebu which is owned by agropastoralists and pastoralists. There have been no studies on livestock diseases that has conducted in Kwimba district regardless of the potentials the district has in terms of cattle. The purpose of this study was to investigate the claw lesions in free range short horn cattle (zebu) in Kwimba district, Mwanza region, Tanzania.

MATERIALS AND METHODS

Study area and animals

The study conducted in Mwanza region, Kwimba district in Nyambiti village. The sample animals were selected from various animal keepers in Nyambiti village during dry and rainy seasons. Kwimba district mostly keep local cattle (Tanzanian Short Horned Zebu). The cattle management system is extensive where cattle are grazed in natural pasture during the day time and are sent back in the kraal during the evening time. There are rare veterinary services offered to the animals. Hoof care also does not exist.

Sample size

The sample size obtained through the following formula:-

$$n = \frac{z2pq}{d2}$$

Where n = expected minimum sample size; z =confidence level; p=proportional of sample; d = degree of accuracy. So a sample size of 384 cattle was calculated. However 367 cattle were examined; 206 cattle during dry season and while during rainy season 161 animals were examined.

Selection of study animals and examination of the cattle for claw lesions

All the cattle keepers in Nyambiti village formed the study population. In each of the cattle keeper, about 10% of the cattle were randomly selected for examination of claw lesions. All animals in the selected herds were observed for the presence or absence of the claw lesions in which the animals were allowed to walk on the hard soil surface where the claw problem can easily be noted. Thereafter, selected animal was restrained by using a rope and where necessary the animal was casted down and well tied to minimize struggles. The leg was being washed with soapy water and a bristle brush up to the level of knee and hock joints. A detailed examination of the hoofs was performed and recorded all the claw lesions encountered as described by Mgasa, (1991) and Mpanduji *et al.* (2012).

RESULTS

General results

During this study, 206 cattle were examined during dry season and 161 cattle during the rainy season making a total of 367 cattle. The prevalence of claw lesions was 7.6%. During the dry season, 13 (6.3%) cattle had claw lesions while during rainy season 15 (9.3%) cattle had claw lesions (Table 1). The lesions seen during dry season were; traumatic injury, claw abscess, hoof overgrowth, inward and outward curved claw, laminitis, hardened groove and swelling of the coronary area (Figure 1 & 3). During the rainy season, the observed claw lesions were foot rot, interdigital dermatitis, laminitis, abscess, heel erosion and double sole. With foot rot and laminitis occurring in relatively more frequent (Table 1). During the rainy season, the animal house environment was always muddy (Figure 2).

Table 1. Claw lesions in adult cattle during dry and rainy seasons of 2014 and 2015 (n=367)

Lesions	Number (%) of claw lesions during		
	Dry season (n=206)	Rainy season (n=161)	Total
Foot rot	0 (0.0)	6 (3.7)	6 (1.6)
Laminitis	2 (1.0)	4 (2.5)	6 (1.6)
Abscess	1 (0.5)	1 (0.6)	2 (0.6)
Interdigital dermatitis	0 (0.0)	2 (1.2)	2 (0.6)
Traumatic injury	1 (0.5)	0 (0.0)	1 (0.3)
Hardship groove	2 (1.0)	0 (0.0)	2 (0.6)
Upward/inward curved claw	3 (1.5)	0 (0.0)	3 (0.8)
Heel erosion	1 (0.5)	1 (0.6)	1 90.3)
Swelling	2 (1.0)	0 (0.0)	2 (0.6)
Double sole	0 (0.0)	1 (0.6)	1 (0.3)
Claw overgrowth	1 (0.5)	0 (0.0)	1 (0.3)
Total affected	13 (6.3)	15 (9.3)	28 (7.6)

Figure 1. A photograph that show some of the lesions encountered during dry season (Left-trauma, right-horizontal claw fissure)

Figure 2. A photograph that show muddy animal house during the rainy season

Distribution of lesions according to sex

Most of the lesions were realized in female during both rainy and dry season accounted for 71.4% of all lesions (Table 2).

Table 2. Distribution of claw lesions in relation to sex

Lesions	Dry season		Rainy season		Total
	M	F	M	F	
Foot rot	0	0	2	4	6
Laminitis	1	0	2	3	6
Abscess	1	0	0	1	2
Interdigital dermatitis	0	0	0	2	2
Traumatic injury	0	1	0	0	1
Hardship groove	1	1	0	0	2
Upward/inward curved claw	0	3	0	0	3
Heel erosion	0	0	0	1	1
Swelling	1	0	0	1	2
Double sole	0	0	0	1	1
Claw overgrowth	0	1	0	1	0
Total affected	4	6	4	14	28

Distribution of claw lesions in relation to legs

The claw lesions encountered during examination were mostly localized on the hind legs and few of them were on the fore legs. The hind legs accounted for 23 (82.1%) of all the lesions (Table 3).

Table 3. Distribution of claw lesions in relation to legs

Lesions	Dry season		Rainy season		Total
	Fore leg	Hind leg	Fore leg	Hind leg	
Foot rot	0	0	3	3	6
Laminitis	0	2	0	4	6
Interdigital dermatitis	0	0	0	2	2
Traumatic injury	0	1	0	0	1
Digital abscess	0	1	0	1	2
Upward/inward curved claw	2	1	0	0	3
Heel erosion	0	0	0	1	1
Swelling	0	1	0	0	1
Double sole	0	0	0	1	1
Hardship groove	0	4	0	0	4
Claw overgrowth	0	1	0	0	1
Total	2	11	3	12	28

DISCUSSION

The claw lesions encountered in the free range short horn zebu in Nyambiti village during dry and wet seasons were traumatic injury on the claw, digital abscess, swelling on the leg, curved claws (upward and inwards), laminitis, horizontal claw fissures, foot rot, heel erosion, double sole and interdigital dermatitis. These lesions give the overall prevalence of 7.6% in which the dry season prevalence is 6.3% and the prevalence in wet seasons was 9.3%. Among the cattle identified with claw lesions during dry season were few animals which did show obvious lameness on walking and most of them did not show the signs of lameness. This may be due to the fact that the intensity of the lesions was not enough to progress the lesions into the stage of showing lameness (Manske, 2002).

The lesions encountered during rainy season were mostly associated by lameness (Mpanduji *et al*., 2012). This was due to the high intensity of the lesions which caused progression of the lesions into showing signs of lameness. Normally the lameness signs are shown when the animal is feeling pain on the leg. During rainy season the lesions were influenced by environmental conditions in which the rain caused the areas to be wet all the time which led to softening of the horny tissue of the hoof of animals. This stimulates destruction of hoofs, breakage and fissure of the horny tissue exposing the sensitive lamina and foot rot (Aliyu *et al.*, 2005). There were other lesions which did not show signs of lameness but were encountered during examination of other obvious lesions.

The study encountered that most of the claw lesions were localized in the hind legs than on the fore legs (Erin, 2004). The results from table 4 show that 28 legs had problems during the study and among which 23 were hind legs (82.1%). It was further suggested that there was no any claw problem in calves; all of the lesions were encountered on the adult cattle. This shows that the lesions are associated with the increase in age (Mishamo and Abebe, 2012). This suggests that the lesions observed were most probably contributed by the long walking distance on hard surface (Manske, 2002; Mpanduji *et al.*, 2012). More effects were realized in the hind legs possible possibly because the claws bear more weight of the particular animal as well as the activities. This also could be due to the weight bearing tendency of the animals on the hind legs in which the hind legs are the one carrying more weight in relation to fore legs.

From the results obtained during dry and rainy seasons show that there is an increase in the claw lesions as previously observed by Manske (2002). This can be due to the environments in which the animals are kept with the presence of rainfall which cause accumulation of mud in cow sheds and since the animals are kept on the same shade for the whole night, this which led to softening of the horny tissue of the hoof of animals. This stimulate destruction of hoofs, breakage and fissure of the horny tissue exposing the sensitive lamina and foot rot, this corresponds with the study done by Aliyu *et al.* (2005). But also wet environment favors the growth of microbes which are responsible for causing some of these claw lesions.

Despite the fact that the environment is favoring the occurrence of the lesions but poor hoof care and animal management by the farmers also was suggested to be among of the factors associated in development of the lesions (Erin, 2004).

It is concluded that claw lesion is a big problem in free range short horn zebu kept in Kwimba district. This calls for the attention that farmers should be given proper information and advices on the claw problems as to how it occurs and the associated effects as well as management on animals.

ACKNOWLEDGEMENT

The authors are indebted to the Buhuri Livestock Training Agency (LITA) for their assistance and cooperation offered on all stages of this work. Mr. Nesphory Mwagala a field officer Nyambiti-kwimba and Mr. Gideon Nghungubaji a field officer Ngudu are thanked for the assistance in data collection. This study was funded by Higher Education Student Loans Board (HESLB).

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Cryopreservation of dog semen as an alternative method to improved fertility in bitches: A review article

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SUMMARY

Cryopreservation of dog semen facilitates the exchange of genes between populations and makes it possible to preserve genes from valuable males for an extended time. Although AI in dogs can help avert sexually transmitted diseases and reducing the chances of inbreeding, the technique is not routinely practised in most developing countries including Tanzania. Among the reasons are problems associated with cryopreservation. Cryopreservation of semen has a number of problems limiting its success and thus, affecting fertility in dogs as a whole. The common assisted reproductive technologies like AI in dogs are constrained by challenges in semen cryopreservation which partly is caused by freezing temperature, cryoprotectants and lifespan of spermatozoa. The purpose of this review is to study the possible ways which can improve cryopreservation of semen in dogs. Improving semen cryopreservation is not a one process; it involves a combination of factors with the ultimate goal of improving the outcome of semen cryopreservation in terms of conception rates in bitches, and thus, fertility in general. Cryopreservation parameters to improve include: type of ejaculate, insemination technique, storage, post thawing procedures, extenders, cryoprotectants, collection method and antioxidants. Several approaches have been recommended, including cryopreservation protocols, for instance freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane, but fast enough to dehydrate the cell without exposing it to lethal salt concentrations. Moreover, the first ejaculates and the ejaculates from old dogs are to be discouraged if we need to improve fertility output under cryopreservation method. Thawing temperature of 70°C and above within a few seconds, have shown promising results. Possibly, this temperature is helpful to reduce the toxic nature of cryoprotectants. However, this review suggests the use of intrauterine method, and possibly innovation of new modification and training expertise would help to improve fertility in bitches. Nevertheless, more work is needed to accurately predict fertility of cryopreserved canine semen.

Keywords: cryopreservation, semen, dogs

INTRODUCTION

The domestic dog (*Canis* familiaris) is one of the first domesticated species (Morey, 1994), and results from at least 14,000 years of domestication that started with the grey wolf (*Canis lupus*). Dogs are used for several purposes, including as hunters, guardians, shepherds, guide dogs for the blind, service and hearing dogs, rescue dogs, and dogs for police and military purposes, e.g. in drug and explosives detection. The largest numbers of dogs are, however, kept as companion animals. The expanding use of dogs for different purposes and the selection by humans have resulted in a large diversity of this animal, with varying appearance and behaviour. The domestic dog can be useful models for research in reproduction of endangered wild *canids*. There is also a great demand to exchange genetic material in domesticated dogs, since many breeds of dogs are small in number and transportation of semen and successful artificial insemination (AI) would help to widen the gene pool of minority breeds. Successful preservation of canine semen is therefore important for improving the results of the main assisted reproductive technologies (ARTs), such as in vitro fertilization and (most commonly) AI in dogs (Luvoni, 2006).

Cryopreservation is a process where cells or whole tissue are preserved by cooling to low sub-zero temperature such as -196ºC. The process usually involves adding cryoprotectants, freezing, storage and thawing. In the modern breeding world use of cryopreserved semen is in demand but there have been a number of challenges with efficiency. The process of cryopreservation is reported to decrease the ability of spermatozoa to fertilize (Bilodeau*et al*., 2000; Cerolini*et al*., 2001), which reduces the balance of antioxidants enzymes of canine spermatozoa and its general biological properties (Strzezek*et al*., 2012).

In an attempt to achieve better conception rates, studies in various species advocate intrauterine deposition of semen (Fontborne and Badinand, 1993: Linde-Forsberg,1999) though in dog anatomical factors limit its use. But the good thing is sperm cell distribution occurred evenly along both horns, independent of the site of semen deposition (Fukushima *et al*., 2009). Moreover, identifying an optimum time of fertilization, remains as a

stumbling block to achieving high conception rates in dogs. Although in other canines such as brown bear, problems of sperm agglutination has been addressed in the ejaculates, the problem which impairs freezability. Gomes-Alves *et al*. (2014) described the usefulness of using Test-tris-fructose-egg-yolk –glycerol as a suitable extender for use in brown bears to reduce agglutination in fresh semen samples.

Ovulation in bitches occurs 1 or 2 days after the preovulatory peak of the luteinizing hormone (LH), still in the beginning of the estrus stage of the estrous cycle, and the ovarian follicles start luteinization before ovulation (Pereira *et al*., 2012). Oocytes are ovulated still immature, in the beginning of the first meiotic division (GV). Therefore, the oocytes require 2 to 5 days to reach maturation after ovulation (Reynaud *et al*., 2012). The environment in the uterine tubes plays an important role in canine oocyte maturation. The uterine tubes in canids, unlike other species, is responsible for maintaining, during an extended period of time, the survival of still immature oocytes until they complete their development, are fertilized and reach the blastocyst stage (Luvoni*et al*., 2005). This necessitates timing of insemination to tally with ovulation.

Additionally, dog's sperms respond differently to cryopreservation as compared to other animal species. Normally, it is associated with high individual differences and poor conception rate post thawing (England, 1993; Yu*et al*., 2002).Other obstacles include poor post thaw semen quality and short lifespan post thawing (Oettle, 1986; England, 1993). Furthermore, it has recently been found that sperm oxidative damage intensifies post thawing (Lucio *et al*., 2016). Therefore, the use of cryopreserved semen is impeded by these setbacks highlights a need for a research on ways to improve cryopreservation. Successful artificial insemination in bitches requires understanding of semen collection method, types of extenders, cryoprotectants, storage, and insemination techniques. This review aimed at discussing the main factors related to cryopreservation of dog's semen as ways of improving artificial insemination in bitches.

Principles of cryopreservation

Spermatozoa cryopreservation is a method for preserving genetic material and maintaining genetic diversity in several species, including dogs. This extends the storage time and facilitates semen transportation over distance. Since the introduction of glycerol as a cryoprotectant agent by Polge*et al*. (1949) and the discovery of dimethyl sulfoxide (DMSO) by Lovelock and Bishop, (1959) many cryopreservation techniques developed, mostly through empirically derived methods. In the principles of cryopreservation, the cooling rate is very important as when it is slow then most of the sperm cells will be functional after thawing.However, cryopreservation has negative effects on sperm viability, which could be related to injury of the plasma membrane, associated to changes in lipid phase transition, mechanical stress, efflux of water and high salt solutions and, possibly, by interfering with intracellular ice crystals formation, and thus decreasing the fertilizing ability of spermatozoa (Bilodeau*et al*., 2000; Cerolini*et al*., 2001). The above could result from enhanced peroxidation of sperm cell membrane lipids which is largely determined by excessive generation of reactive oxygen species (ROS) in semen (Alvarez and Storey, 1992; Wang *et al*., 1997). The membrane of a sperm cell contains large amounts of polyunsaturated fatty acids (PUFAs), which make them highly susceptible to lipid peroxidation (LPO) in the presence of ROS, resulting in impaired sperm function (Aitken *et al*., 1996). The main factors which influence the survival of cryopreserved spermatozoa are: gamete's osmotic properties, cooling and warming rates and formation of intracellular ice crystals (Mazur *et al*., 1972; Stănescu and Bîrţoiu, 2012). Since canine spermatozoa act as a perfect osmometer(Songsasen*et al*., 2002), the responsiveness of spermatozoa to osmotic challenge and the ability to regulate cell volume is critical to cryopreservability(Petrunkina*et al*., 2005). Different canine semen cryopreservation techniques include as a first step the centrifugation of the sperm. The goals of centrifugation are to remove the excess of prostatic fluid which has a negative effect on motility and vitality of frozen-thawed spermatozoa (England and Allen, 1992; Rota *et al*., 1995; Sirivaidyapong*et al*., 2001) and to standardize the extension of the semen to a controlled final sperm and glycerol concentration. The most used regimen in the cryopreservation protocols is centrifugation at 600 – 700 g for 5 – 10 minutes (Rijsselaere*et al*., 2002; Schäfer-Somi *et al*., 2006). The main disadvantage of this method is the elimination of natural antioxidants from seminal plasma. Thus, freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane, but fast enough to dehydrate the cell without exposing it to lethal salt concentrations.Therefore, for better results in cryopreservation, freezing and thawing procedures have to be optimally maintained to

ensure maintenance of pH, osmolality, as well as energy provision and cryoinjury prevention.

Semen collection and evaluation

Prior to semen collection a thorough and complete historical review of the dog's previous health and breeding experiences should be obtained.Inaddition, information regarding the medication or supplements administered over the previous 6 months (at the minimum) and on the genetic or familiar background are important (Johnson, 2006). There should also be collection of information about the status of vaccinations, dewormings and heartworm protection history, as well as on the duration of the ownership and accuracy of the history of the animal even before the establishment of the ownership (Freshman, 2002; Ettinger*et al*., 2010). Many factors influence semen quality, including the animal's age, the size of the testicles, the degree of sexual arousal, the frequency of ejaculation, the collection procedure and the amount of seminal fluid collected (Kutzler, 2005; Johnson, 2006).Different techniques are used to collect semen in dog and these include the use of electroejaculator, artificial vagina andpenis massage (Ortega-Pacheco *et al*., 2006).

For preservation reasons, either semen freezing or chilling, or for fresh semen AI, it may be of benefit to perform two collections, with an interval of 45 to 75 minutes among them. Although the number of spermatozoa is rather low in the second collection comparing with the first, the amount of both collections is in average, 70% more than if the collection is performed only once (England, 1999). The total volume of a dog's ejaculate may vary from 1.0 mL up to 30.0 mL (England *et al*., 2010; Ettinger*et al*., 2010). The canine ejaculate is composed of 3 distinct fractions (Johnston *et al*., 2001; Kustritz, 2007). The first or pre-spermatic fraction is composed of clear seminal plasma, devoided of sperm cells, originates from the prostatic gland and its main function is to flush the urethra (England *et a*l., 2006; Nelson *et al*., 2009; Ettinger*et al*., 2010). The volume of the pre-spermatic fraction usually varies between 0.5 and 2.0 mL (Freshman, 2001). The second fraction, also denominated sperm-rich fraction, has a cloudy and opalescent appearance with an opaque consistency, varying in volume between 0.5 and 5.0 mL, depending on the testicular size and on the individual variation; moreover, in its composition there should be no cellular components besides sperm cells (England, 1999; Nelson et al., 2009). The dog can take up to 2 minutes to achieve the emission of the sperm-rich fraction (Kutzler, 2005).

Type of ejaculates

The type of ejaculate collected can be influential in terms of improving post thawing viability in dog's semen. For instance, the first ejaculates have been found to have lower post-thaw motility and velocity as compared to subsequent ejaculates that were collected two days apart (Dobrinski *et al.*, 1993). Interestingly, motility pre-process of all the ejaculates were almost similar but after thawing motility is different. Showing that, for better results the first ejaculates has to be discarded especially the ejaculate collected after long period of reproductive or sexual rest, and the subsequent ejaculates are the ones to be used. Moreover, it is also found out that the best ejaculates come from dogs below eight years of age (Thomassen *et al*., 2006).These studies suggest that time and what semen to collect, as well as age of the animal from which we collect our ejaculates can improve cryopreserved semen. Hence, the first ejaculates and the ejaculates from old dogs are to be discouraged if we need to improve fertility output under cryopreservation method.

Individual dog difference

Individual dog difference in terms of post-thaw sperm quality has been advocated in many studies (Alhaider & Watson, 2009; Dobrinski *et al*., 1993). InDobrinski *et al*. (1993) the difference were obtained in terms of interaction with the extender, as well as the number of ejaculates. Yu *et al*. (2002) captured differences in terms of cooling and warming rates. Individual difference had also been captured in terms of age. Whereas using frozen semen of dogs older than eight years, the whelping rate was found to be lower (than those below eight) (Thomassen *et al*., 2006). Individual differences were also captured by Zakosek *et al*. (2012). The scenario was when dogs showed different results when the same extender was used. But when they tried to use human extenders, the results post-thaw was better. The concept shows that we can manipulate extenders based on the breed of dogs we have at the time. Moreover, increasing sperm number has been advocated as one of the means to compensate differences in the fertility among males (Saacke*et al*., 2000). Hence, these studies reveal that there are individual differences in terms of post-thaw semen quality. Therefore, for better results in cryopreservation choosing as a donor needs a lot of study and examination of the particular stud dog which provides good results in terms of post-thaw viability of spermatozoa. Possibly, there can be development of individual freezing extenders for

those dogs that show problems in terms of semen freezing, and yet are considered as superior breeds.

Insemination technique

Insemination techniques are an influencing factor in improving results after cryopreservation of semen in dogs. The technique can be in terms of manipulating the route of insemination, number of spermatozoa, and number of insemination. Intrauterine route of insemination is found to result in a 85% pregnancy rate when fewer number of insemination dose (30-35 $\times 10^6$) were used using the same dose and the route being non-surgical intrauterine (Linde-Forsberg et al., 1999; Kong *etal*., 2003). Contrary to the above findings, Nothling, Gertsenberg, and Volkmann (1995) found higher whelping rate in intravaginal as compared to intrauterine. But in Nothling's study, bitches were inseminated on average more than five times, therefore, this could not reflect the real case under field situation. Hence, from these studies it shows that in order to improve fertility under the cryopreservation, the technique used for insemination is vital (Table 1 and 2). This research suggests the use of intrauterine method, and possibly innovation of new modification and training expertise would help to improve fertility.

Table 10: Comparisons of vaginal AIs and intrauterine AIs

Total number of spermatozoa (x 10^6)	Vaginal AIs			NIU AIs		
	Number of AIs	Whelping rate (%)	Litter size	Number of AIs	Whelping rate (%)	Litter size
≤100	7	28.6	3.5 ± 2.1	8	100.0	3.0 ± 2.0
101 - 200	24	45.8	2.8 ± 2.1	31	90.3	4.8 ± 3.0
201 - 300	24	50.0	4.5 ± 2.0	30	77.4	5.3 ± 2.5
301 - 400	19	63.2	4.5 ± 3.2	21	76.2	6.4 ± 3.9

Table 11. Litter size and whelping rate under intrauterine insemination versus intravaginal insemination

Items	Intravaginal	Intrauterine
No. of bitches inseminated[a]	15	26
No. of bitches whelping (%)*	6 (40.0)	21 (86.6)
No. of pups born	17	89
No. of pups per litter (Mean±S.E.)	2.8±1.2	4.2±1.6

Semen storage

Semen to be used for AI can be prepared in different ways. It can either be used fresh, or extended fresh or chilled, or frozen-thawed (FT). Fresh semen for AI is usually used when both the male and the female dog are present at the site of semen collection and the AI can be performed straight away. The freshly ejaculated semen can be extended in order to maintain viability or even improve motility, e.g. in male dogs with prostate problems. The fresh semen has the advantage of not being processed and therefore of not being damaged by chilling or freezing procedures. A great disadvantage is of course that the semen has to be used immediately after semen collection. For any further transport or short storage, the semen has to be chilled, while for long-term storage, freezing is the only option. Today AI in dogs is routinely used, with acceptable pregnancy results both with fresh, chilled and with FT semen, although chilled dog semen have given better results for AI, compared withfrozen and thawed semen.

Cryopreservation of spermatozoa is well studied in various species including the dog, and the first pregnancies resulting from FT spermatozoa were

achieved with semen diluted in lactose and Tris (hydroxymethyl) aminomethane (Tris)-based extenders. However, cooling of dog spermatozoa appears to damage the spermatozoa less than freezing and thawing do, and compared with FT spermatozoa, semen quality (expressed as motility, sperm morphology, acrosome status, hypo-osmotic swelling, and longevity at 39ºC) has been reported to be superior for up to 4.9 days of cool storage despite some deterioration during cold storage. As with motility, acrosome reaction in dogs is more affected by freezing and thawing than by cold storage (Oettlé, 1986). Changing of the means of storage of dog semen has been recommendedelsewhere. For instance, use of ultra-freezers (UF) at -152°C rather than liquid nitrogen(Álamo *et al*., 2005). Moreover, the use of the dry shipper for short time storage of dog's semen has also been advocated (Batista *et al*., 2012). However, studies investigating conception rates of sperm stored under these methods have not been done. Possibly, they could result in the improvement it is not yet known. Hence, there can be a possibility that improving the method of storage can help to improve the semen quality of the cryopreserved semen. Overall, more research is needed to establish the best storage improvement for cryopreserved semen.

Post thawing procedures

Various manipulations regarding post thawing procedures are advocated to improve post-thaw motility. They included use of certain extenders such as Equex STM paste though timing in terms of thawing is seen to be more influential where survival and motility was favoured first hour post-thawing (Alhaider and Watson, 2009). Moreover, individual dog interaction with Equexhas been reported, particularly in terms of concentration of intracellular free calcium and membrane fluidity (Alhaider and Wats, 2009). In some studies, thawing temperature has been captured to influence post-thaw spermatozoa quality. Nothling and Shuttleworth (2005a)established that thawing at 70ºC in water was the best approach other than 37ºC, and this was better in terms of having few abnormal acrosomes. Moreover, it is further supported that when 0.5mls straw were put at 70 for 8 seconds, provided better results in terms of post-thaw survival as compared to 30ºC for 15 to 60 sec(Peña and Linde-Forsberg, 2000; Rota *et al*., 1998). Further supported by Lyashenko (2015) that temperature range of 65 ºC to 70 ºC with time range of 6-7 seconds provided better results. Though there seem to be an association between freezing rate and thawing rate where the freezing rate was fast then the thawing rate has to be faster as well. Similarly, a slower freezing rate has to be followed by a slower thawing rate (Farstad, 1996). The scenario can imply that one has to be aware of the freezing procedure, or follow the manufactures manual on the freezing protocol used. Therefore, few studies involving thawing temperature of 70C and above within a few seconds, have shown promising results (Figure 1). Possibly, this temperature is helpful to reduce the toxic nature of cryoprotectants. Hence, this research recommends further studies and modifications to be done to qualify the use of higher temperatures.

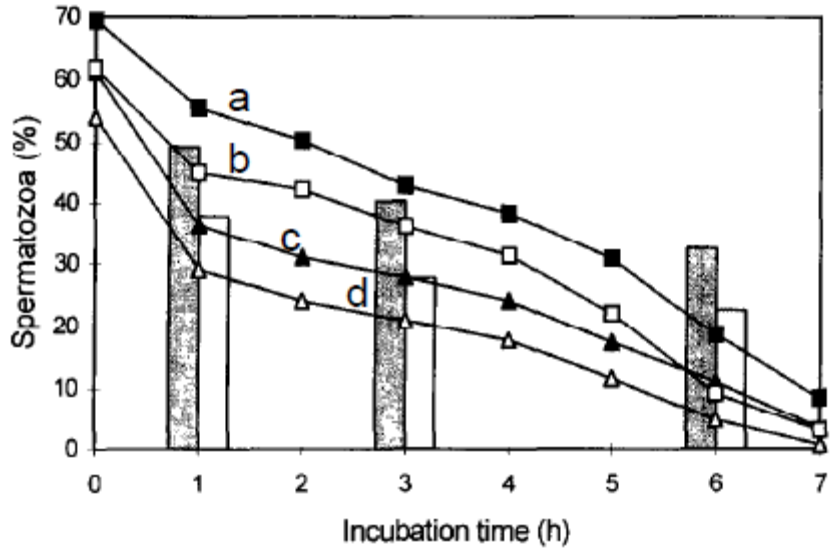

Figure 3. Percentages of sperm motility and live spermatozoa at 37ºC and 78ºC

Extenders

For a long time, the standard semen extenders used in dogs were made with chicken egg yolk. However, egg yolk presents numerous disadvantages. It is associated with a health risk as egg yolk is an excellent microbial culture medium and can promote the onset of uterine infection following insemination of the bitch. Egg yolk also poses problems for analyses using a CERROS type image analyser or during biochemical assays (Wall and Foote, 1999). Lastly, the components of egg yolk can vary as a function of the diet of the chicken, and are not necessarily all beneficial for spermatozoa; the granules are actually deleterious for the respiration and motility of spermatozoa (ksmpshmidt *et al*., 1953; Pace and Graham, 1974).

Currently Tris being extenders is commonly used and found effectively in dog semen (Hewitt *et al*., 2001: Ponglowhapan and Chatdarong, 2008: Martins *et al*., 2012).However, some researchers (Bencharif *et al*., 2010) suggest the 6% low-density lipoproteins (LDL) extender to be the best. While other studies show that equex and 6% LDL extenders are most successful (Bencharif*et al*., 2010). Studies on extenders are still controversial as most extenders present some interesting individual features which are not in the others. Such features are: post-thaw fertility, motility, acrosomal integrity, DNA integrity, etc. Likewise, efficiency of most extenders is still dependent on the concentration and dilutions levels. Hence, achieving the balance of all these parameters allow for determining the best extender. Similarly, (Bencharif et al., 2012) found that each extender to have better results in a specific aspect and its own advantage. For instance, the mixture of 6% LDL with glutamate medium was found to improve the aspect of spermatozoa motility.the aspect of where the semen is collected is also captured. In canine epididymal sperm ACP-106c extender is found useful especially immediately after sperm recovery (Filho*et al*., 2014).

Furthermore, it has found that when extenders and enhancers are added to cold stored semen help to improve spermatozoa viability and progressive motility (Kmenta *et al*., 2011). Hence, the argue is to find more in terms of fertility of these spermatozoa likewise as to whether enhancers can be helpful in other extenders other than lecithin-based extenders. Nevertheless, this is supported by studies comparing commercial versus laboratory prepared extenders. In addition, laboratory preparedextenders are found to have better preservation characteristics as compared to the commercial ones (Iguer-ouada and Verstegen, 2001)(Figure 2). Therefore, this shows that the modification in terms of extenders can provide a way to improve cryopreservation. Possibly use of own prepared extenders other than commercial ones will be useful as they will consider the need of the breeder. They might take note of individual dog difference and the environment in question as the commercial ones were made under different settings.

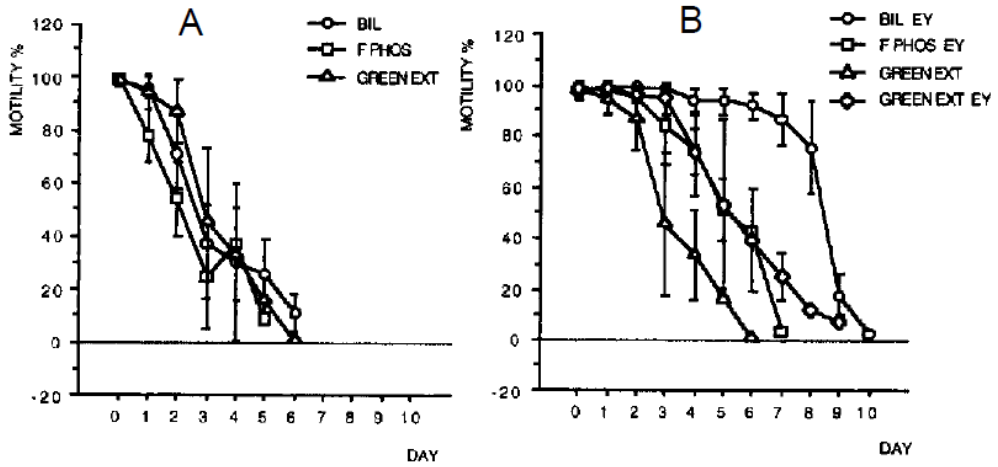

Figure 4. Percentages of spermatozoa motility over time

Figure 2: (A) shows changes in percentages of spermatozoa motility over time when semen was diluted in commercial extenders (Biladyl, fresh-phos, and green-extender). B shows improved motility when modifications were done on the three commercial extenders especially under biladyl. The modification in this case was addition of 20% egg

yolk (taken with modification from Iguer-ouada and Verstegen (2001).

Cryoprotectants

Semen cryoprotectants improve cell survival after the freezing process. Cryoprotectants are divided in to two groups. First are intracellular cryoprotective agents like glycerol and dimethylsulfoxide. Second are extracellular ones such as proteins and sugars. Glycerol is the cryoprotectant often used to freeze semen of different species (Silva *et al*., 2003). As a permeable cryoprotectant, glycerol prevents the formation of ice crystals inside the cells. However, glycerol has been reported to has toxic effects on spermatozoa, such as physicochemical alterations that can lead to rupture of the plasma membrane or removal of important membrane proteins, and cause acrosomal damage, which will be reflected in reduced fertility (Curry, 2000; Holt, 2000).

The ideal cryoprotectant should have a low molecular weight with high water solubility and low toxicity (Medeiros *et al*., 2002). However, there is a great diversity in cryobiological responses of different cell types or given cells among different mammalian species. Cryosurvival requires that cell freezing and thawing be done within certain biophysical and biological limits defined by the following cryobiology principles: cells should be frozen in such a way that little or none of their intracellular water freezes. They should be warmed in such a way that unfrozen intracellular water remains unfrozen during warming, or that small ice crystals formed during cooling remain small during warming. Even the aforementioned conditions are important; most cells will not survive unless substantial concentrations of cryoprotectants are used. It is well known that milk and egg yolk are nonpermeable cryoprotectants. They prevent formation of ice crystals outside the cells, provide enough energy for the spermatozoa and also play the role of phosphate buffer for them (England *et al*., 2000).

Egg yolk has been an important ingredient in extenders used for preservation of dog semen. The low density proteins in egg yolk provide protection of thesperm membranes against cold-shock and cryo-induced cell damages (Farstad 2009). Egg yolk is, however, a biological product with associated disadvantages of a high risk of contamination with bacteria (Bousseau*et al*., 1998). Furthermore, the fears of interspecies transmission of avian influenza, hygienic risks associated with the use of egg yolk and the difficult to standardize the quality of the extenders (Bousseau*et al*., 1998), there is an interest in using animal-free protein extenders for preserving semen from different species. Recently, it has been demonstrated that soya bean lecithin might be an interesting alternative to egg yolk for liquid cold preservation as well as cryopreservation of dog semen (Beccaglia*et al*., 2009a, Beccaglia*et al*., 2009 b; Kmenta*et al*., 2011; Kasimanickam*et al*., 2012). However, Soya lecithin contains different phospholipids concentrations which have negative effects on post-thaw quality of canine sperms (Axner and Lagerson, 2016).

Usually with the use of cryoprotectants agents (CPAs) there are certain levels or concentration that are advised to be used around 2-8%, but beyond which, the cryoprotectants become toxic. In dogs using 0.5ml straw, it has been found that the use of CPA-free freezing is difficult to be achieved. But there is the possibility of CPA-free methods of cryopreservation to be used in large volume of sperm in dogs. Therefore, it is suggested to use methods that involve low concentration of glycerol without cooling stages to 6^0C. Likewise, a brief exposure to the liquid nitrogen vapour can be used as it provides better post freezing and thawing sperm characteristics (Kim *et al*., 2012). Concentration of glycerol of about 4 to 11% has been widely used (Peña and Linde-Forsberg, 2000). However, high glycerol concentrations of from 4 to 8% have been studied (Cardoso*et al*., 2003) providing better results like inclusion of 20% coconut and egg yolk 20% extenders. Since there is limited research to whether CPA-free can be a solution in dogs then, the use of low glycerol concentration can be adopted. Hence, this review recommends the use of concentration of about 4 to 8% for better results. This might be helpful to control toxicity levels of cryoprotectants.

Method of collection

The most commonly used method of semen collection in dogs is manual. Sperm can also be collected from the epididymis after surgical sterilization, post-mortem or vaginal lavage after natural mating. Dogs ejaculate in three fractions. The first fraction is termed as pre-sperm fraction which originates from the prostate gland. Normally it is clear or slightly cloudy and volume ranging from 0.5 to 20 ml or more (Freshman, 2001). The second fraction is called as sperm-rich fraction which is normally opaque, milky-white in colour and ranging from 0.5 to 2.0 ml (Johnston *et al*., 2001). The third or prostatic fraction is normally clear and may consist of more volume, depending on how long pressure is maintained proximal to the bulbusglandis (Johnston *et al*., 2001). This prostatic

fraction is also useful for evaluating the prostate diseases. Interestingly, the method of collection can limit sperm quality. For instance, dog's semen quality varies between manualand electro-ejaculation methods.Although spermatozoa motility is almost the same in both manual and electro-ejaculation but individual sperm motility does not last longer in manual collection as compared to electro-ejaculation method (Christensen *et al*., 2011). Moreover, direct collection of ejaculates to the extender in sought as an attempt to reduce agglutination (Gomes-Alves *et al*., 2014). Additionally, modification such as collection into a warm extender help to reduce the impact of pH shift and cold shock (Johnson, 2000). Hence, improve parameters such as viability and motility (Figure 3).

The site of where semen is collected for instance in the epididymal spermatozoa when fluids from accessory glands has much improvement in terms of semen quality. Addition of prostatic fluid has also been found to improve sperm fertilising ability (Nothling*et al*., 2005), conception rate (Nothling*et al*., 2007), motility (Hori *et al*., 2005; Korochkina*et al*., 2014), viability (Hori *et al*., 2005), and distance average path and velocity (Korochkina*et al*., 2014). These positive effects of prostatic fluid could be due to secretions produced by the prostate which contains citric acid, calcium, and a number of enzymes that can protect the sperm cell and improve the survival, motility and fertilizing ability.

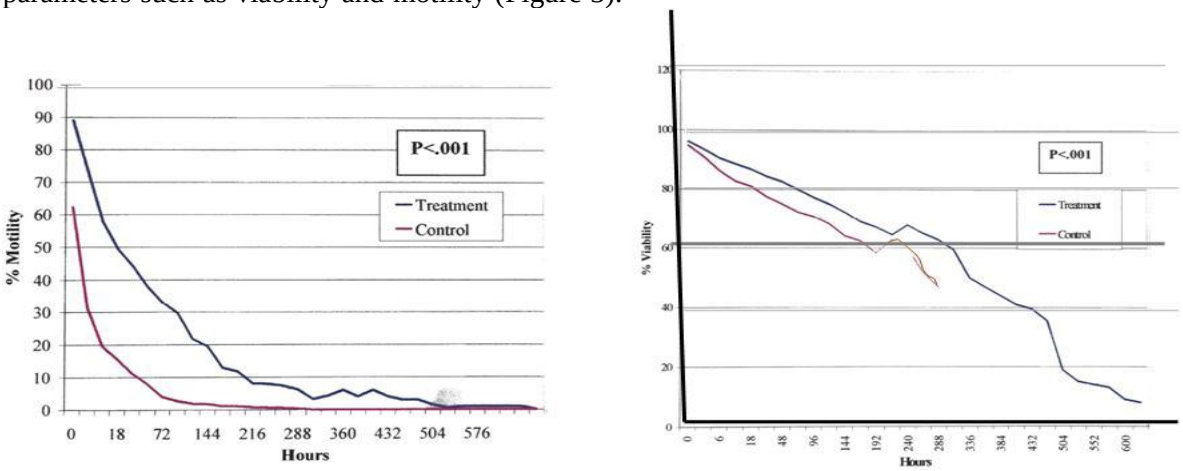

Figure 5. Motility and viability when collection of semen is modified

Figure 3: Treatment group are dogs where modification in collection was undertaken i.e. the sample was collected into warmed extender. The results were better in the treatment group than those where semen were collected into dry container without any modification (Control). Hence, improved motility and viability of spermatozoa (source: Johnson, 2000).

Anti-oxidants

Antioxidants are found to control the balance between the reactive oxygen species and hence, control the sperm cell membranes and DNA. It is worthwhile noting that normally the physiological concentration of antioxidant in the spermatozoa cytoplasm is not that sufficient to protect against the cryopreservation procedures (Bansal, 2010). Antioxidants like catalase were reported to have a pronounced effect on the improvement of sperm quality after thawing (Michael e*t al*., 2007). Kmenta *et al.* (2011) used antioxidant catalase as an extender with 0.8% lecithin and reported superior preservation of cold stored spermatozoa in canine (Kmenta *et al.*, 2011). Though still the concentration of these antioxidants is found to be necessary to achieve certain results in terms of improvement of sperm quality that would also vary from one antioxidant to another. For instance, in studies involving glutathione and ascorbic acid, glutathione at 5mM was found to give better results when supplemented (Monteiro *et al*., 2009) (see table 3). Farstad, (2009) suggested that when these extenders are supplemented with defined compounds such as cryoprotectants and antioxidants they can be helpful to protect sperm membrane damage and also DNA damage. Therefore, addition of antioxidant is very useful towards protecting sperm from damage and hence, improving cryopreservation. The use of antioxidants can be of value if the right type of antioxidant and the right concentration of antioxidant can be applied in order to give better results. Hence, this area requires a need for further research to ascertain the best antioxidant and their respective concentration.

Table 3: Showing forward progressive velocity of sperm (SFPV 0-5). Glutathione (Glu) at concentration of 5 had a higher velocity in almost all time intervals. Varying concentration of both

ascorbic acid (AA) and glutathione gave different results in terms of SFPV. Sperm involving glutathione at a concentration of 5mM showed higher forward progressive velocity (taken from (Monteiro *et al*., 2009).

Table 12. Showing forward progressive velocity of sperm

Sperm forward progressive velocity (0–5)					
Groups	0 min	30 min	60 min	90 min	120 min
Control	2.9 ± 0.1	2.4 ± 0.3*	1.5 ± 0.6^{ad}	0.8 ± 0.9^{b}	0.2 ± 0.3
AA-50	3.1 ± 0.1	2.7 ± 0.3^{c}	2 ± 0.4^{d}	1.4 ± 0.6^{d}	0.4 ± 0.7
AA-250	3.1 ± 0.1	2.8 ± 0.3^{c}	2.1 ± 0.4^{c}	1.1 ± 0.7	0.3 ± 0.7
Glu-1	3.2 ± 0.4	2.6 ± 0.4^{c}	2.1 ± 0.7^{d}	1.3 ± 1.2^{d}	0.4 ± 0.9
Glu-5	3.4 ± 0.5	3.2 ± 0.5^{c}	2.6 ± 0.7^{ad}	1.8 ± 1.2^{bd}	0.8 ± 0.8

Conclusion

Cryopreservation of dog semen facilitates the exchange of genes between populations and makes it possible to preserve genes from valuable males for an extended time. However, cryopreservation of dog semen has a number of limitations which are partly caused by freezing temperature, cryoprotectants and post thawing lifespan of spermatozoa. As an attempt to improve the output of cryopreservation, the time, type of semen to collect, as well as age of the animal from which we collect our ejaculates are important. Nevertheless, storage is still a stumbling block but there seems a possibility that improving the method of storage, can help to improve the semen quality. Therefore, more research is needed to find out the appropriate storage improvement for cryopreserved semen.

There are individual differences in terms of post-thaw semen quality. Involvement of thawing temperature of 70ºC and above within few seconds presents promising results. Modification in terms of extenders can provide a way to improve cryopreservation. There is a need for further study and examination of the particular stud dog which provides good results in terms of post-thaw viability of spermatozoa. Possibly, there can be development of individual freezing extenders for those dogs that show problems in terms of semen freezing and yet are considered as superior breeds. Alternatively, increasing sperm number can be one of the options to compensate differences in fertility among males. Moreover, we recommend further studies and modifications to be done to qualify the use of higher temperatures as an attempt to improve results under the cryopreservation.

Modification in terms of extenders can provide a way to improve cryopreservation. Possibly, use of own prepared extenders other than commercial ones will be useful as they will consider the need of the breeder. They might take note of individual dog difference and the environment in question as commercial ones were made under different settings. Since there is limited researchon the use of CPA-free in cryopreservation in dogs then, therefore, we recommend the use of glycerol at 4 to 8% concentration for better results. Possibly, as the concentration of cryoprotectants increases also the thawing temperature has to increase as well. This might be useful for controlling toxicity levels of cryoprotectants, and thus improving conception rates.

The collection method can be a tool to improve the results of the cryopreservation process. Therefore, use of methods that do not confer to long exposure of sperms to prostate fluid will be of value here. Furthermore, collection of ejaculates into extension media together with modification of the environment of extension media can be of value. The addition of antioxidants is very useful for protecting sperm from damage and thus, improving cryopreservation. The use of antioxidants can be of value if the right type of antioxidant and the right concentration of antioxidant can be applied in order to give better results. Therefore, the review recommends further research to ascertain the best antioxidant and their respective concentrations.

Finally, improving cryopreservation is not one process. It involves a combination of a lot of factors with the ultimate goal of improving the outcome of cryopreservation. This is in terms of conception rates in bitches and thus, fertility in general. Factors

to be improved include: type of ejaculate, insemination technique and storage also, post thawing procedures, extenders, cryoprotectants, collection method and antioxidants.

ACKNOWLEDGEMENTS

The authors acknowledge all academic members of staff in the Department of Veterinary Surgery and Theriogenology at Sokoine University of Agriculture for their contributions during writing of this review work.

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Magnitude of foetal wastage and the monetary losses in sheep and goats slaughtered in Morogoro selected slaughter facilities, Morogoro Tanzania

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SUMMARY

Foetal wastage in slaughter animals not only causes a loss to farmers and livestock traders, it's against animal welfare still information on the magnitude of slaughter of pregnant goats and sheep is scarce. This study evaluated the level of fetal wastage in goats and sheep slaughtered at Morogoro slaughterhouse and Mkongeni slaughter slab in Mvomero district between December 2016 and January 2017. Of the 351 goats slaughtered, 80.1% were female and 40.2% of them were pregnant. Likewise, 97 sheep were slaughtered, 80.4% were female of which 57.7% were pregnant. Of all the fetuses (n=163) recovered, 61.5% had ≥2 months of age and 61 (37.4%) were female. Four out of 113 (3.5%) of pregnant does and 1/45 (2.2%) of pregnant ewes had twins. A loss of 951,000 Tanzania shillings was calculated due to fetal wastage in 30 days. The results of this study demonstrate that there is significant flock wastage and losses which limits availability of animal protein to the people in Morogoro and elsewhere in Tanzania. It is therefore recommended that appropriate measures including legislation enforcement, capacity building on pregnant diagnosis for slaughter facilities staff be put in place to control the slaughter of pregnant stock. Stock owners and traders should be sensitized on the implication on losses of genetic materials and sustainability of meat production associated with continued slaughtering of pregnant animals.

Key words: sheep, goats, foetal wastage, slaughter facility, monetary loss

INTRODUCTION

The human population is increasing along with the increase for food rich protein. This rapid growth gives challenges to food security especially in developing counties like Tanzania. Tanzania has a population of 44.9 million people which has increased from 34.4 million in 2002 with an average growth rate of 3% per year (PHCT, 2013). Meanwhile, the livestock population growth shows that in 2006, there were 18.5 million cattle, 13.1 million goats, 3.6 million sheep and 1.2 million pigs (Njombe and Msanga, 2009). Currently there are 25.8 million cattle, 17.1 million goats, 9.2 million sheep, 42.0 million local chicken, 34.5 million commercial chicken and 2.7 million pigs (URT, 2016). Demand for animal protein which is propelled by population growth, urbanization, eating behavior and increased income is not matching with the slow annual growth rate of domestic livestock production, currently estimated at 2.5% compared to 2.7% human growth (URT, 2012).

Furthermore, there has been a global trend of human population towards preference to proteins of animal sources. The FAO projects a 73% increase in meat consumption by 2050 and that much of the future demand for livestock will be met by large-scale/intensive operations (Alexandratos and Bruinsma, 2012). To meet this demand, more intensified livestock keeping coupled with increased number may be important. As a result of this slow growth and high demand, such trend attracts movement and trade of small stock within and outside the country and undesirable practices including slaughtering of breeding stock and pregnant animals is common (Nonga, 2015).

Meat is one of the sources of protein of animal origin which is obtained from slaughter of livestock. The common meat animals in Tanzania include cattle, sheep, goat, pigs and poultry. Sheep and goat production in Tanzania is under small scale farmers. Many households especially in rural areas keep goats and sheep (URT, 2012). Sheep and goat are the second major source of meat after cattle in Tanzania, but full utilization of these animals is not achieved due many reasons including losses which occur during slaughter in particular fetal wastage. Slaughtering of pregnant does and ewes has been a common practice which is unethical and also it cause fetal losses. In developing countries this practice has been noted in countries such as in Tanzania where by 38.9% of does and ewes slaughtered at Tanga abattoir were pregnant (Swai *et al.,* 2015). Kashoma and Melkiory (2016) also reported foetal wastage of up to 51.8% in sheep and goats slaughtered at Dodoma Municipal abattoir. In Sahel region in Nigeria foetal wastage of 22.8 % and 17.9 % was recorded in ewes and goats

slaughtered respectively (Bokko, 2011). Hence there is therefore an urgent need to salvage this unaccepted practice of slaughtering useful pregnant animals.

In Tanzania local breeds of sheep and goats are the mostly slaughtered and these animals are raised by pastoralist and agro pastoralists who sell their animals at the livestock market or to business people who ultimately send the animals to slaughter facilities. On average 2.71 million goats and 1.008 million sheep are slaughtered in Tanzania annually (NBS, 2012). It is required by law that all animals sent for slaughter in slaughter facilities have to undergo ante mortem examination. Unfortunately, pregnant diagnosis is not normally done and this has been among the reasons of slaughter of pregnant animals.

Morogoro slaughterhouse slaughters up to 15 sheep and goats per day. Similarly, at Mkongeni livestock market in Mvomero district, the slaughter of sheep and goats goes up to 40 on each Saturday. According to the Mvomero District Veterinary Officer, each slaughter is accompanied with a number of foetal wasted. Due to this reason, the study was conducted so as to establish the magnitude of foetal wastage and estimate economic losses due to slaughter of pregnant ewes and does at Morogoro slaughterhouse and Mkongeni livestock market in Mvomero district in Morogoro, Tanzania. The study has generated a baseline data that will be useful to different stakeholders including policy makers to combat the problem of slaughtering pregnant animals.

MATERIALS AND METHODS

Study area and study animals

This cross sectional study was conducted at Morogoro slaughterhouse and Mkongeni livestock market in Mvomero district, Morogoro Tanzania. The Morogoro region is located 196 kilometers west of Dar es Salaam. The Morogoro slaughterhouse provides the daily meat requirements of the inhabitants of Morogoro with the population of 2,218,492 (PHCT, 2013). The slaughterhouse serves as a slaughter place for cattle and small ruminants where the average of 100 cattle and up to 15 sheep and goats are slaughtered daily. Mkongeni livestock market in Mvomero slaughters between 30 and 40 sheep and goats per Saturday. Animals slaughtered at Mkongeni slaughter facility are obtained mainly from livestock markets including Mkongeni and Melela and mostly are local breeds. The study animals were sheep and goats brought for slaughter from different districts of Morogoro. Some animals were transported to the slaughterhouse using vehicles and others were trekked.

Data collection

This prospective study was undertaken for 30 days (December 2016 to Jan 2017). It involved daily visiting at the Morogoro slaughterhouse and at Mkongeni livestock market every Saturday and participated in meat inspections. After evisceration the uterus of slaughtered sheep and goats were thoroughly examined for pregnancy. All gravid uteruses were opened in order to age and sex the foetuses. The recorded information also involved total number of slaughtered animal in that particular day, number of ewes and does slaughtered, number of pregnant ewes and does, presence of twins or not, the slaughter area, source of slaughter stock, age and sex of fetus and the breed of an animal.

Monetary loss was estimated using Babatunde *et al.* (2011), which calculates the monetary loss at birth (MLB) MLB = N0 x P0; where N0 = Number of fetuses at birth, P0 = average price of kid and lamb at birth. The average price of a lamb was 5000/= and a male kid was 6000/= and for the ewe lamb was 6000/= and a female kid was 8000/= (Personal communication, Mr. Wera, 2017).

Data analysis

The collected data were entered, stored in Microsoft Excel spread-sheet and analysed by EPI-info statistical software. Descriptive statistics such as the proportion of all slaughters, frequency of pregnant slaughtered small ruminants and the extent of fetal wastage were generated.

The percentage of fetal wastage was calculated as the total number of fetuses recovered divided by the total number of ewes and does slaughtered. For the purpose of this study, the number of male kids was 75X6000=450000 while that of male lambs was 25X5000=125,000. The female kids were 32X8000=256000 and the ewe lambs were 20X6000=120000. A total loss of 951,000 Tanzania shillings was encountered due to fetal losses during the study.

RESULTS

General results

A total of 448 sheep and goat were slaughtered between December 2016 and January 2017.Of the slaughtered small ruminants 351 (78.4%) were goats

and 97 (21.6%) were sheep. The results of the proportion of males, females and pregnant does and ewes slaughtered are shown in Table 1. Out of 351 slaughtered goats 281 (80.1%) were females of which 113 (40.2%) were found to be pregnant. Consistently, of 97 slaughtered sheep 78 (80.45%) were females and 45 (57.7%) were also found to be pregnant. In total, (sheep and goats) 359 female animals were slaughtered during this one month and 44.01% were pregnant. Four out of 113 (3.5%) of pregnant does and 1/45 (2.2%) of pregnant ewes had twins. Overall, (n=359; 80.1%) female goats and sheep were slaughtered than males (n=89; 19.9%) during the study period.

Table1. Proportions of small stock (goats and sheep) slaughtered at Morogoro slaughter facilities

Species	Parameter	Frequency	Percent
Goat	Total goats slaughtered	351	78.3
	Male goats slaughtered	70	19.9
	Does slaughtered	281	80.1
	Pregnant does slaughtered	113	40.2
	Twin pregnant does	4	3.5
Sheep	Total sheep slaughtered	97	21.7
	Male sheep slaughtered	19	19.6
	Ewes slaughtered	78	80.4
	Pregnant ewes slaughtered	45	57.7
	Twin pregnant ewe	1	2.2

Proportions of fetuses retrieved by age and sex

Results of the proportions of male and female fetuses and stage of gestation are shown in Table 2. In goats, 38 (33.6%) and 75 (66.4%) of the fetuses were females and males respectively. A total of 62 (60.2%) of the retrieved fetuses were estimated to be in their advanced stage (≥2 months) of their gestation while 41 (39.8%) were in their early stage of gestation (≤2 month). In sheep, 20 (44.4%) of the fetuses were females and 25 (55.6%) males. Up to 32 (71.7%) pregnant sheep slaughtered were at the advanced (≥2 month) stage of gestation while 13 (28.3%) were in their early stage of gestation (≤2 month). In both species, there were more male fetuses than females.

Table 2. Proportions of fetuses retrieved by age and sex at Morogoro slaughter facilities

Species	Parameter	Frequency	Percent
Goat	Male fetuses	75	66.4
	Female fetuses	32	28.3
	Male fetuses ≤ 2 months	35	31.0
	Female fetuses ≤ 2 months	27	23.9
	Male fetuses ≥ 2 months	40	35.4
	Female fetuses ≥2 months	11	9.7
Sheep	Male fetuses	25	56.9
	Female fetus	20	43.1
	Male fetuses ≤ 2 months	10	22.2
	Female fetuses ≤ 2 months	9	20.1
	Male fetuses ≥2 months	15	33.3
	Female fetuses ≥2 months	11	24.4

Financial losses incurred due to fetal losses

The summary of financial losses incurred due to fetal losses is in Table 3. It was found that 951,000 Tshs is lost in the two slaughter facilities due to fetal loss in one month period. This implies that in one year a total of 11,412,000= is lost due to fetal wastage.

Table 3. Financial loss due to fetal loss

Species	Sex	Number of fetus	Price (Tshs)	Monetary loss (Tshs)
Goat	Male	75	6000	450,000
	Female	32	8000	256,000
Sheep	Male	25	5,000	125,000
	Female	20	6,000	120,000
Total loss				951,000

DISCUSSION

The results in this study show that more female goats and sheep (80.1%) were slaughtered than males. This scenario also was noted by Swai *et al.* (2015) where 57.1% slaughtered small ruminants were female. The results of the current study are also in line with other studies in Tanzania and elsewhere (Kashoma *et al.*, 2016; Bokko, 2011; Borji *et al.*, 2011; Simenew *et al.*, 2011; Zulu *et al.*, 2013). The reasons could be female animals were being culled due to different reasons including infertility. Since the study was done during season, female animals may have appeared good and shiny because of pregnancy but farmers selected them for sell since they fetched good prices at the market.

Also it was noted that slaughter of pregnant does and ewes was rampant since (44.1%) of slaughtered does and ewes were found to be pregnant. This high incidence was also reported in a study done by Swai *et al.* (2015) were 38.5% of slaughtered does and ewes were found to be pregnant. The incidence of slaughter of pregnant sheep and goats was very high in the study done in West Africa where 60% of the 1,248 female goats slaughtered at an abattoir over a period of 1 year were pregnant (Goossens *et al.*, 1998). Differences in magnitude of foetal wastage in slaughter animals may be due to differences in sample size and the duration of study. The possible reasons could be pregnancy diagnoses is not routinely conducted during ante-mortem inspection in the slaughter facilities due to various reasons including poor infrastructures and lack of diagnostic tools for pregnancy diagnosis. Also another reason could be that the pregnant animals had good looking appearance compared to others hence sent for slaughter at higher numbers as the study was done during the dry period.

Slaughtering of pregnant ewes and does at Morogoro slaughterhouse and Mkongeni livestock market has been noted during the study. This causes fetal losses, it is against the Animal Welfare Act in Tanzania (URT 2008) and has a direct impact to the future sheep and goat flocks in Tanzania. Slaughter of pregnant animals provides meat of poor quality to the consumers but also causes losses to traders since the carcass weight of a pregnant animal is less by ten percent (Nonga, 2015).

Another observation was that up to 60.2% of the retrieved fetuses in the two species were in their advanced stage (≥2 months) of pregnancy, which was also noted by Swai *et al.* (2015). The possible reasons for this could be that these animals seemed to be fatter than others (good looking) and this is easily noted at advanced stages of pregnant which make owners to select them for sell on thought of having more weight.

Also male fetuses were retrieved more than female fetuses. This observation was different from the study done by Swai *et al.* (2015) where more female (57.2%) fetuses were retrieved in slaughter small ruminants at Tanga, Tanzania. These observations may have happened by chance.

In Morogoro region more goats 351 are slaughtered than sheep 97 in a span of one month. The reason is that more goats are kept in Morogoro region than sheep. Nevertheless, the other possible reason could be on consumer preferences on goat meat against sheep meat. This could be supported by the price of these two species which indicates that goat meat is more expensive than sheep meat.

The average fetal wastage rate of 40.2% in goats and 57.7% in sheep revealed in this survey are more or less similar to that reported by Swai *et al.* (2015) in Tanga and Muhammad *et al.* (2009) in Nigeria. The reason for the rates observed is that pregnancy diagnoses are not routinely conducted during antemortem inspection in the slaughter premise due to various reasons including poor infrastructures and staff competency in carrying out pregnancy diagnosis.

The losses due to slaughter of pregnant does and ewes were about 951,000 Tanzania shillings due to fetal wastage in one month. If it is assumed that the same levels of losses are incurred each month, 11,412,000 Tanzania shillings are lost per year only from the two slaughter facilities in Morogoro region. The risk of this loss to increase is high as the

number of animals slaughtered increases in festival seasons. Therefore, it is important that such losses are minimized through routine antemortem examination of all slaughter sheep and goats.

Based on the findings from this study it is concluded that there is high fetal wastage in Morogoro region. Most of the wasted fetuses are were at the advanced stage of pregnancy and were males. The losses associated with such a high foetal losses are also high. It is important that the government and all stakeholders in livestock industry should make sure the habit of slaughtering pregnant animals is stopped immediately.

ACKNOWLEDGEMENTS

The authors acknowledge the Higher Education Student Loan Board for financing this study. The cooperation shown by the staff at Morogoro slaughterhouse and Mkongeni livestock market is appreciated.

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Microbiota prime leukocyte response for intestinal innate immunity

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SUMMARY

The number of microbiota in the intestinal lumen is overwhelmingly larger than the total number of an individual's cells. This alludes to a significant contribution of the microbiota to the life of an individual, particularly priming the immune system. The diverse microbiota antigens are encountered by leukocytes that drive non-specific innate immunity. While these leukocytes are insensitive to harmless agents in terms of inciting inflammatory response, they respond to noxious agents by immediately destroying and eliminating them from the body. This is done through phagocytosis, granulocytosis, degranulation to release enzymes and inflammatory mediators, antigen presentation, and activation of adaptive immune system. These functions are influenced by microbiota whose disruption impairs the optimal functioning of leukocytes and predisposes the intestine to diseases like allergy, inflammatory bowel diseases, and irritable bowel syndrome. Maneuvers that restore disrupted microbiota benefit patients with these diseases. Gaining insight into the microbiota-leukocyte crosstalk and its subsequent role in maintaining intestinal immunity is pivotal to designing medicaments against microbiotal dysbiosis-mediated illnesses.

Key words: Microbiota, natural killer cells, innate lymphoid cells, mast cells, eosinophils, intestinal innate immunity

INTRODUCTION

Cells of the innate immune system are mainly leukocytes that include natural killer (NK) cells, mast cells, eosinophils, basophils and the phagocytic cells including macrophages, neutrophils and dendritic cells (DCs). Their functions involve identifying and eliminating antigens and pathogens that might cause disease. Intestinal luminal antigens must activate these cells to cause inflammation which is subsequently promoted and maintained through stimulation of the adaptive immune response. To carry out their functions, these cells are equipped with all necessary ability to combat the microorganisms including expression of receptors, secretion of inflammatory mediators like cytokines, histamine, heparin, proteolytic enzymes, reactive oxygen species, eicosanoids, and growth factors (Malago, 2015) As they execute their functions, they initiate inflammation by secreting these mediators and antimicrobial agents leading to phagocytosis of microbes and infected cells, antigen presentation, and activation of the adaptive immune system (Nakanishi, 2010; Smith *et al.*, 2012; Noti *et al.*, 2014).

The intestinal environment is enriched with diverse commensal microbes and oftentimes challenged by pathogens. While the pathogenic microbes are recognized and selectively eliminated from the body by the cells of the immune system, commensal microbes contribute significantly to supporting the immune system fight the pathogens. Disturbances to the microbiota may fail this support and could lead to diseases like colitis (e.g. chemical, spontaneous, and that occurring in inflammatory bowel disease, IBD), irritable bowel syndrome (IBS), cytomegalovirus, and allergy. Interventions aimed at restoring microbiota profile often ameliorate in part or complete the severity of these diseases. The mechanisms involved in this amelioration involve improvement in the functioning of cells of the innate immune system (Wang *et al.*, 2013; Brenner *et al.*, 2014; Kobayashi *et al.*, 2014). Indeed, the influence of microbiota to these cells is fairly evident. Amongst others, the microbiota plays role in regulation of granulocytosis, neutrophil phagocytosis, degranulation and homeostasis, and host resistance to conditions caused by disruption of leukocytes (Deshmukh *et al.*, 2014; Knoop *et al.*, 2015). Gaining insight into the microbiota-innate immune cell interaction could be a prelude towards designing successful therapeutics against disorders caused by abnormalities in this relationship. This work describes the contribution of microbiota to the functioning of NK cells, mast cells and eosinophils for gut innate immune system during health and disease.

INFLUENCE OF MICROBIOTA ON INNATE LYMPHOID CELLS

The microbiota primes innate lymphoid cell functioning

Natural killer (NK) cells belong to innate lymphoid cells (ILCs), some of which are analogous to T helper (Th) cells while others to cytotoxic NK cells. ILCs regulate immune homeostasis and inflammation and play role in gut immunity. The intestinal microbiota regulates expression of NK cell ligands on intestinal epithelial cells (IECs) to influence NK cell responses. In this regard, it decreases the expression magnitude of NK group 2 member D (NKG2D) ligands on IECs. Absence of microbiota in germ-free and ampicillin-treated mice increases the NKG2D ligand expression while propagation of *Akkermansia muciniphila* by vancomycin which reduces the level of interferon (IFN)-γ and interleukin (IL)-15, decreases the NKG2D ligand expression. Similarly, dietary xylooligosaccharides-induced increase in *A. muciniphila* decreases the expression of NKG2D ligand while IL-10 deficiency increases NKG2D ligand expression (Hansen *et al.,* 2013).

Furthermore, the microbiota promotes a crosstalk between macrophages and ILCs that leads to immune homeostasis in the intestine. This occurs when macrophages sense microbial signals and produce IL-1β that drives transcription factor retinoic acid orphan receptor (ROR)γt$^+$ ILCs to produce granulocyte-macrophage colony-stimulating factor (GM-CSF) that maintains intestinal immune homeostasis (Figure 1). Deficient GM-CSF production alters mononuclear phagocyte effector functions resulting in reduced regulatory T (Treg) cell numbers and impaired oral tolerance (Mortha *et al.,* 2014).

The ILCs also control microbiota composition through modulation of IgA production. This occurs when RORγt$^+$ ILCs produce either soluble lymphotoxin α (sLTα3) which controls T cell-dependent IgA induction via regulation of T cell homing to the gut or membrane-bound lymphotoxin β (LTα1β2) which drives T cell-independent IgA induction by controlling DC functions (Figure 1). Ablation of LTα in RORγt$^+$ cells abrogates IgA production and alters intestinal microbiota composition (Kruglov *et al.,* 2013). In addition, both LTi cells and IL-22-producing NKp46$^+$ cells of the ILCs constitutively produce most of the intestinal IL-22. The microbiota represses this production by inducing epithelial expression of IL-25. This function plays an essential role in fine tuning the equilibrium among intestinal symbionts, adaptive immunity and RORγt$^+$ ILCs (Sawa *et al.,* 2011).

Some specific microbiota strains like *Bifidobacterium longum* BB536 maintain or increase the activity of NK cells in elderly which under normal circumstances decreases with aging (Akatsu *et al.,* 2013). Increased growth of intestinal *Bifidobacterium* spp by galacto-oligosaccharides (GOS) also increases the percentage of NK cells in mesenteric lymph nodes (MLNs) and reduces severity of *Helicobacter hepaticus* colitis. Further, *Lactobacillus salivarius* salivarius CECT5713 isolated from breast milk and taken orally at 2 x 10^8 colon forming units daily for 4 weeks increases the percentage of NK cells and the regulatory cytokine IL-10 (Sierra *et al.,* 2010).

Microbiota-driven ILC functions during disease

A study by Klose and others (2013) reported that during ontogeny CCR6$^+$RORγt$^+$ ILCs do not express transcription factor T-bet. Instead, T-bet expression occurs in postnatal period and is controlled by signals from microbiota and IL-23. Its expression activates T-bet target genes including IFN-γ and natural cytotoxicity receptor NKp46 to produce NK-22 cells (i.e. IL-22 producing NK cells). The produced IFN-γ protects the gut against *Salmonella* enteric infection through the release of mucus-forming glycoproteins (Figure 1). The microbiota also promotes production of type I IFN by macrophages and the subsequent IFN-priming of NK cells. The primed NK cells confer protection against mouse cytomegalovirus and lymphocytic choriomeningitis virus (Ganal *et al.,* 2012). Furthermore, the microbiota confers benefits against NK cell leukemia, a condition characterized by an expansion of these cells. The condition develops from an immune response signaling through platelet-derived growth factor (PDGF) and IL-15 to constitutively activate the NK cell expansion while dysregulating apoptosis and favoring their survival. A dysregulated sphingolipid metabolism with increased glucosylceramide synthase (GCS) expression is the cause for the disease. Maneuvers that induce NK cell apoptosis such as suppression or inhibition of GSC expression have been earmarked as potential therapeutics against the disease (Liu *et al.,* 2010; Watters *et al.,* 2013).

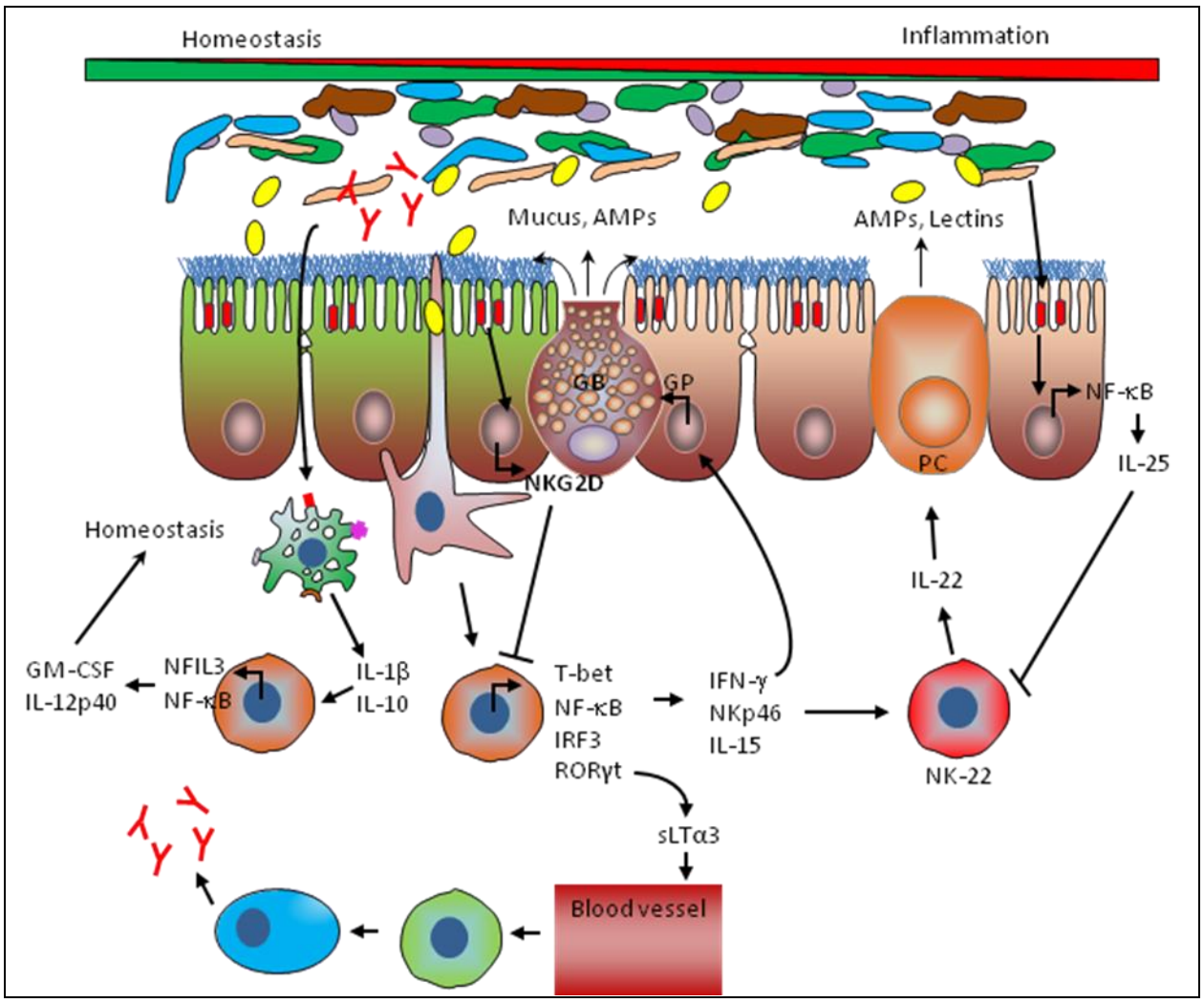

Figure 1. Influence of microbiota to innate lymphoid cells. Sampling of microbiotal antigens by dendritic cells or macrophages induce activation of various transcription factors whose effector products generate homeostatic and inflammatory responses to maintain intestinal immunity. Responses for homeostasis include production of GM-CSF, immunoglobulins following intestinal homing of B and plasma cells, mucus, and AMPs. To curb inflammation, the microbiota suppresses the inflammatory responses by suppressing the activities of ILCs such as production of IL-22, AMPs and lectins. AMPs, antimicrobial peptides; GB, goblet cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; GP, glycoproteins; IRF, interferon-regulatory factors; IL, interleukin; NF-κB, nuclear factor kappa B; NK, natural killer; NKG2D, natural killer group 2 member D; PC, Paneth cell; ROR, retinoic acid orphan receptor; sLT, soluble lymphotoxin.

SHAPING OF MAST CELL FUNCTIONING BY MICROBIOTA

The microbiota primes mast cell functioning for intestinal immunity

During early life, the microbiota sends appropriate signals that are critical for intestinal mast cell immunoregulation to protect from IgE-induced anaphylaxis. This has been observed in germ-free mice and those with low-diversity microbiota. Such animals develop high serum levels of IgE in early life due to B cell isotype switching to IgE at mucosal sites in a CD4 T-cell- and IL-4-dependent manner. The elevated IgE levels lead to increased mast-cell-surface-bound IgE and exaggerated oral-induced systemic anaphylaxis (Figure 2). This anaphylaxis can be prevented by maintaining a critical level of microbial diversity that inhibits IgE induction (Cahenzli *et al.*, 2013).

The microbiota influences the number and activity of mast cells with impact to intestinal health. A great increase in number of mast cells in the jejunum, allergic diarrhea and elevated ovalbumin (OVA)-specific IgG1 and total IgE in blood occur in mice sensitized with OVA. These changes are coupled with disruption of gut microbiota involving

a decrease in *Lactobacillus* spp in the colon (Figure 2) (Wang *et al.,* 2013). A separate study indicated that treatment of humanized mouse model with *Lactobacillus rhamnosus* GG (GG) and *Propionibacterium freudenreichii* spp. *shermanii* JS lowers the numbers of intestinal mast cells and down-regulates intestinal pro-inflammatory changes induced by a high-fat diet (Oksaharju *et al.,* 2013). The potency of microbiota in lowering the number and activity of mast cells has also been shown in antibiotic-induced alterations of microbiota in rats. These rats exhibit an increased mast cell protease II levels and higher mast cell numbers in their intestines (Nutten *et al.,* 2007). The alterations appear to involve almost-complete eradication of *Lactobacillus* in the whole intestine and in a drastic reduction of colonic total aerobic and anaerobic bacteria, in particular *Enterobacteriacae* and *Enterococcus* (Figure 2). Recovery of these microbes could lower the number of mast cells and their activity (Schumann *et al.,* 2005).

Microbiota-driven mast cell function during disease

Microbiota dysbiosis, such as small intestinal bacterial overgrowth (SIBO) and changes in the composition of the luminal and mucosal colonic microbiota have been linked to IBS. Subsets of patients with IBS show high numbers of mucosal mast cells in the small and large intestine (Ortiz-Lucas *et al.,* 2010; Barbara *et al.,* 2011; Barbara *et al.,* 2012). The increase in mast cells could partly be due to both psychological and physical stress. Stress induces degranulation of mucosal mast cells whose released products increase intestinal epithelial permeability. The increased permeability allows bacteria and/or their products like peptidoglycan to translocate from the lumen to the interior of the body where they prime the innate immune system to respond to antigenic stimulation. Treatments modulating the microbiota and those targeting activated mast cells may benefit IBS patients. Several probiotics have shown a reduction in IBS symptoms by an immunomodulatory and analgesic effects while others have demonstrated possible benefit of microbiota-mast cell interaction to IBS. The benefits of microbiota could be related to its influence on mast cell degranulation since exposure of mast cells to microbiota down-regulates degranulation. This effect requires a direct contact between bacteria and mast cells (Wesolowski and Paumet 2011).

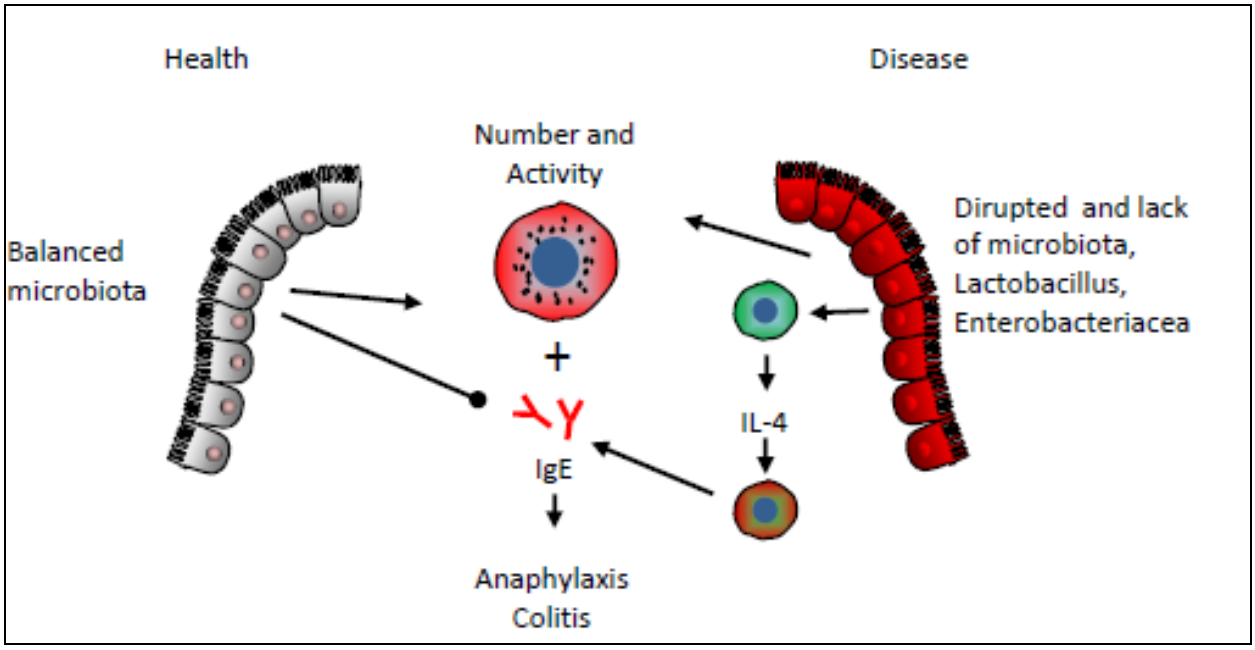

Figure 2. Influence of microbiota to mast cell functioning during health and disease. A balanced intestinal microbiota lowers the number and activity of mast cell and IgE. This reduces the mast cell binding to IgE and prevents diseases like anaphylaxis and colitis. Microbiotal disruption or absence increases mast cell number and activity as well as IgE and leads to diseases. IgE, immunoglobulin E; IL, interleukin.

MICROBIOTA INFLUENCE ON EOSINOPHILS

The microbiota primes eosinophil funtioning

The microbiota drives DCs to present tolerogenic signals by expressing TNF receptor (TNFR)-associated factor (TRAF)6 molecule. DC expression of TRAF6 is pivotal to gut-microbiota dependent immune tolerance which, in part, involves inactive eosinophils. Deletion of TRAF6 results in loss of mucosal tolerance exhibited by development of Th2 cells in the lamina propria and eosinophilic enteritis and fibrosis in the small intestine. This loss of tolerance is microbiota-dependent (Han *et al.*, 2013). The microbiota also promotes T-independent IgA class switching. Mice deficient in eosinophils exhibit impaired intestinal IgA production, accompanied by a disrupted mucosal layer and alterations in microbiota density and composition (Chu *et al.*, 2014). The role played by eosinophils in maintaining immune homeostasis in the gut revolves around influencing functional crosstalk between microbiota, IgA, T cells, and DCs (Figure 3).

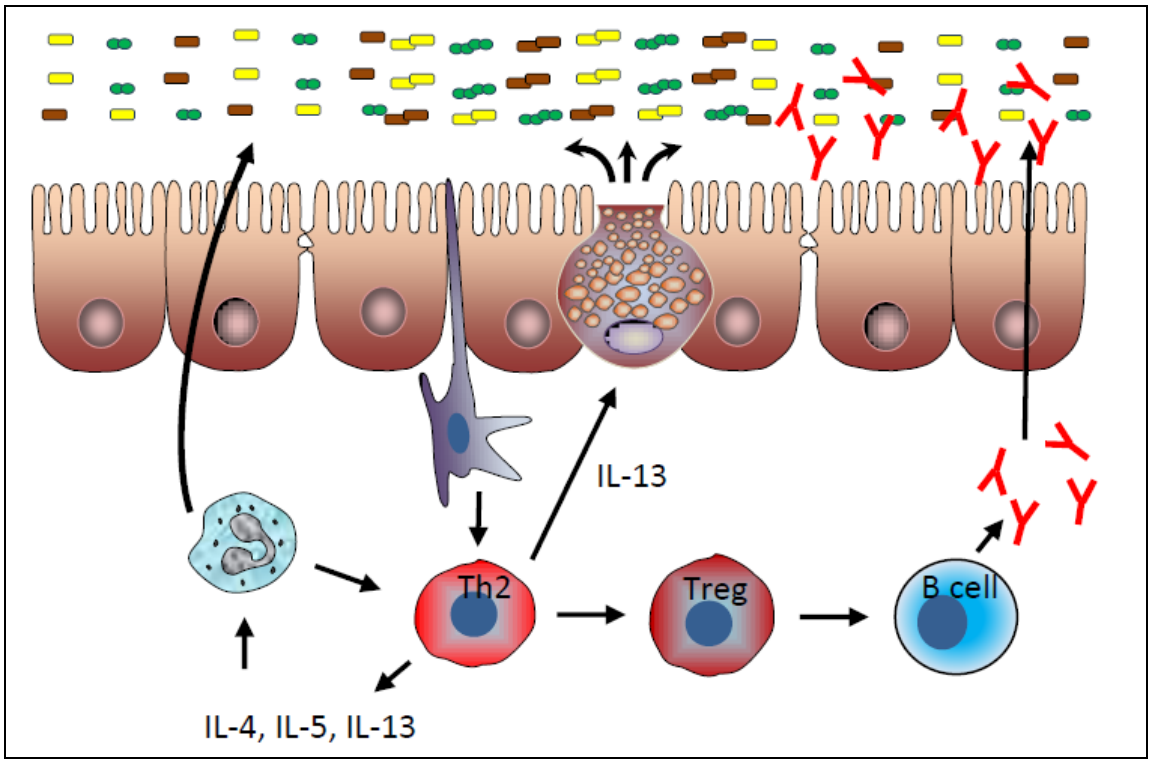

Figure 3. **Microbiota-eosinophil interaction for intestinal immunity**. Microbiota, sampled by dendritic cells influence eosinophil production and functions. Part of eosinophils' functions includes regulation of luminal microbiota profile directly or through production of immunoglobulins. IL, interleukin; Th, T helper; Treg, T regulatory.

Microbiota-driven eosinophil functions during disease

There is enough evidence on the influence of microbiota to eosinophils on controlling allergic reactions. A study by Bisgaard and others (2011) showed that microbiotal diversity of neonates at 1 and 12 months of age is inversely associated with the risk of allergic sensitization, peripheral blood eosinophils, and allergic rhinitis in the first 6 years of life. The increase in eosinophilia due to poor microbial diversity could result in serious inflammatory reactions in various tissues. Specific microbes have been implicated in affecting eosinophil production and functions to alleviate allergic reactions in animal models and humans. For example oral administration of microbiotal bacterial strains *Bifidobacterium breve* M-16V and *Lactobacillus plantarum* NumRes8 to OVA-sensitized BALB/c mice reduces the number of eosinophils in the bronchoalveolar lavage fluid as well as OVA-specific IgE and IgG1 and prevents development of allergic reactions in these animals (Hougee *et al.*, 2010). Similarly, combined administration of *Lactobacillus acidophilus* (*L. acidophilus*) NCFM and *Bifidobacterium lactis* (*B. lactis*) Bl-04 prevents pollen-induced infiltration of eosinophils into the nasal mucosa of children. In these children, pollen season induces a reduction in

Bifidobacterium, *Clostridium* and *Bacteroides*. Although the reduced commensals are not recovered by this treatment, the administered microbiota member species are able to prevent the allergic reactions by suppressing eosinophil production and function (Ouwehand *et al.*, 2009).

CONTRIBUTION OF MICROBIOTA TO NEUTROPHIL FUNCTIONING

Microbiota-neutrophil crosstalk for gut immunity during health and disease

There is enough evidence indicating the beneficial role of microbiota-driven neutrophil function against pathogens. Animals without endogenous intestinal microbiota are highly susceptible to primary intracellular infection by pathogenic *Listeria monocytogenes* with impaired activation and accumulation of phagocytes to the site of infection (Mittrücker *et al.,* 2014). Clarke and others (2010) observed that microbiota peptidoglycan signaling through Nod1 systemically primes the innate immune system to enhance killing of pathogenic *Streptococcus pneumoniae* and *Staphylococcus aureus* by neutrophils (Clarke *et al.*, 2010). Another study indicated that *L. rhamnosus* GG potently dampens production of reactive oxygen species and phagocytic capacity of the neutrophils while protecting against cell cytotoxicity. By so doing, the microbiota confers desensitization towards luminal antigens, favors survival of beneficial microbes that contribute to maintenance of gut immunity (Vong *et al.,* 2014). Another microbiota member *Lactobacillus rhamnosus* GG inhibits both phorbol 12-myristate 13-acetate- and *Staphylococcus aureus*-induced formation of neutrophil extracellular traps. This inhibitory regulation could favor appropriate destruction of the pathogen by neutrophils. Furthermore, the microbiota regulates neutrophils to curb late-onset sepsis (LOS). The regulation is depicted by induction of IL-17 production by intestinal ILCs, increased plasma granulocyte colony-stimulating factor (G-CSF) levels and neutrophil numbers in a toll-like receptor 4- and MyD88-dependent manner and restoration of IL-17-dependent resistance to LOS. Deletion of ILCs prevents IL-17- and G-CSF-dependent granulocytosis and resistance to LOS (Deshmukh *et al.,* 2014).

CONCLUSION

The influence of microbiota to the functioning of leukocytes for intestinal immunity is vital to the maintenance of immunity. Gaining insight into how the microbiota shapes these cells is a great potential to developing medicaments that will treat intestinal disorders caused by disrupted commensals. Although at the moment there is no substantial therapeutic regimen based on microbiotal-mediated shaping of innate immune cells, the microbiota-leukocyte interaction described herein indicates that such an approach is possible. The approach can focus on strengthening the influence of microbiota on innate immune cells. This could subsequently improve intestinal immunity during health and control inflammatory reactions during disease without much tissue damage. Furthermore, maneuvers geared at maintaining a proper and balanced microbiota profile should be promoted to prevent immune breakage and occurrence of intestinal disorders.

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Microbiotal shaping of antigen presenting cell signaling during intestinal immune response

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SUMMARY

Macrophages, dendritic cells (DCs) and to less extent B cells are professional antigen presenting cells (APCs). They sample antigens from the intestinal lumen, process, and present them to cells of the adaptive immune system. While DCs are capable of priming T cell responses, macrophages do polarize the responses. The intestinal lumen contains diverse range of antigens from food proteins, microbiota, and pathogenic microbes that constantly challenge the immune system. While antigens from food proteins and microbiota are not harmful, those from pathogenic microbes are detrimental to the body. The immune system detects both types of antigens and drives immune responses geared at inducing tolerance or reaction to maintain immune homeostasis. Uptake of these antigens is done by the APCs. These cells present antigens to effector cells of the adaptive immune system which generate responses corresponding to particular antigen. The microbiota influences the nature and type of responses generated by APC-activated effector cells by shaping the APC signaling to the adaptive immune system. In a steady state gut environment, the shaping is towards tolerogenic responses while a protective inflammatory reaction results from antigens sampled from harmful microbes. Understanding the interaction between microbiota and APCs in driving immune responses would pave way to improving human and animal health through promotion of maneuvers that maintain immunity.

Keywords: Microbiota, intestine, immunity, dendritic cells, macrophages

INTRODUCTION

The gastrointestinal tract is constantly exposed to an array of non-harmful antigens from food proteins and microbiota and harmful antigens from pathogenic organisms, mainly bacteria. These antigens are accommodated through the tightly regulated gut immune response that maintains immunological tolerance to non-harmful antigens or elicits protective immune responses against pathogenic organisms (Mann *et al.,* 2007; Amu *et al.,* 2010; Lee *et al.,* 2011). Antigen sampling, processing and subsequent presentation to cells of the immune system is done by professional antigen presenting cells (APCs) that include macrophages, dendritic cells (DCs) and to a lesser extent B cells (Batista and Harwood, 2009; Steinman, 2012). These cells are pivotal to initiating, maintaining, shaping and linking innate and adaptive immune systems in the gut (Fujimoto *et al.,* 2011). Specifically, APCs recognize enteric antigens through their Toll-like receptors (TLR) (Fujimoto *et al.,* 2011), drive regulatory responses such as differentiation, expansion, and maintenance of regulatory T cell (Treg) populations and induce immunoglobulin (Ig)A against the microbiota (Manicassamy and Pulendran, 2009).

The microbiota influences APC functions pertaining to intestinal immune homeostasis and responses to foster gut immunity. Imbalances of microbiota and the subsequent defective APC signaling lead to intestinal disorders. The cascade of events involves induction of TLR activity that activates innate immune response exhibited by sampling of gut antigens by APCs, APC-mediated differentiation of naïve T cells into effector T helper (Th) cells, alteration of the gut homeostasis and induction of diseases (Bermudez-Brito *et al.,* 2014; Janelsins *et al.,* 2014). On the contrary, during homeostasis, the microbiota drives a balance between pro- and anti-inflammatory mechanisms for immunity. This is through microbiotal-potentiated generation of pro-inflammatory Th17 cells or anti-inflammatory Treg cells expressing Foxp3 (Rivollier *et al.,* 2012; Arpaia *et al.,* 2013).

Besides influencing APC functions, the microbiota shapes the signaling of these cells to influence effector responses (Rodes *et al.,* 2013; Trapecar *et al.,* 2014). An intact normal microbiota profile shapes the signaling towards homeostasis by inducing tolerogenic responses towards harmless antigens or reactive responses against noxious antigens. A disturbed microbiota may shape the APCs signaling towards inflammatory responses that usually predispose to diseases (Abdelouhab *et al.,* 2012; Baker *et al.,* 2012). Restoring normal microbiota profile to such patients treats the

diseases due partly to re-shaping the distorted APC signaling during disease.

MACROPHAGES

Macrophages and gut tolerance

Gut macrophages are mostly located in the LP of the gut mucosa throughout the entire gastrointestinal tract (Hume *et al.,* 1984). A small population is also present in the smooth muscle layers of the gastrointestinal tract (Mikkelsen and Rumessen, 1992; Tajima *et al.,* 2012). They are involved in maintaining microbiota-host homeostasis, intestinal epithelial renewal and protective immunity. The contribution of macrophages to gut tolerance can be through their constitutive production of anti-inflammatory IL-10, generation and maintenance of Tregs, and suppression of effector Th1, Th2 and Th17. The cytokine IL-10 produced by $CX3CR1^{+}$ macrophages drives the expansion and differentiation of $FoxP3^{+}$ Treg in the intestinal mucosal LP (Hadis *et al.,* 2011). Tolerance can also be mediated through their F4/80 molecules (Lin *et al.,* 2005). Furthermore, Treg can directly target APCs to induce gut tolerance (Shevach 2009). It has been shown that constitutive surface expression of cytotoxic T lymphocyte antigen (CTLA)-4 enables the interaction with co-stimulatory molecules CD80 and CD86 on macrophages. This interaction leads to down-regulated expression of CD80 and CD86 resulting in tolerance (Onishi *et al.,* 2008; Shevach 2009). Individuals without CTLA-4 lack tolerance and develop multi-organ inflammation and premature death (Read *et al.,* 2006; Wing *et al.,* 2008).

Influence of microbiota on macrophage functioning during health and disease

The effect of microbiota to the macrophage functioning is depicted during development and differentiation of these cells as well as functioning for tolerance and active immune response. Trapecar and others (2014) observed that microbiota affects early development of macrophages. From the weaning time, the microbiota drives a process of replacing intestinal macrophages by chemokine receptor CCR2-dependent influx of $Ly6C^{hi}$ monocytes that differentiate locally into mature, anti-inflammatory macrophages. This microbiota driven process continues throughout adult life to maintain a normal intestinal macrophage pool and tolerance (Bain *et al.*, 2014).

At steady state, the microbiota maintains tolerance partly by inhibiting $CX3CR1^{hi}$ macrophages from transporting commensal and pathogenic bacteria from the intestinal lumen to MLNs. However, the trafficking of non-invasive bacteria to the MLNs is resumed during dysbiosis. This transport is in a CCR7-dependent manner and leads to induction of immune reaction mediated by both T cell responses and IgA production (Diehl *et al.,* 2013). The microbiota can also engender immune homeostasis by promoting a crosstalk between innate myeloid and lymphoid cells. This phenomenon depends on the ability of macrophages to sense microbial signals and produce IL-1β that triggers production of granulocyte macrophage colony-stimulating factor (GM-CSF) by retinoic acid-related orphan receptor $(ROR\gamma t)^{+}$ innate lymphoid cells (ILCs). Deficient production of GM-CSF alters mononuclear phagocyte effector functions leading to reduced number of Tregs and impaired tolerance (Mortha *et al.,* 2014). Such scenario predisposes to intestinal diseases like IBD.

The microbiotal shaping of macrophage production of cytokines has been observed in experiments involving specific microbiota species. *Lactobacillus reuteri, L. rhamnosus, L. plantarum, Bifidobacterium animalis, B. bifidum, B. longum,* and *B. longum* subsp. *infantis* have been demonstrated to suppress macrophage production of pro-inflammatory TNF-α and IL-1β but increase production of anti-inflammatory cytokines IL-4 and IL-10. Both macrophage-shaped responses protect against intestinal lipopolysaccharide (LPS)-induced colitis (Rodes *et al.,* 2013). Under disease conditions, the anti-inflammatory IL-10 produced by intestinal macrophages maintains the expression of Foxp3 on Treg to suppress colitis (Murai *et al.,* 2009). Furthermore, the microbiota negatively regulates macrophage production of pro-inflammatory cytokines via production of IL-10 to maintain intestinal immune homeostasis and prevent intestinal diseases (Ueda *et al.,* 2010) (Figure 1).

The shaping of macrophage signaling towards protective direction has been observed to occur during recovery of previously disrupted microbiota. For instance, small bowel resection (SBR) that disrupts colonic microbiota by decreasing the *Firmicutes*, increases the number of macrophages and their pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-18 and TNF-α in colonic mucosa leading to clinical short bowel syndrome (SBS) (Lapthorne *et al.,* 2013). A similar decrease in *Firmicutes* occurring in mice predisposes the animals to *Citrobacter rodentium* infection. This infection causes acute colitis characterized by increased colonic mucosal macrophages, pro-inflammatory macrophage inflammatory protein (MIP)-2α,

inducible nitric oxide synthase (iNOS), IFN-γ, IL-22, and TNF-α expression (Baker *et al.*, 2012).

Maneuvers that specifically enrich colonic *Firmicutes* and decrease γ-*Proteobacteria*, and those manipulating the microbiota in general suppress macrophage induced pro-inflammatory responses and protect against infectious and chemical colitides (Abdelouhab *et al.*, 2012; Baker *et al.*, 2012).

DENDRITIC CELLS

DCs are bone marrow derived APCs. Although they are present in small numbers in the intestine, they have a large influence on immune responses. For example, one DC can influence the function of 300 to 1000 T cells (Stagg *et al.*, 2003). Their main functions are acquisition of antigen and stimulation of lymphocytes.

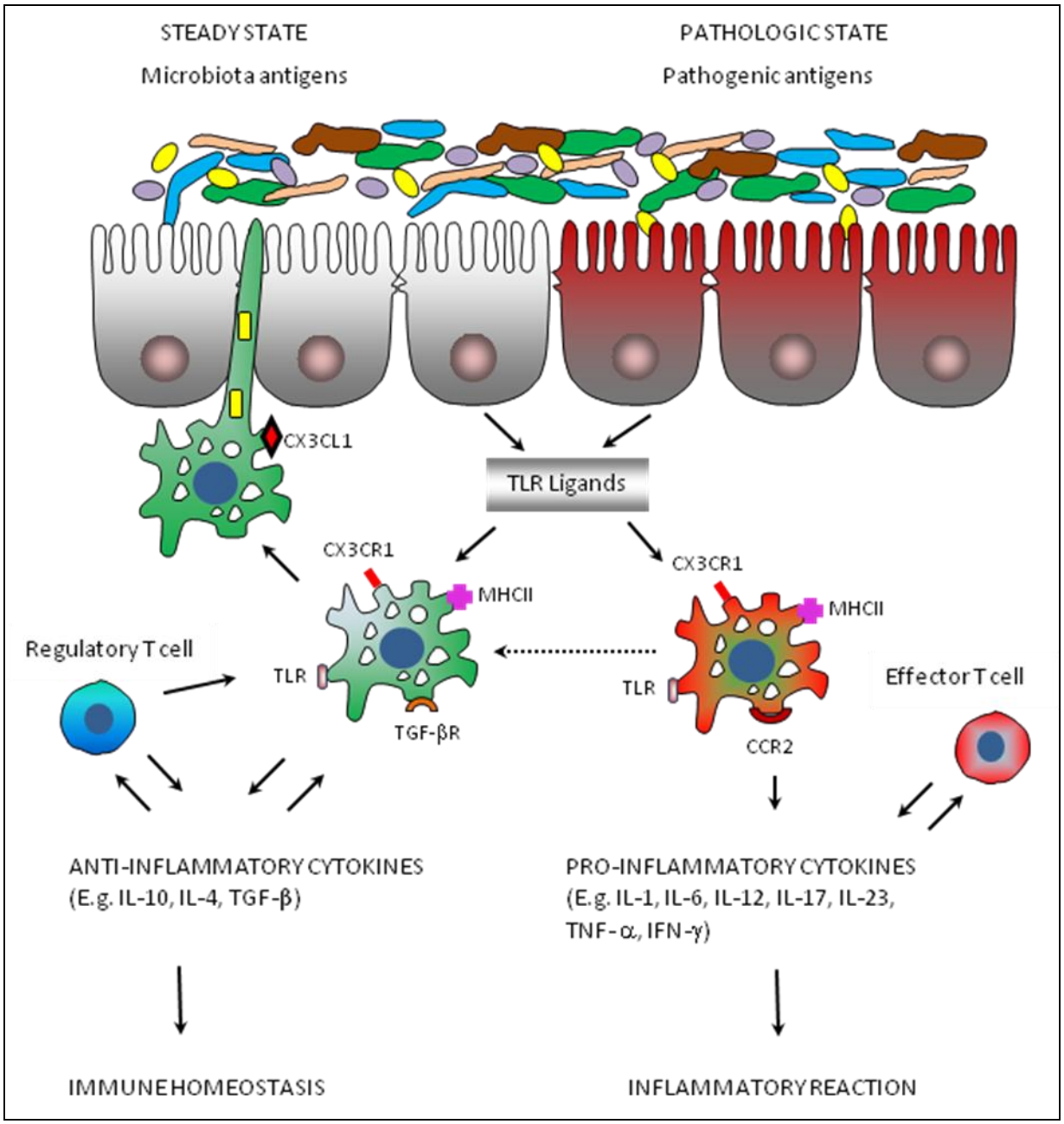

Figure 1. Microbiotal influence on macrophage functioning during health and disease. During intestinal steady state, macrophages sample microbiota antigens, produce anti-inflammatory cytokines, and polarise regulatory T cells to induce immune homeostasis. On the contrary, during infections, macrophages sample pathogenic antigens, produce pro-inflammatory cytokines and polarise effector T cells to cause inflammation. IFN, interferon; IL, interleukin; MHC, major histocompatibility class; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumour necrosis factor.

Influence of microbiota on antigen sampling and presentation by DCs

Intestinal DCs continually sample luminal antigens and present them to the immune cells in the LP. This can be via M cells of the follicle-associated epithelium of Peyer's patches (PP). The M cells take up luminal antigens and translocate them to subepithelial dome regions of PP where they deliver them to DCs. In addition, DCs found at the base of villi and those residing in lymphoid aggregates sample luminal antigens (Mowat, 2003; Iwasaki, 2007). After sampling, DCs present the antigens directly to GALT or migrate to MLNs to induce immune responses for tolerance or reaction (Niess, 2008). Microbiotal uptake by DCs limits bacterial penetration through the intestinal mucosa, neutralizes LPS, and hinders the microbiota from activating the immune system in the LP (Macpherson and Uhr, 2004). Antigen sampling and presentation by DCs can be enhanced by specific microbiota species, *Bifidobacterium* species, in IBD patients (Strisciuglio *et al.,* 2014). Similarly, food derived *Lactobacillus* species (*L. plantarum* WCFS1, *L. salivarius* UCC118, and *L. lactis* MG1363) upregulate the activity and numbers of $CD11c^+$ $MHCII^+$ DCs in the immune-sampling PP of healthy mice (Smelt *et al.,* 2013).

Influence of microbiota on DC-mediated tolerance

The microbiota, through pathogen-associated molecular patterns (PAMPs) is recognized by DC TLRs that are highly expressed by DCs. Subsequently, DCs are activated via intracellular signaling molecule TNF receptor associated factor (TRAF)6 to institute gut immune homeostasis. A loss of intestinal mucosa tolerance characterized by development of Th2 cells, eosinophilic enteritis and fibrosis occurs following DC-specific deletion of TRAF6 Han *et al.,* 2013). The microbiota can also maintain gut homeostasis by activating DCs to express low levels of MHCII that avoid damage to commensal bacterial (Bell *et al.,* 2001). The MHCII production profile changes during diseases associated with disruption of microbiota in which case DCs express high levels of MHCII (Sun *et al.,* 2013).

Influence of microbiota on DC-mediated induction of T cells and implication during disease

The influence of microbiota to DC-driven T responses has been observed in naïve T, effector Th1, Th2, and Th17 as well as Treg cells involved in gut immunity. Specific microbiota species that have been observed to activate DC to prime naïve T cells include *Lactobacillus acidophilus* that drives DCs to polarize production of Th1, *L. reuteri* and *Bifidobacterium bifidum* for generation of Th2, *L. rhamnosus* that induce production of Th17, and *Bifidobacterium breve* that facilitates production of Tr1 (Figure 2) (Jeon *et al.,* 2012; Dongarrà *et al.,* 2013). Polarization of Th1 response by DCs responding from microbiota is depicted from a combination of *L. casei* and TLR3 ligand poly(I:C) that selectively induce DCs to produce substantial amount of IL-6, IL-1β and IL-23 and enhanced levels of IL-12p70. These DCs prime T cells to generate IFN-γ-producing T-bet-positive cells (Th1 cells) without driving a Th17 response. The *L. casei* plus poly (I:C) is considered an *in vitro* model of viral intestinal infection. As such, the ability to polarise Th1 responses could have protective role in intestinal viral diseases. Besides *L. casei*, *Bifidobacterium infantis* 35624, acting through retinaldehyde dehydrogenase (RALDH) also influences DC signaling by inducing production and increase in the number of DCs which in turn, increases production of Treg $Foxp3^+$ cells but reduce that of Th1 and Th17 in the intestinal LP. The *B. infantis* 35624-induced decrease in Th1 and Th17 reduces the severity of dextran sulphate sodium (DSS)-induced colitis (Konieczna *et al.,* 2013).

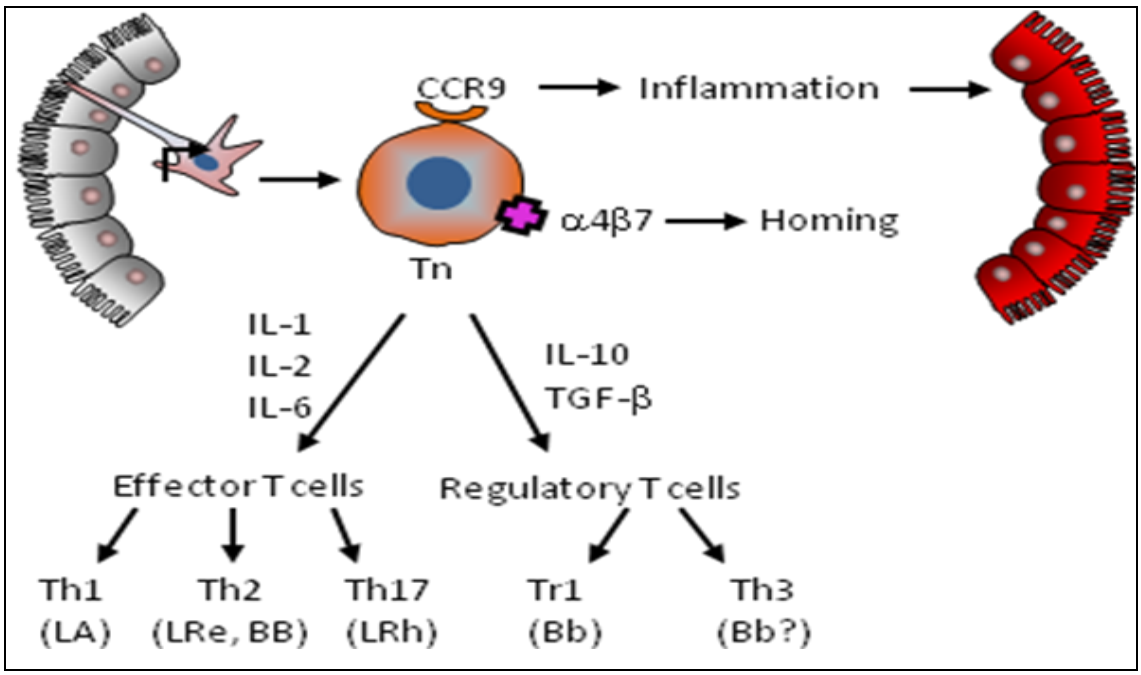

Figure 2. Dendritic cell-mediated microbiotal influence on T cells. Presentation of microbiotal antigens by dendritic cells to naïve T (Tn) cells induces expression of CCR9 and α4β7 integrin receptors on Tn cells for inflammatory and homing responses respectively. The antigen presentation also drives differentiation of effector (e.g. Th1. Th2, Th17) and regulatory (e.g. Tr1, Th3) T cells. Different microbiotal antigens may have different T cell type differentiation. Bb, *Bifidobacterium breve*; BB, *Bifidobacterium bifidum*; IL, interleukin; LA, *Lactobacillus acidophilus*; LRe, *Lactobacillus reuteri*; LRh, *Lactobacillus rhamnosus*; TGF, tumour necrosis factor.

According to Goto and others (2014), commensal segmented filamentous bacteria (SBF) sampled by DCs, primes and induces generation of Th17 cells in a MHCII dependent antigen presentation manner. The generated Th17 contributes to intestinal protection and is essential for immunity. Deficiency of MHCII promotes production of Th17 through different pathways independent from SBF while dysregulation of Th17 cells results in autoimmunity. The ability of DCs to polarize Th17 cells is affected by microbial products like bacterial LPS. DCs express acyloxyacyl hydrolase (AOAH) enzyme that inactivates microbiota LPS to polarize and generate Th17 effector cells (Janelsins *et al.*, 2014). Another way by which DCs can shape effector responses is by expressing myeloid C-type lectin receptors (CLRs) that enable them bind to glycan structures present on self or foreign antigens. Clinically, the significance of CLRs stems from their involvement in IBD pathogenesis. Both the macrophage-restricted C-type lectin (MCL) and the DC immunoreceptor (DCIR) bind to intestinal microbiota to modulate production of pro-inflammatory cytokines by these cells and impact subsequent T cell responses. MCL-/- as well as DCIR-/- mice exhibit an increased severity of colitis compared to wild-type counterparts indicating a role for MCL and DCIR in the regulation of intestinal immunity (Hütter *et al.*, 2014)

Effect on Treg

Mucosal DCs express high levels of TLR5 protein in a steady state gut. This expression is downregulated following treatment with various bacterial ligands. The DC signaling through TLR5 restrains Treg cell generation whereas mice lacking TLR signaling ($TLR5^{-/-}$) have increased $Foxp3^{+}$ Treg cells in the intestinal LP (Feng *et al.*, 2012). The microbiota also influences DC-mediated Treg cell functioning via its dietary fiber fermentation products, butyrate and niacin. These chemicals promote the anti-inflammatory activity of DCs by stimulating them to induce differentiation of Treg cells and IL-10-producing T cells through their receptor GPR109A (Singh *et al.*, 2014). Furthermore, the microbiota maintains balance between pro- and anti-inflammatory mechanisms through the fermentation products by inhibiting histone deacetylase (HDAC) (Arpaia *et al.*, 2013) to facilitate extrathymic differentiation of Treg cells in an intronic enhancer CNS1 (conserved non-coding sequence 1, essential for extrathymic Treg-cell differentiation)-dependent manner. A crosstalk between intestinal DCs and *Bifidobacterium breve*

induces development of Tr1. The crosstalk starts with *B. breve* activating intestinal $CD103^+$ DCs to produce IL-10 and IL-27. The produced cytokines drive generation of Tr1 which subsequently produce IL-10 as a potent anti-inflammatory mediator, and express cMaf, IL-21, and Ahr (aryl hydrocarbon receptor) in the large intestine. $CD103^+$ DCs from IL-$10^{-/-}$, Tlr$2^{-/-}$, and Myd$88^{-/-}$ mice exhibit defective *B. breve*-induced Tr1 cell development and may predispose to intestinal diseases. IL-10 (Jeon *et al.*, 2012).

CONCLUSION

The intestinal microbiota influences macrophage and DC development, differentiation and functioning for tolerance and active immune response geared at maintaining immune homeostasis. In the absence of microbiota or during dysbiosis, macrophages and DCs drive inflammatory responses leading to diseases that can be deleterious to the host. The microbiota-mediated macrophage and DC protective responses are manifested by the ability of the commensals to shape the effector products of these cells. As a result, the microbiota prevents intestinal diseases that would otherwise develop from activated macrophages and DCs. Insight in this understanding could pave way to establishing medicaments against intestinal diseases by employing the microbiota.

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The use of fetal femur length for estimation of gestational age in cattle

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SUMMARY

The aim of the present research was to study the relationship between the femur and gestational age and thus generate an equation which would be able to estimate fetal age in cows. 23 dairy cows were examined every after one week transrectally using B−mode real−time ultrasound scanner with a 7.5MHz linear probe (Mindray DP 6600, Mindray Szechuan, China). The dairy cows were examined at Large Animal Research Unit of Massey University New Zealand between day 60 and day 120 of pregnancy. Ultrasonography examination commenced the 20th of July 2015 and was finished in September 2015. The predicted gestation age in dairy cows had a chance of being 21.7 days greater and 21.6 days less than actual gestational age. The findings from this study show there is a moderate positive linear correlation between femur length and gestational age in the dairy cows (R^2 =0.64). However, this relation did not have good agreement to justify the use of equation developed to be used in estimation of gestational age.

Key words: dairy cows, gestational age, agreement, femur length

INTRODUCTION

Gestation is the period between conception and birth, and gestational age is therefore the time since conception. In cattle, gestational age is most commonly estimated using rectal palpation or transrectal ultrasonography. In the present study, the focus is onthe use of transrectal ultrasonography. This is a quick, safe and non-invasive technique that can be used to diagnose pregnancyas early as 28 days after conception (Racewicz and Jaskowski, 2013). Transrectal ultrasound can also be used to determine gestational age, based on the relationship between time since conception and size of the conceptus (Varol *et al*., 2001) and fetal viability based on the presence of a beating heart (Lamb*et al*.,2015).Importantly transrectal ultrasound has not been found to affect embryonic or fetal viability(Kahn, 1992; Ball and Logue, 1994).

The key rationale for estimating (or confirming) gestational age is that doingsowill allow prediction of expected calvingdate; in dairy cattle this prediction can be used toidentify drying-off date and to plan for calving (e.g. identifying labour requirements) (Doize*et al*., 1997). Inextensivesystems, drafting cows based on fetal age at particular time points during the year may be easierthan trying to locate and remove all bulls from a paddock (Jephcott, 2009).

A huge range of parameters are potentially measureable using transrectal ultrasound including biparietal diameter, femur length, crown-rump length, thoracic diameter and abdominal diameter and non-fetally-based measures such as placentome diameter or corpus luteum size.

Femur length measurements

The femur is is the most proximal bone in the hind limb of the cow, articulating with the acetabulum in the pelvic bone to form the hip joint whereas distally there is a knee joint. As for BPD, studies in multiple species have shown a very strong correlation between gestational age and femur length including goats (Rihab *et al*., 2012), sheep (Noia *et al*., 2002), buffalo (Terzano *et al*., 2012) and hyena (Place *et al*., 2002) as well as cattle (Kahn, 1989). In cattle the association is seen in all breeds though there can be significant differences between them (Table 1)

A wide range of parameters that can also be measured by ultrasonography have been shown to be highly correlated with gestational age, including: crown rump length (r=0.91), head (biparietal) diameter (r=0.95), head length (r=0.94), trunk diameter (r=0.95), nose diameter (r=0.95), uterine diameter (r=0.93) (all results from White *et al*., 1985) and femur length (r=0.99; Kahn 1989). Femur measurements are illustrated in Figure 1. Femur measurement is considered to be accurate only when the image shows two blunted ends, i.e. the extension to the greater trochanter and the head of the femur are not included.

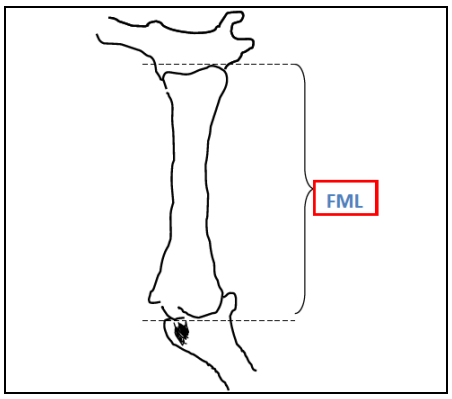

Figure 1. Representation of reference points for femur length (FML) used when measuring using ultrasound

Table 1. Fetal legs traits during various stages of gestation in cattle

	Gestational age		
Breed	3 months	6 months	9 months
German Angus	4.3	19.7	32.7
Galloway	3.2	17.1	33.3
Holstein Friesian	5.6	21.3	38.5
Belgian Blue	4.6	19.2	36.4

Table 1: Effect of breed on change in leg length (cm) with gestational age (Source: Mao *et al*., 2008).

Postmortem data has shown that significant cartilaginous development of the long bones has begun by ~7 weeks of gestation with ossification beginning a few weeks later being detectable from around 74 days of gestation (the first identification of calcium phosphate deposits), with ossification centres being seen in all bones by day 81 (Trujillo *etal*., 2011). So although femur measurement is possible from 49 days, measurement using bobly landmarks is only feasible only from ~80 days onwards Femur measurement is much less practicable in older fetuses. Kahn (1989) reported that by month 4 of gestation only 60% of fetuses could have measurements made of their hind limb area. By month 7 this figure had decreased to 25%, while in months 9 and 10 no measurement of the hind limbs was possible. This means that femur measurement as an estimate of gestational age needs to be restricted to fetuses <160 days.

MATERIALS AND METHODS

Study animals

The animals used in this study belonged to the large animal teaching unit (LATU) of Massey University. Sixtynon-lactating 2.5-year-old dairy cows (Friesian and Friesian cross Jersey) were used.

In order to ensure that the exact date of conception was known, allthe cows used in this study had been synchronised using an intravaginal progesterone plus GnRH-PGF$_{2\alpha}$-GnRH program (Adeyinka *et al*., 2014), with pregnancy diagnosis undertaken 6 weeks after synchronisation. The cows were all inseminated on 14th May 2015.

Ultrasound equipment

The uteri of the selected cows were examined transrectally, using a B-Mode real-time ultrasound scanner with a 7.5 MHz linear probe (Mindray DP6600, Mindray Szechuan, China). Fetal femur length measurements were made in the dairy cattle from 20th July to 1st October 2015. This meant they were scanned between days 60 to 130. Measurements were made in weekly basis.

Measurement of femur length

Femur length was measured and defined as the length of the diaphysis of the femur (see Figure 1) diaphysis at both ends (line x to x) as shown in figure 1. Once a suitable image was obtained it was recorded digitally before transfer to a desktop computer for image analysis using the image processing and analysis programme Image J (Figure 2).

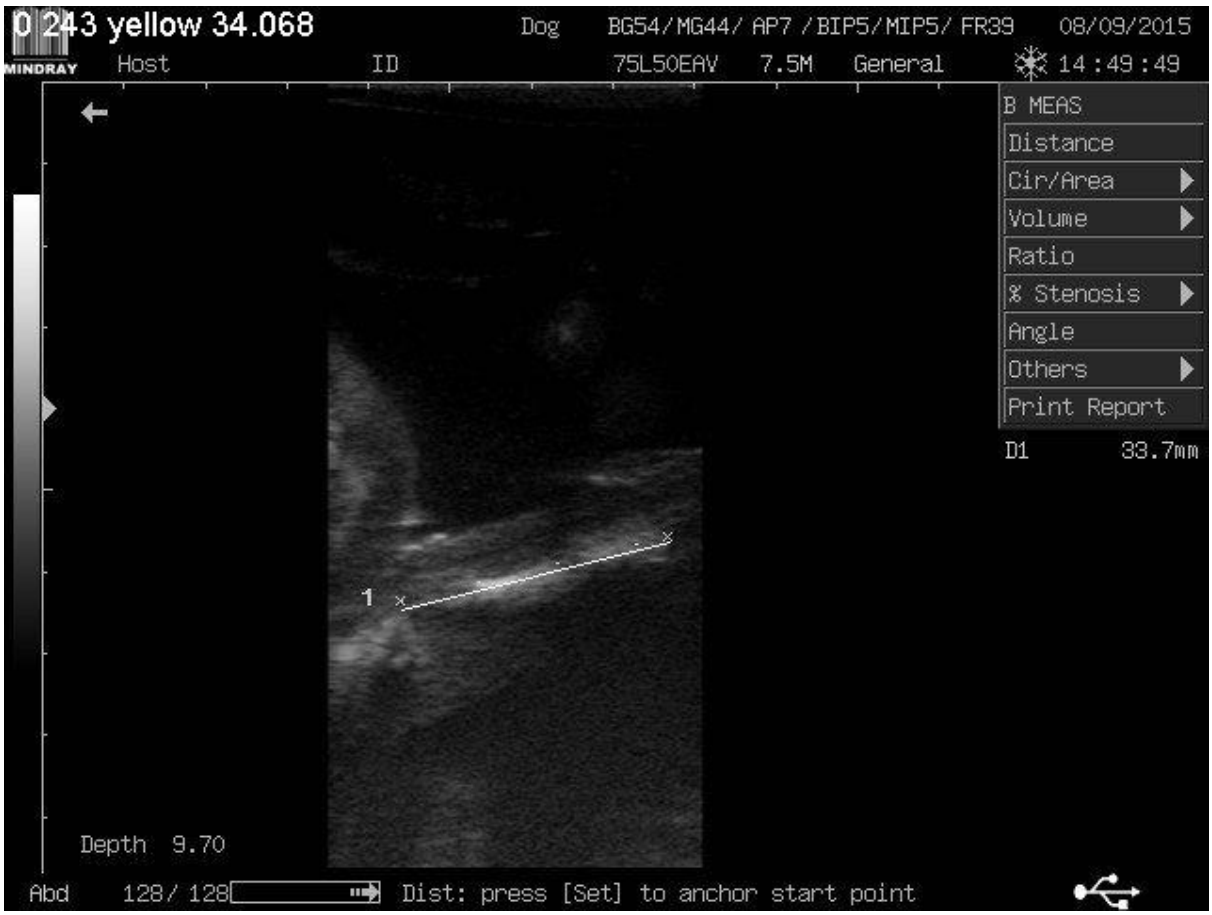

Figure 2. Example of a digital image showing measurement of femur length

Statistical analysis

All analysis were undertaken using SPSS 24 (IBM, USA)

Regression analysis

A regression analysis of gestational age against femur length was undertaken to establish the strength of the association between the measure and the best equation for predicting gestational age from the measurements.

Limits-of-agreement analysis

Predicted age (based on the regression equation) was calculated from the femur length, and a mean/difference plot (Bland *et al*., 1990) created. Regression analysis was then undertaken to identify whether there was a significant association between mean and difference (and the variance of that difference), and the limits-of-agreement then calculated (Bland and Altman 1999). Femur length for each cow at each given time point was used to create the predicted gestational age. The agreement between the predicted and the actual gestational age in terms of femur length for dairy cows was identified by the limits of agreement. The two versions of limits of agreement were used. One by (Bland and Altman, 1999) where the limits of agreement were calculated using the standard deviation of the difference between the predicted and the actual gestational age. The other one as suggested by (Bland and Altman, 2007) for repeated measurement where the true value varies, taking into consideration the association between method difference and gestational age.

RESULTS

Regression analysis

The association of fetal femur length with gestational age is illustrated in Figure 3

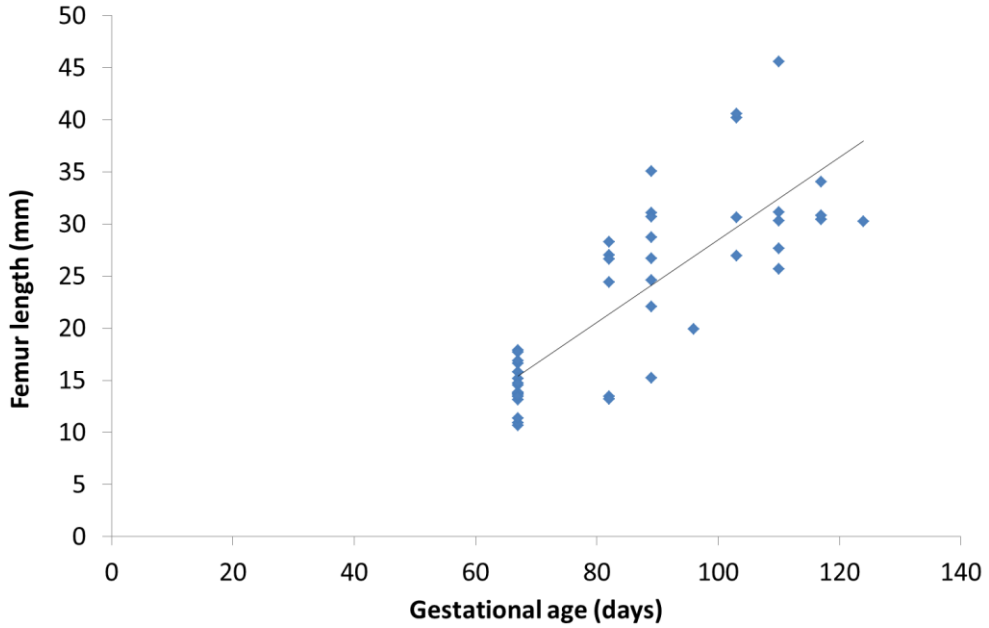

Figure 3. Relationship between femur length (mm) and gestational age for dairy cows measured using transrectal ultrasonography. Solid line: line of best fit.

The results of the regression analyses are summarised in Table 2

Table 2. Association between gestationalage andfemur length measured using transrectal ultrasound

Measure	Type	Prediction equation from gestational age (days)	R^2
Femur (mm)	Dairy	0.40*age - 11.1	0.64

Limits-of-agreement analysis

The limits-of-agreement plot based on the femur size is shown in Figure 4. Overall there was no evidence of bias (mean difference [SEM] was -0.025 [2.05]). There was a moderate association between mean and difference (R^2=0.124; p=0.018) and a strong association between mean and variance of the difference (R^2=0.447; p<0.001). The limits-of-agreement analysis thus took account of these associations and suggests that at 80 days, 95% of the differences between predicted and actual gestational age will be between -19 and +16 days whereas at 120 days the equivalent figures will be -29 and +46 days. So at 80 days ~74% of differences will be ≤10 days from the line of best fit, whereas at 120 days ~21% of differences will be ≤10 days.

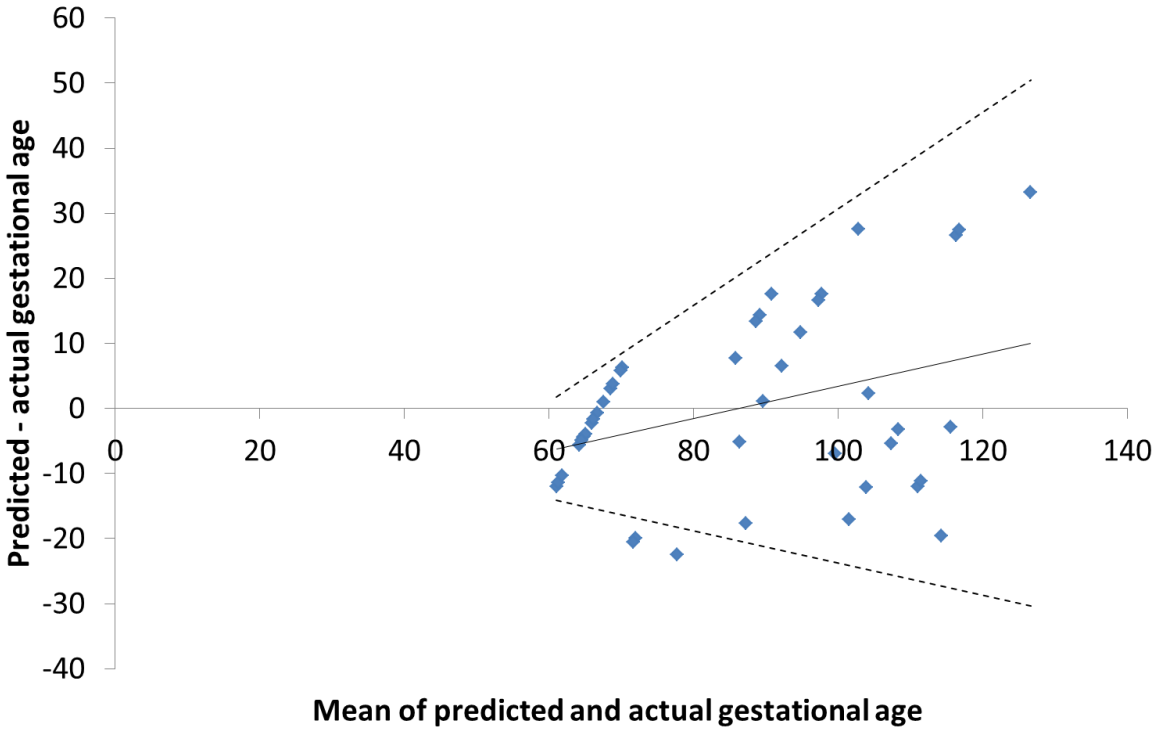

Figure 4. Showing scatter plot of the difference between predicted and actual gestational age and the mean of predicted actual gestational age (days). The dark blue line is the best-fit line; the doted line is for unadjusted limits of agreement.

DISCUSSION

In cattle, measurement of femur length is one of the commonly used methods of estimating gestational age; however very few studies have properly assessed the agreement between femur length and gestational age. Many studies have focussed on correlation and relationship between means which are not appropriate for assessing agreement as they ignore the variation between individual results (variance of the difference) and the change in that variance as measurements increase.

In this study there was a significant association between femur lengthand gestational age. The good association in this study (R^2=0.64) was poorer than some previous results (e.g. Kahn 1989, White *et al*., 1985), but other studies reported similar good values (Terzano2012). It is not clear why correlations between studies. In this study, in an attempt to mimic what was feasible under New Zealand conditions, measurement was undertaken as soon as the measure could be identified, so it is likely that measurement time was short relative to previous studies. In addition, the beef cattle used in this study were unused to handling, so were difficult to scan safely, again increasing the likelihood of errors. Another possibility which could have decreased correlations in this study is the relatively high gestational age of many of the fetuses when they were first scanned which would again reduce association.

This is the first study that has specifically focussed on the agreement between estimates of gestational age from femur and actual gestational age. Previous papers (e.g. White *et al*., 1985 and Kahn 1989), have principally focussed on correlation and when they have included measures of agreement have not taken account of change in agreement with time; e.g. White *et al*. (1985) reported residual standard deviations ranging from 4.5 days for crown-rump length to 12.6 days for uterine diameter, but did not take into account the increase in standard deviation with time shown on their graphs. The impact of time on agreement is particularly important under New Zealand conditions as, although in intensive systems most cows are scanned before 42 days (Fitzgerald *et al*., 2015), in New Zealand the majority of pregnant cows are >80 days when scanned with many cows scanned at a later stage (Brownlie *et al*., 2015).

The limits-of-agreement analysis showed that for femur length (in dairy cows) there was a large increase in the predicted differences with increasing gestational age; i.e. estimation of gestational age became less precise as pregnancy progressed. This is consistent with the results shown by previous

studies of fetal size such as Khan (1989) and White *et al*., (1985) where graphical representation of the data from the fetus showed that variance increased with gestational age, and Adeyinka*et al*., (2014) who found no effect of gestational age on the precision of its estimation using placentome length. This lack of association was observed irrespective of whether the regression equation from this dataset or that from Adeyinka*et al*., (2014) was used. Femur size became increasingly difficult to access as gestational age increased.

It is concluded that measurement of gestational age is a crucial part of the pregnancy diagnosis process. This study is the first to compare the agreement between predicted and actual gestational age for fetal femur length. However, this study has confirmed that the precision of these measurements decreases significantly as gestation progresses and by 120 days of gestation. Measuring femur length was by far the most difficult measure and by 120 days was much less precise than other fetal measurements studied before like placentome size and biparietal diameter. Femur measurement should be restricted to use only in early gestation and then it should be used alongside with other fetal measurements.

ACKNOWLEDGEMENTS

The study was financially supported by the New Zealand Ministry of Foreign Affairs and Trade

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OPENING SPEECH OF THE 35TH TVA SCIENTIFIC CONFERENCE BY THE DEPUTY MINISTER OF LIVESTOCK AND FISHERIES, HONOURABLE, ABDALLAH H. ULEGA (MP), ON 5TH DECEMBER 2017 AT AICC - ARUSHA

- Chairman of TVA,
- Regional Commissioner for Arusha
- Permanent Secretary for Livestock
- Permanent Secretary for Fisheries
- Members of TVA Executive Committee
- Directors and other officials from the Ministry of Livestock and Fisheries
- Foreign Delegates
- Members of TVA
- Other conference participants
- Ladies and gentlemen

Good morning.

Dear Conference Participants, allow me, in the first place, tojoin the Chairman of TVA in welcoming you to the 35th TVA Scientific Conference; your Annual General Meeting planned for tomorrow and to our magnificent city of Arusha. It is my hope that you will make of use of your time in Arusha appropriately in order to achieve the overall objectives of your conferenceand I also expect that you will find time to explore the beauty of the city and the surrounding areas.

I would also like to take this opportunity to sincerely express my gratitude for the honourand privilegeyou have bestowed on me by allowing me, on behalf of the my ministry, to be associated with your 2017 TVA Scientific Conference, but more notably to link up with you, TVA members and other stakeholders in discussing issues of pertinence to the livestock sector.

Dear Conference Participants, I am informed that the main thrust of your conference is to exchange scientific results or other forms of evidences amongst researchers and field staff. BUT, I have also been made aware that your annual conference also often endeavours to nest presentations of policy papers that stimulate useful discussions that are the centre stage of helping you to generate recommendations for uptake by the Ministry, other stakeholder Institutions or other Parties. Further to this, you also normally have Annual General Meetings, where members discuss issues of interest to the association as well as matters that influence or impact on the sector. These options definitely provide you with an expanded opportunity for discussing issues of importance to the sector. So, it is my expectation that at the end your conference you will be able to do us justice by coming up with sound and tangible but doable suggestions that address key challenges of the sector. We expect this, from you given the fact that your professional strand is core to the standing and performance of the animal health service delivery system, which is key to livestock development.

Ladies and Gentlemen, in our pursuit to addressing the key challenges of the sector, we need to take cognisance of the fact that in Tanzania, animal agriculture is one of the dependable lifelines of rural economies and prosperity as approximately 95% of resource poor rural communities depend on crop and/or animal agriculture for their daily livelihood.

Available data also show that 97% of rural households are involved in crop farming, whereas60% of people are engaged in livestock keeping. Animal agriculture indeed contributes to the employment of the able population segment, for which crop and livestock subsectors jointly provide various forms of employment opportunities to around 65.5% of the national population. For this matter, the livestock sub-sector is and will continue being at the centre stage of rural economic development and prosperity.

Therefore, it is of utmost importance to ensure that all the potentials of animal agriculture are properly harnessed in order to accelerate and deepen livestock sector performance and its contribution to the livelihood of value chain actors and GDP.

Ladies and Gentlemen, as you navigate through your conference schedule and deliberations, I kindly request you to take note that the Government of The United Republic of Tanzania is committed to transform the livestock sub-sector as implied in the National Livestock Development Policy of 2006 and the National Development Strategy and Programme. You may also wish to note that the same aspiration is enshrined in the National Fisheries Development Policy of 2016 and National Agriculture Development Policy 2013. The three policies envision anchoring on transformative change of the sub-sectors as implied in the [2015/16-2020/21] Five Year Development Plan [FYDP].

With the current FYDP, the Fifth Phase Government aspires to buildan industrial based economy, attaina middle income earning country status and reduces unemployment, notably amongst youths mainly through unlocking agribusiness opportunities in various agricultural value chains. You should therefore endeavour to anchor your reflections on these facts and to support our national aspiration for economic growth and prosperity through agricultural sector transformative change as an innovative game changer.

Dear Conference Participants, looking at the theme and subthemes of this year's conference, it appears that you will be touching base on a number of key subjects which are of great concern to the sector and which have some influence on the roadmap towards our national aspiration for putting up an industry-based economy.

Therefore, I am inclined to believe that these subthemes surely compel you to have serious debates that will provide inputs for catalysing and deepening the contribution of animal agriculture to industrial development and allowing for effective engagement of youths in agribusiness.

Dear Conference Participants, I am also confident that you are also aware of the fact that the national population has expanded so much in the recent past and because of this expanded population, ecosystem resources have started showing signs of dwindling. Indeed, they have started being limiting. One notable limiting resource is land that has been one of the drivers for increased encroachment of wildlife ecosystems; conflicts between crop farmers and livestock farmers as well as with other land resource users such as those involved in mining. Unfortunately, in a number of situations, such conflicts have often been bitter; with some leading to loss of human life and property as well as deliberate and inhumane killing of animals.

Ladies and Gentlemen, you will also agree with me that these conflicts have been on-going for some time and in fact they have persisted for too long. For this matter and given theimmense social, economic and political dimensions and for the interest of community cohesions needed in fostering economic development, stakeholders now need to come together to address the root causes in a participatory manner, comprehensively and for the benefit of the sector.

Although, these debates need to be organised and led by my ministry, this does not, in any way, limit other stakeholders such as TVA from discussing these issues. I therefore, request you, Mr Chairman and TVA members to allocate time during this conference to initiate dialoguesthat can lead to identification of sound inputs to our ministry.

Dear Conference Participants, as we ponder on the issues related to conflicts emanating from uncontrolled animal movements across the regions and hence the country, we also need to take cognisance of the impacts of some of the known root causes of animal mobility, which relate to limitations of water and pasture resources.

We all know that water and pasture resources have increasingly become scarce because of the negative effects of climate change and variability; poor resource use patterns; continued tendency of keeping large herds sizes of animals; poor land allocation strategies as well as our failure to promote and build capacities for dry season feeding system using locally available feed resources.

Ladies and Gentlemen, when you reflect carefully on water and pasture seasonal scarcity trends, you will also agree with me that we have also possibly been part of the problem because we have made inadequate investment in rallying livestock farmers behind the desire to participate in the establishment of water sources for their animals.

Experts, especially those working in local governments have also invested minimal efforts designed to facilitate formulation of community-based strategies for building technical capacities in, for instance, dry season feeding system using feed resource such as use of crop residues. Livestock farmers have also not been assisted to harvestpastures during and soon after the rains using low cost technologies. Generally, farmers training schemes are inadequate. This implies that we have an obligation of sorting out some of the challenges that our farmers face.

Dear Conference Participants, I would like to acknowledge the belated kick-start of vaccination programmes for important diseases as initiated, advocated and argued by TVA following the consultations with field vets that were done in 2013/14. It is thus disappointing to learn thatstakeholders including you, have acted rather slowly on this matter as most animal diseases as of recent most diseases have become rampant and endemic.

I would like to inform you that my ministry has of recent had serious reflections on this matter and to this effect, we have already signed circular that spells out the need to initiate vaccination programmes for selected diseases. This circular was indeed signed by the former Minister for Agriculture, Livestock and Fisheries in August 2017. Based on this, the ministry is now ready to initiate vaccination programmes.

Our plan is expected to anchor on public-private partnered arrangement, with vaccinations being done on the basis of cost recovery by private veterinary practitioners. In pursuit of this, may I take this opportunity to request the Permanent Secretary to ensure that the roadmap for vaccination regimens /schedule for selected diseases is firmed up and put in place by end of February 2018.

Ladies and Gentlemen, the other concerns of the Chairman of TVA pertaining longstanding complaints of poultry farmers about poor feed quality and disease burdens are very pertinent. Similarly, the disruption of the veterinary structure which is the lifeline of quality animal health service delivery needs to be relooked into for the purpose of reviving it within the framework of the Decentralisation by Devolution.

Dear Conference Participants, as allude before, since the livestock sector faces a multitude of problems, I support the idea of TVA Chairman of allowing TVA to assemble all inputs needed in engaging the ministryaccordingly. However, for the purpose of coming up with evidence-based suggestions and based on expanded consultations, I therefore urge TVA to team up with Tanzania Society for Animal Production [TSAP] and Tanzania Veterinary Paraprofessional Association [TAVEPA] to come up with comprehensive proposals. In order for this to be done promptly and properly, I request the Permanent Secretary to take the lead by convening a team of experts from TVA, TSAP, TAVEPA and my Ministry to prepare a working paper that will tabled in an expanded meeting involving ministry officials. I suggest that this be done in January 2018.

Dear Conference Participants, as we work towards this realisation, allow me also to express my disappointments for veterinarians who are working with PO-RALG. Much too often we have witnessed the shear lack of livestock development initiatives that focus on adoption of innovative animal husbandry practices. It is also apparent that animal health service delivery system has remained unregulated, unchecked and chaotic in some areas.

In other areas, the outcry has been the lack of animal health services despite extension agents being deployed up to ward/village level.To make matters worse, we have not witnessed veterinarians utilising professional mandates, notably legal powers to close down abattoirs and livestock auction markets when their operations are not up the set standard and contravene legal requirements. We have only been witnessing the Tanzania Food and Drugs Authority (TFDA) providing leadership for the closure of such entities. On the other hand, quarantine measures intended to curtail disease spread is also rarely executed and if put in place, operational activities are in no way matching with the standard operating procedures.

Therefore, **Dear Conference Participants**, you will agree with me that silence and passiveness of veterinarians in these critical issues are liabilities to your profession. I therefore urge all of you to re-examine your professional mandates and obligations. More importantly, it is prudent for you to stand to the principles of your profession despite the pressure you face from grass root politicians. The important issue is not run away from political pressures by being passive BUT to work around the political complexity to sort out issues

as per your professional mandates. So, please I urge you to play your party and avoid you being one of the root causes for the failure of the sector.

Dear Conference Participants, I know that this is scientific conference which also provides a room to meet friends and colleagues who you may have missed since you last met them in December 2016. It is thus my hope that you will all make use of stay in Arusha effectively including enjoying your stay including exploring the beauty of Arusha City and the magnificent and multitude of tourist attractions within Northern Circuit of Tanzania.

Ladies and Gentlemen, on behalf of my Ministry and livestock sector stakeholder family, may I take this opportunity to thank you once again for your support, in particular for allocating your precious time to come to this conference and in accepting to have dialogues on important challenges of the sector. For this matter, I request you to participate effectively and with the highest degree of commitment so that your conference and dialogues become instrumental in the operational change of the livestock sector.

Dear Conference Participants, having said this, my I now declare that the 35th TVA scientific Conference is officially graced.

THANKS FOR LISTENING

PROGRAMME FOR THE 34TH TVA SCIENTIFIC CONFERENCE

Day 1: Tuesday 6th December, 2017		
Conference Opening Session in Mbayuwayu hall Chaired by Dr A. Hayghaimo		
Time	Event	Responsible persons
08:00-08:45	Registration	ALL
08:45-09:00	Participants and Invited Guests seated	ALL
09:00-09:10	Welcoming Remarks	TVA Chairman
09:10-09:15	Invitation of the Guest of Honour	TVA Chairman
09:15-09:45	Official Opening of the 35th TVA Conference	Guest of Honour
09:45-10:00	Vote of Thanks	TBI
10:00-10:15	Group Photo	ALL
10:15-10:45	Tour of Exhibition Pavilions	Guest of Honour/TVA Chairman
10:45-11:00	HEALTH BREAK	All

SESSION 1: MBAYUWAYU	**CHAIR: Prof . M.J. Kipanyula**	
11:00-11:30	**Key Note Paper 1:** Control of Livestock Diseases in Tanzania: Challenges and the Way Forward	R.R. Kazwala & L.S.B Mellau
11:30-12:00	**Key Note Paper 2:** Control of Livestock Diseases at a Global Perspective	F. Bonto
12:00-12:30	**Key Note Paper 3:** Livestock Production Performance and constraints under different farming systems in Tanzania	S.W. Chenyambuga
12.30 - 13.00	**Key Note Paper 4:** The Impact of Antimicrobial Resistance (AMR) to Health and Value Addition	R.H. Mdegela
13:00-14:00	LUNCH BREAK	All

SESSION 2: MBAYUWAYU	**CHAIR: Dr. E.S. Swai**	
14.00-14:30	**Key Note Paper 4:** Challenges Associated with Value Addition in the Livestock Sector	G. Mbassa
14:30-15:00	**Key Note Paper 5:** Welfare and Contribution of Draft Animals to the Transformation of the Agricultural Sector	T.K. Kahema
15:00-15:30	**Key Note Paper 6:** Euthanasia in Animals with Special focus to Developing Countries	A.P. Muhairwa
15:30-15:40	Evaluation of stress hormone and biochemical parameters of pigs kept under intensive management systems in Morogoro, Tanzania	G. G.Bakari *et al*.
15:40-15:50	An assessment of donkeys' welfare using physical and emotional parameters: a case of Mkwinda EPA, Bunda area, Lilongwe, Malawi	T.J. Namangale *et al*.
15:50 -16:00	Assessment of health status, handling and management of working animals in Tanzania: A case study of Donkeys in Kilosa district, Tanzania	W.H. Kimaro & M.J. Kipanyula
16:00-16:10	Studies on Gastrointestinal Helminth of Donkeys at Huacheng International Abattoir, Dodoma, Tanzania	M.R. Galibona *et al*.
16:10-16:30	Mapping on Bachelor of Veterinary Medicine Curricula: Kansas State University in USA and Sokoine University of Agriculture in Tanzania	T. Sebhatu
16:30-16:40	HEALTH BREAK	All

SESSION 3: MBAYUWAYU	**CHAIR: Dr C. Uronu**	
16:40- 16:50	Animal disease surveillance: gaps identified and introduction of mobile technology to address them in selected districts in Tanzania	C. Sindato *et al*.
16:50-17:00	Trend in Diagnosis of Marek´s Disease (MD) in Poultry at Central Veterinary Laboratory in Dar-es-Salaam, Tanzania	A.M. Sailen *et al*.
17:00-17:10	Assessment of Livestock Keepers' Perception on Post-	G.H. Mbata *et al*.

	Intervention on Tsetse Control in Southern Tanzania	
17:10-17:20	Microbiota Prime Leukocyte Response For Intestinal Innate Immunity	J.J. Malago
17:20-17:30	Organ Condemnations and Financial Losses in Cattle Slaughtered at Mahenge Slaughter Facility in Ulanga District, Morogoro, Tanzania	E.G. Lyimo *et al*.
17:30-17:50	General discussion	Chair
17:50-	END OF DAY1	All

DAY 2: Wednesday 7th December 2017

08:30-13:00	TVA Annual General Meeting	TVA Members and Invited Guests only
13:00-14:00	LUNCH	All

SESSION 4: MBAYUWAYU **Chair: Dr M. Makungu**

14:00-14:20	Towards the application of digital technologies to enhance early detection, timely reporting and prompt response and long term strategic management of animal diseases in Tanzania	E.D. Karimuribo & N. Mtui-Malamsha *et al*.
14:20-14:30	Molecular characterisation of foot-and-mouth disease virus recently detected in Tanzania	C.J. Kasanga *et al*.
14:30-14:40	Livestock Movements as Determinants of Foot-and-Mouth Disease Virus Circulation in Northern Tanzania	D. Ekwem *et al*.
14:40 -14:50	Measuring Value Added in Livestock Sector in Tanzania	G.K. Simbila
14:50-15:00	Sustainability of control of CBPP in Tanzania: lessons from the Southern highlands	S. Nong'ona & L.J.M. Kusiluka
15:00-15:20	Anthrax in the Ngorongoro Conservation Area: a major issue for livestock health and human livelihoods	T. Forde *et al*.
15:20-15:30	General Discussion	Chair

SESSION 5: MBAYUWAYU **Chair: Dr Z.E. Makondo**

15:30-15:40	Sero-prevalence of *Brucella abortus* in cattle and antibiotic residues in raw milk in Bukombe district Tanzania	M. Mhozya & H.E. Nonga
15:40-15:50	Brucellosis in northern Tanzania: One Health research findings and implications for national strategy development	A.H. Lukambagire *et al*.
15:50-16:00	Studies on seroprevalence and risk factors for occurrence of Bovine brucellosis in cattle in Lindi district, Tanzania	J.E. Sijapenda *et al*.
16:00-16:10	Knowledge, perceptions and practices regarding brucellosis in pastoral communities of Kagera Region, Tanzania	J. B. Ntirandekura *et al*
16:10-16:20	Seroprevalence of Brucellosis in Dairy Cows and KAPs Among Dairy Farmers in Dodoma Municipality, Tanzania	I. Mengele *et al*.
16:20-16:30	Epidemiological study of *Mycobacterium* species in cattle carcasses and the abattoir environment in Iringa Municipality, Tanzania	G. Paul *et al*.
16:30-16:40	Porcine Cysticercosis – An Emerging Neglected Food Borne Parasitic Zoonosis in Urban settings in Tanzania: Need for Immediate Control Strategies	E. M. Mkupasi *et al*.
16:40-16:50	Is deep frying of Lake Victoria sardine (*Rastrineobola argentea*) a value addition process at the expense of nutritionally valued long chain polyunsaturated fatty acids (LC-PUFAs)?	D.N. Chaula
16:50-17:10	General discussion	Chair

SESSION 6: MBAYUWAYU	**CHAIR: Dr S. Nong'ona**	
17:00-17:10	Endocrine Disrupting Estrogens a Potential Threat to Fishing Industry in Tanzania	E. Moshiro *et al*.
17:10-17:20	Molecular characterisation of infectious bursal disease virus detected in Morogoro, Tanzania	A. Msomi *et al*.
17:20-17:30	Thermal stability study of I-2 Newcastle vaccine	F. Makoga *et al*.
17:30-17:40	Possible involvement of *Dioscorea* species in human poisoning at Bwakila Juu in Morogoro Rural District, Tanzania	A. Issae *et al*.
17:40-18:00	General discussion	Chair
18:00-	END OF DAY2	All

DAY 3: Thursday 7th December 2017		
SESSION 7: MBAYUWAYU	**CHAIR: Prof. S.I. Kimera**	
08:30-08:50	Supporting the National Action Plan on AMR in Tanzania (SNAP-AMR)	R. Zadoks *et al*.
08:50-09:00	Current situation for Antimicrobial use, Antimicrobial Resistance and Antimicrobial residues in the food and agriculture sectors in Tanzania	Y. M. G. Hounmanou & R.H. Mdegela
09:00-09:10	Antimicrobial resistance patterns of extended-spectrum beta-lactamase producing *Escherichia coli* in low quality water in Morogoro, Tanzania	O.J. Mhongole *et al*.
09:10-09:20	Effect of freezing on stability of oxytetracycline residues in beef from Dodoma region, Tanzania	F. Mgonja *et al*.
09:20-09:30	Prevalence and antimicrobial resistance patterns of *Salmonella* spp. in Nile perch and water of Lake Victoria, Tanzania	Z. Baniga *et al*.
09:30-09:40	Prevalence of non-typhoidal *Salmonella* in different red meat value chains in Moshi, Tanzania	R. Zadoks *et al*.
09:40-09:50	Pesticides usage and safety of fish from Lake Victoria, Tanzania	A. Wenaty *et al*.
09:50-10:00	Prophylactic antibiotics in augmenting Surgical wound healing	C.W Werema & D.G Ndossi
10:00-10:10	Occurrence and distribution of sulfonamides, tetracyclines and quinolones in livestock manure in Morogoro Municipality, Tanzania	H.S.A. Mohamed *et al*.
10:10-10:30	General Discussion	Chair
10:10-10:40	HEALTH BREAK	All

SESSION 8: MBAYUWAYU	**CHAIR: Prof. R. Zadoks**

10:40-10:50	Tilapia lake virus threatens tilapines farming and food security: socio-economic challenges and preventive measures in Sub-Saharan Africa	Y.M.G. Hounmanou *et al*.
10:50-12:00	Molecular detection of tilapia lake virus (TiLV) genome in Nile tilapia (*Oreochromis niloticus*) from Lake Victoria, Tanzania	A. A. Chengula *et al*.
12:00-12:10	A cross-sectional and retrospective study of *Pseudomonas aeruginosa* in chicken feeds and carcasses in Dar es Salaam, Tanzania	S. Mdemu *et al*.
12:10-12:20	Assessment of awareness degree on mycotoxins among commercial poultry feed handlers in Morogoro, Tanzania	B. A. Temba
12:20-12:30	Epidemiological study of *Edwardsiella* infections in farmed fish in Morogoro, Tanzania	E. Mkemwa *et al*.
12:30-12:40	Lake Victoria Sardine Business Environment Analysis: Macro-Environment Factors	J. Mkunda *et al*.
12:40-13:00	General discussion	Chair
13:00-14:00	LUNCH BREAK	All

CONFERENCE CLOSING SESSION: MBAYUWAYU		
Chair: Hon. Abdallah H. Ulega (MP)		
14:00-4:10	Participants and Invited Guests Seated	ALL
14:00-4:30	Presentation of Recommendations from the Taskforce	Dr Melewas et al
14:30-5:30	General discussion	Chair
15:30-5:40	Invitation of the Guest of Honour	TVA Chairman
15:40-6:00	Official Closing of 35th TVA Conference	Guest of Honour
16:00	END OF 35TH TVA SCIENTIFIC CONFERENCE (*Bon Voyage)*	

POSTER PRESENTATION		
1	Caudal mediastinal abscessation in an adult East African black headed Ewe – a case report	M. Makungu & J. Malago
2	Seroimmune Responses to Strategic Vaccination in Chickens Against Newcastle Disease Using Commercially Available Vaccines	I. Mengele *et al*.

RESERVE LIST		
1.	Possible involvement of *Dioscorea* species in human poisoning at Bwakila Juu in Morogoro Rural District, Tanzania	A. Issae *et al*.
2.	Comparative effectiveness of Aloe vera crude extracts and ivermectin for treatment of gastrointestinal nematodes infection in goats	J. Nzalawahe & H. Ibrahim
3.	Relationship Between Faecal Egg Count and Chronic Status of Liver Fasciolosis of Cattle in Tanzania	G. A. Minga *et al*.
4.	Effective Microorganisms for the Control of Mixed Internal Parasitic Load of Afar Sheep	W. Chernet
5.	Analysis of different biomaterials as scaffolds in bone tissue engineering	W. Chernet
6.	Supporting Evidence Based Interventions: Causes and extent of mortality of domestic ruminants in Tanzania	T. Kibona *et al*.
7.	Endocrine Disrupting Estrogens a Potential Threat to Fishing Industry in Tanzania	S.C. Msigala *et al*.

8.	Can Effective Microorganisms Improve Nutrient Contents, feed Intake, N-Balance, Digestibility of Feeds and improve Growth of Local Sheep?	W. Chernet
9.	Magnitude of foetal wastage and the monetary losses in sheep and goats slaughtered in Morogoro selected slaughter facilities, Morogoro Tanzania	L.A. Kilumbi & H.E. Nonga
10.	Prevalence of Claw Lesions in Tanzania Short Horn Zebu Cattle in Kwimba District, Tanzania	E.M Ndaki & D.G Mpanduji
11.	Retrospective Study of African Swine Fever Disease in Tanzania	J.S. Chang'a *et al*.
12.	Microbiota Prime Leukocyte Response for Intestinal Innate Immunity	J.J. Malago
13.	Capacity Building for Training and Research in Health of Aquatic Resources in East and South African Region: A Case of Sokoine University of Agriculture	R. Mdegela et al.
14.	Towards Formulation of Herbal Disinfectants from *Synadenium glaucescens* Root Extracts as Promotional Tool for Organic Farming	A. Mpanyakavili et al.
15.	Understanding Bushmeat Trade and Consumption: It's Role in Novel Pathogen Spill-Over and Spread in Tanzania	A. Hayghaimo et al.
16.	Quantification of *Enterococci* species isolated from apparently healthy human animal-waste attendants, cattle and cattle wastes in Tanzania	B. P. Madoshi et al.
17.	Studies on Brucellosis in Lactating Cows in Babati District, Tanzania	G. Makingi et al.

TANZANIA VETERINARY JOURNAL

INSTRUCTIONS TO AUTHORS (EDITED 2013)

The Journal

The Tanzania Veterinary Journal (The Tropical Veterinarian) is a biannual Journal, which publishes original contribution to knowledge on Veterinary Science, Animal Science and Production, and allied sciences including new techniques and developments in Veterinary Medicine. The target readers of the Journal are the Research Workers, Veterinary Clinicians, Animal Scientists, Field Officers, as well as Policy Makers. Papers are accepted on the understanding that their content has not been published elsewhere and that they are subject to editorial revision. The Editors reserve the right to make minor alterations in the lay out or phraseology without reference to the author.

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Assemble the manuscript in the following order: Title Page, Summary, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables and Figure Legends. Subtitles may be used within sections. The total number of words in the whole manuscript should be between 2500 and 3000 excluding Tables, Figures, and References.

The ***Title Page*** should include the authors' initials of first and any other names followed by surnames, and affiliations, a running title of fewer than 60 characters and spaces, the name of the corresponding author along with email information.

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Reviews must be invited by the Editor prior to preparation and submission.

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Short communications like case reports are limited to a maximum of three Figures and one Table. They should present a complete study that is more limited in scope than is found in full-length papers. They are not designed for presentation of preliminary or fragmentary data. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Summary is limited to 200 words; (2) instead of a separate Materials and Methods section, experimental procedures should be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion are combined into a single section.

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Journal article, up to 8 authors
Mdegela RH, Tiringa P, Ngowi HA, Kazwala RR, Mtambo MMA. Prevalence of ectoparasites in domestic and feral cats in Morogoro municipality and Serengeti district in Tanzania. *Tanz Vet J* 22: 125-131, 2005.

Journal article, more than 8 authors
Mbassa GK, Singine EK, Pereka AE, et al. Differential features of erythrocytes of African free ranging chicken (Gallus gallus domesticus) and the greater flamingo (*Phoenicopterus ruber roseus*) of Lake Manyara National Park. *Tanz Vet J* 22: 102-111, 2005.

Article in a book
Mwakitumbi SM, Ramadhan Y, Makale JJ. Interactions Maasai cattle and wildebeests of Ngorongoro. In: The Epidemiology of Malignant Catarrhal Fever in Tanzania, ed Shoo AJ, Mushi KL, Aloo HJ. Mzumbe Book Project, Morogoro, 2001.

Book
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