OCCURRENCE OF TICK-BORNE HAEMOPARASITES IN CATTLE IN THE MUNGWI DISTRICT, NORTHERN PROVINCE, ZAMBIA

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Highlights

- Identification of tick-borne pathogens in cattle in Mungwi District, Zambia, using a reverse line blot hybridization assay.
- Identification of ticks associated with tick-borne infections in cattle in Mungwi District, Zambia.
- Findings on the occurrence of tick-borne haemoparasites in cattle in Mungwi.

ABSTRACT

Little is known about the occurrence of haemoparasites in cattle in communal grazing areas of Mungwi District of Northern Province, Zambia. Clinical signs and post mortem lesions are pathognomonic of mixed tick-borne infections especially babesiosis, anaplasmosis and East Coast fever. The main objective of this study was to screen selected communal herds of cattle for tick-borne haemoparasites, and identify the tick vectors associated with the high cattle mortalities due to suspected tick-borne diseases in the local breeds of cattle grazing along the banks of the Chambeshi River in Mungwi District, Northern Province, Zambia. A total of 299 cattle blood samples were collected from July to September 2010 from Kapamba (n=50), Chifulo (n=102), Chisanga (n=38), Kowa (n=95) and Mungwi central (n=14) in the Mungwi District. A total of 5,288 ticks were also collected from the sampled cattle from April to July 2011. DNA was extracted from the cattle blood and the hypervariable region of the parasite small subunit rRNA gene was amplified and subjected to the reverse line blot (RLB) hybridization assay. The results of the RLB assay revealed the presence of tick-borne haemoparasites in 259 (86.6%) cattle blood samples occurring either as single (11.0%) or mixed (75.6%) infections. The most prevalent species present were the benign *Theileria mutans* (54.5%) and *T. velifera* (51.5%). *Anaplasma marginale* (25.7%), *Babesia bovis* (7.7%) and *B. bigemina* (3.3%) DNA were also detected in the samples. Only one sample (from Kapamba) tested positive for the presence of *T. parva*. This was an unexpected finding; also because the tick vector, *Rhipicephalus appendiculatus*, was identified on animals from Kowa (14.0%), Chisanga (8.5%), Chifulo (6.0%) and Kapamba (1.4%). One sample (from Kapamba) tested positive for the presence of *Ehrlichia ruminantium* even though *Amblyomma variegatum* ticks were identified from 52.9% of the sampled animals from all study areas. There was significant positive association between *T. mutans* and *T. velifera* ($p<0.001$)

infections, and between *A. marginale* and *B. bovis* (p=0.005). The presence of *R. microplus* tick vectors on cattle was significantly associated with *B. bovis* (odds ratio, OR = 28.4, p<0.001) and *A. marginale* (OR=42.0, p<0.001) infections, while *A. variegatum* presence was significantly associated with *T. mutans* (OR=213.0, p<0.001) and *T. velifera* (OR=459.0, p<0.001) infections. *Rhipicephalus decoloratus* was significantly associated with *B. bigemina* (OR=21.6, p=0.004) and *A. marginale* (OR=28.5, p<0.001). Multivariable analysis showed a significant association between location and tick-borne pathogen status for *A. marginale* (p<0.001), *T. mutans* (p=0.004), *T. velifera* (p=0.003) and *T. taurotragi* (p=0.005). The results of our study suggest that the cause of cattle mortalities in Mungwi during the winter outbreaks is mainly due to *A. marginale, B. bovis* and *B. bigemina* infections. This was confirmed by the clinical manifestation of the disease in the affected cattle and the tick species identified on the animals. The relatively low prevalence of *T. parva, B. bigemina, B. bovis* and *E. ruminantium* could indicate the existence of endemic instability with a pool of susceptible cattle and the occurrence of disease outbreaks.

Key words: Haemoparasites, Tick-borne diseases, *Theileria, Babesia, Anaplasma, Ehrlichia*

INTRODUCTION

Tick-borne diseases (TBDs) are among the most important constraints to livestock production in developing countries (Kivaria, 2006). The most important TBDs of cattle in sub-Saharan Africa are theileriosis caused by *Theileria parva*, babesiosis caused by *Babesia bovis* and *B. bigemina*, anaplasmosis caused by *Anaplasma marginale* and heartwater caused by *Ehrlichia ruminantium* (Makala et al., 2003).

Theileriosis poses a major constraint to the Zambian livestock industry with losses of about 10 000 cattle per annum (Nambota et al., 1994). While East Coast fever (ECF) occurs in the Eastern and Northern provinces of Zambia, Corridor disease is widespread in the Southern, Central and Lusaka provinces and has been reported in the Copper-belt province (Makala et al., 2003). However, ECF is moving towards the Southern Province; 683 clinical cases were recorded in 2002 (Chisembele, 2005) and 377 cases and deaths of 87 head of cattle were reported in 2017 (Zambia Farmers Hub, 2017). The highest number of ECF cases occurs from January to March in the Northern and Eastern provinces, and the highest number of Corridor disease cases are recorded during the month of January in the Southern province (Samui, 1987). The epidemiology is complicated by, among other factors, the wide distribution of the tick vector, *Rhipicephalus appendiculatus*, which is found all over the country (Nambota et al., 1994).

Bovine babesiosis is an economically important TBD of cattle in tropical and subtropical regions of the world (McCosker, 1981). *Babesia bovis* and *B. bigemina* are present in all the Zambian provinces (Jongejan et al., 1988; McCosker, 1981) and are recognized as being of economic importance in cattle and small ruminants (Luguru, 1985; Pegram et al., 1989; Pegram and Banda, 1990). In Africa, *Rhipicephalus microplus*, *R. annulatus* and perhaps *R. geigyi* transmit *B. bigemina* and *B. bovis*. In addition, *B. bigemina* is transmitted by *R. decoloratus* and *R. evertsi evertsi* (Bock et al., 2004; Walker et al., 2003). *Babesia bovis* and *B. bigemina* are transovarially transmitted (Bock et al., 2004; Chauvin et al., 2009).

Heartwater, caused by *E. ruminantium*, is a rickettsial disease that affects domestic and wild ruminants in Zambia (Jongejan et al., 1988; Makala et al., 2003). In the agricultural areas of

Zambia, *Amblyomma variegatum* is the main vector of heartwater (Makala et al., 2003). In Zambia, heartwater is mainly a disease of cattle, although outbreaks in sheep and goats have been reported and recorded. Records from the Central Veterinary Research Institute (CVRI) for the period 1986–1997 revealed that the disease occurred throughout Zambia (Makala et al., 2003; Mangani, 1997). Heartwater is believed to be responsible for numerous deaths occurring throughout the year, but especially during the rainy season from March to September.

Bovine anaplasmosis (formerly known as gall sickness), caused by *A. marginale*, is an infectious but noncontagious disease that occurs in tropical and subtropical regions worldwide (Aubry and Geale, 2011). *Anaplasma centrale*, a less pathogenic but closely related organism, is used as a live vaccine for cattle in Israel, South Africa, South America and Australia (de la Fuente et al., 2005). Notably, there are strains of *A. centrale* with intermediate morphology (approximately half of the organisms touching the edge of the red blood cells, instead of some 70% in typical *A. marginale* and some 30% in *A. centrale*), and such strains are not particularly mild (FAO, 1994). *Anaplasma marginale* is present in all the provinces of Zambia (Jongejan et al., 1988); it is regarded as the only *Anaplasma* species of importance to cattle in Zambia (McCosker, 1981). Transmission experiments have listed up to 19 different ticks as capable of transmitting *A. marginale* (Kocan et al., 2004).

Despite the importance of TBDs, little is known about the occurrence and prevalence of haemoparasites in cattle in the communal grazing areas of Mungwi District of Northern Province, Zambia (Marufu et al., 2010). Mungwi District is located in an area of Zambia where ECF is thought to be present, and vector control using acaricides has proved to be very costly for the

small-scale farmers. Also, Mungwi experiences increased cattle mortalities from December to March and from May through to July. All age groups of cattle are affected. This study was conducted to screen selected communal herds of cattle for tick-borne haemoparasites, and identify the tick vectors associated with the high cattle mortalities due to suspected TBDs in the local breeds of cattle grazing along the banks of the Chambeshi River in the Mungwi District, Northern Province, Zambia.

MATERIALS AND METHODS

Blood samples

Bovine blood samples were collected from July to September of 2010. The sampling areas were all in Mungwi District of Northern Province, Zambia. Kapamba, Chifulo, Chisanga and Kowa are located along the Chambeshi flood plains while Mungwi central is on the upland (Fig. 1). Although Mungwi District experiences increased mortalities in cattle due to TBDs between December to March and May to July, samples could not be collected during this time period as farmers are busy with agricultural activities (i.e., land preparation, planting, weeding and fertilizer application from December to March). Also, some cattle are used for draught power and are, therefore, not available for sampling during this time of cultivation. However, we did manage to collect ticks from April to July. Information on the owner, age, sex and color of each animal sampled was captured. Cattle of different age groups were sampled and all were indigenous breeds (Angoni). The sampled cattle included 84 males and 215 females. The animals were restrained in a crush pen during routine deworming and treatment. Blood samples were collected from the ear vein and 250 µl was spotted onto Whatman® filter paper (Merck, Darmstadt, Germany). The filter papers containing the dry blood spots were stored in silica gel before being transported to the Molecular Biology Laboratory,

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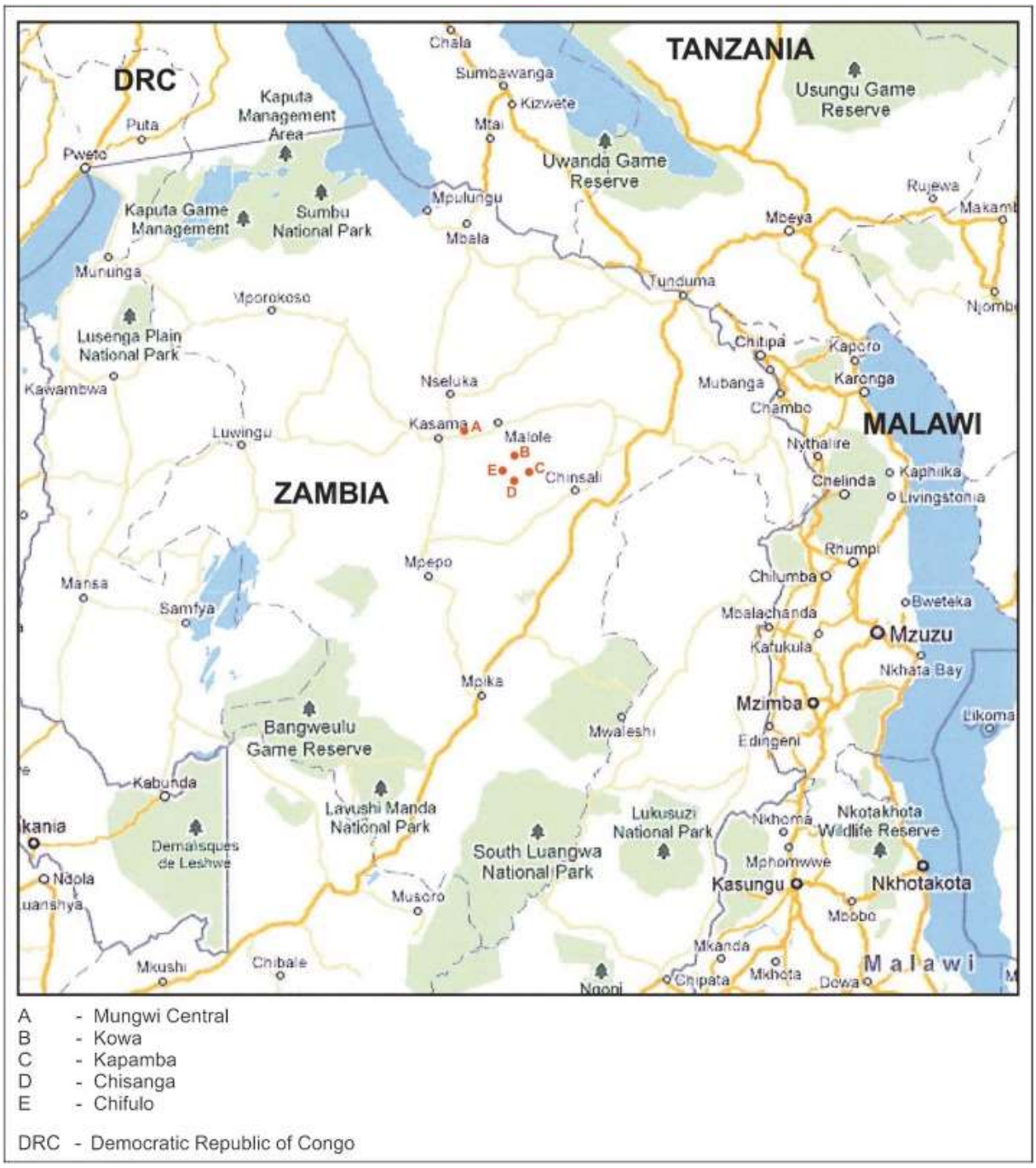

Fig. 1: Map showing the five localities (red dots) of Mungwi, in Zambia, from which the blood samples were collected from cattle.

Tick counts and identification

Half body tick counts were conducted on all the 299 cattle that had been sampled for blood. To ensure that the same animals be sampled during re-visitations, animals were ear-tagged, and individual identification records were kept. The objectives, time frame and benefits from the study were also discussed with the farmers. Regular visits were also made to the study areas to conduct routine animal husbandry practices to avoid drop out of the animals from the study.

Ticks were identified to genus and species level using a stereomicroscope and dichotomous identification keys of ticks as illustrated by Walker et al. (2003). The identified ticks were compared to species descriptions and distribution (Walker et al., 2003). The tick counts and identifications were done once on each animal from April to July 2011.

DNA extraction

DNA was extracted from dried blood spots using the QIAamp® DNA Mini kit (QIAGEN, Southern Cross Biotechnology [Pty] Ltd, Cape Town, South Africa) following the manufacturer's instructions. Extracted DNA was eluted in 100 µl elution buffer and stored at -20°C until further analysis.

PCR amplification and Reverse Line Blot (RLB) hybridization assay

Separate PCR master mixes were prepared for the amplification of *Theileria*/*Babesia* species (Nijhof et al., 2003, 2005) and *Ehrlichia*/*Anaplasma* species (Bekker et al., 2002). *Theileria* and *Babesia* group-specific forward primer, RLB F2 [5'-GAC ACA GGG AGG TAG TGA CAA G-3'] and biotin-labelled reverse primer, RLB R2 [5'-Biotin-CTA AGA ATT TCA CCT CTA ACA

GT-3’] were used to amplify the V4 hypervariable region of the parasite 18S rRNA gene (Nijhof et al., 2003). *Ehrlichia* and *Anaplasma* spp. were detected by amplifying the V1 hypervariable region of the 16S rRNA gene using the *Ehrlichia* and *Anaplasma* group-specific forward primer Ehr-F [5’-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3’] (Schouls et al., 1999) and biotin-labelled reverse primer Ehr-R [5’-Biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3’] (Bekker et al., 2002).The PCR reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Celtic Molecular Diagnostics, South Africa), 8 pmol/µl of each of the forward and reverse primers and 2.5 µl of DNA template in a total volume of 25 µl. Positive controls for the 16S rRNA PCR (*Anaplasma*/*Ehrlichia*) and 18S rRNA PCR (*Theileria*/*Babesia*) were DNA extracted from *A. centrale* blood vaccine (Onderstepoort Biological Products, Pretoria, South Africa) and from blood of a known *T. parva* infected buffalo (KNP102, Sibeko et al., 2008), respectively. Negative controls (master mix without DNA template) were included to monitor false positive and false negative results. A touch down thermal cycler program was used to amplify the DNA (Nijhof et al., 2005). The PCR products were then analysed using the RLB hybridization technique as previously described (Bekker et al., 2002; Gubbels et al., 1999; Nijhof et al., 2003, 2005). Genus- and species-specific probes included on the membrane are listed in Table 1.

Table 1: Genus- and species-specific probes and their sequences present on the in-house prepared membrane used for detecting pathogen DNA. Symbols used to indicate degenerate positions: R=A/G, W=A/T.

Oligonucleotide probe identification	Probe Sequence (5'--> 3')	Reference
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A	Bekker et al. (2002)
Anaplasma centrale	TCG AAC GGA CCA TAC GC	Georges et al. (2001)
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	Bekker et al. (2002)
Anaplasma sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC	Bekker et al. (2002)
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG	Schouls et al. (1999)

Babesia genus-specific probe 1	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
Babesia genus-specific probe 2	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
Babesia bicornis	TTG GTA AAT CGC CTT GGT C	Nijhof et al. (2003)
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG	Gubbels et al. (1999)
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG	Gubbels et al. (1999)
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT	Butler et al. (2008)
Babesia canis	TGC GTT GAC CGT TTG AC	Matjila et al. (2004)
Babesia divergens	ACT RAT GTC GAG ATT GCA C	Nijhof et al. (2003)
Babesia felis	TTA TGC GTT TTC CGA CTG GC	Bosman et al. (2007)
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G	Bosman et al. (2007)
Babesia leo	ATC TTG TTG CTT GCA GCT T	Bosman et al. (2007)
Babesia major	TCC GAC TTT GGT TGG TGT	Georges et al. (2001)
Babesia microti	GRC TTG GCA TCW TCT GGA	Nijhof et al. (2003)
Babesia occultans	CCT CTT TTG GCC CAT CTC GTC	He et al. (2011)
Babesia rossi	CGG TTT GTT GCC TTT GTG	Matjila et al. (2004)
Babesia sp. (sable)	GCT GCA TTG CCT TTT CTC C	Oosthuizen et al. (2008)
Babesia vogeli	AGC GTG TTC GAG TTT GCC	Matjila et al. (2004)
Ehrlichia/Anaplasma group-specific probe	GGG GGA AAG ATT TAT CGC TA	Bekker et al. (2002)
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	Schouls et al. (1999)
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	Schouls et al. (1999)
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	Bekker et al. (2002)
Theileria/Babesia group-specific probe	TAA TGG TTA ATA GGA RCR GTT G	Gubbels et al. (1999)
Theileria genus-specific probe	ATT AGA GTG CTC AAA GCA GGC	Nijhof (unpublished)
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG	Matjila et al. (2008)
Theileria annulata	CCT CTG GGG TCT GTG CA	Georges et al. (2001)
Theileria bicornis	GCG TTG TGG CTT TTT TCT G	Nijhof et al. (2003)
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT	Gubbels et al. (1999)
Theileria sp. (buffalo)	CAG ACG GAG TTT ACT TTG T	Oura et al. (2004)
Theileria equi	TTC GTT GAC TGC GYT TGG	Butler et al. (2008)
Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG	Nijhof et al. (2005)
Theileria lestoquardi	CTT GTG TCC CTC CGG G	Nagore et al. (2004)
Theileria mutans	CTT GCG TCT CCG AAT GTT	Gubbels et al. (1999)
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG	Bekker et al. (2002)
Theileria parva	GGA CGG AGT TCG CTT TG	Nijhof et al. (2003)
Theileria sp. (sable)	GCT GCA TTG CCT TTT CTC C	Nijhof et al. (2005)
Theileria separata	GGT CGT GGT TTT CCT CGT	Schnittger et al. (2004)
Theileria taurotragi	TCT TGG CAC GTG GCT TTT	Gubbels et al. (1999)
Theileria velifera	CCT ATT CTC CTT TAC GAG T	Gubbels et al. (1999)

Statistical analyses

The prevalence estimates at individual level, defined as the number of animals that tested positive for various tick-borne infections, using the RLB assay, were derived for each location, sex and age categories. To determine associations between tick-borne infections as a result of joint occurrence, the distribution of observed prevalences for each pathogen over the five sub-locations was compared to the distribution of expected prevalences under the null hypothesis that the prevalences were the same across the study area. Univariate followed by multivariable analyses of significant associations between potential risk factors and the major tick-borne pathogens of cattle (*T. parva*, *A. marginale*, *B. bovis* and *B. bigemina*) and parasites for benign theileriosis (*T. mutans*, *T. velifera* and *T. taurotragi*) were carried out considering the tick-borne pathogen status as a binary outcome (positive or negative). *Ehrlichia ruminantium* was not included in the analysis because only one animal was detected as positive from the sampled cattle. The variables considered were sex, age category (4-12 months, 13-24 months and >24 months), location (Chisanga, Chifulo, Kapamba, Kowa and Mungwi central), and the presence or absence of the respective tick vectors. The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 23.0 (IBM SPSS, 2014).

RESULTS

A representative reverse line blot (RLB) demonstrating hybridization of PCR products generated from samples collected in this study, and the positive and negative controls, with RLB probes is shown in Figure 2. Results from the RLB assay indicated that 11.0% (n = 33) of the sampled animals tested positive for single infections of haemoparasites, while 75.6% (n = 226) of the sampled animals tested positive for mixed infections (Table 2). The most prevalent haemoparasites

present as single infections were *Theileria mutans* (4.7%) and *T. velifera* (4.0%). Three samples tested positive for single infections of *Ehrlichia canis* while two tested positive for single infections of *Theileria* sp. (sable). Other samples with single infections were positive for *B. bovis* and *T. equi*. The majority of the single infections were from Chifulo (17.66 %) followed by Chisanga (15.8 %) (Table 2). The samples with mixed infections exhibited a combination of one or two *Theileria* species and another species (85.0%) and occurred in all of the five areas sampled.

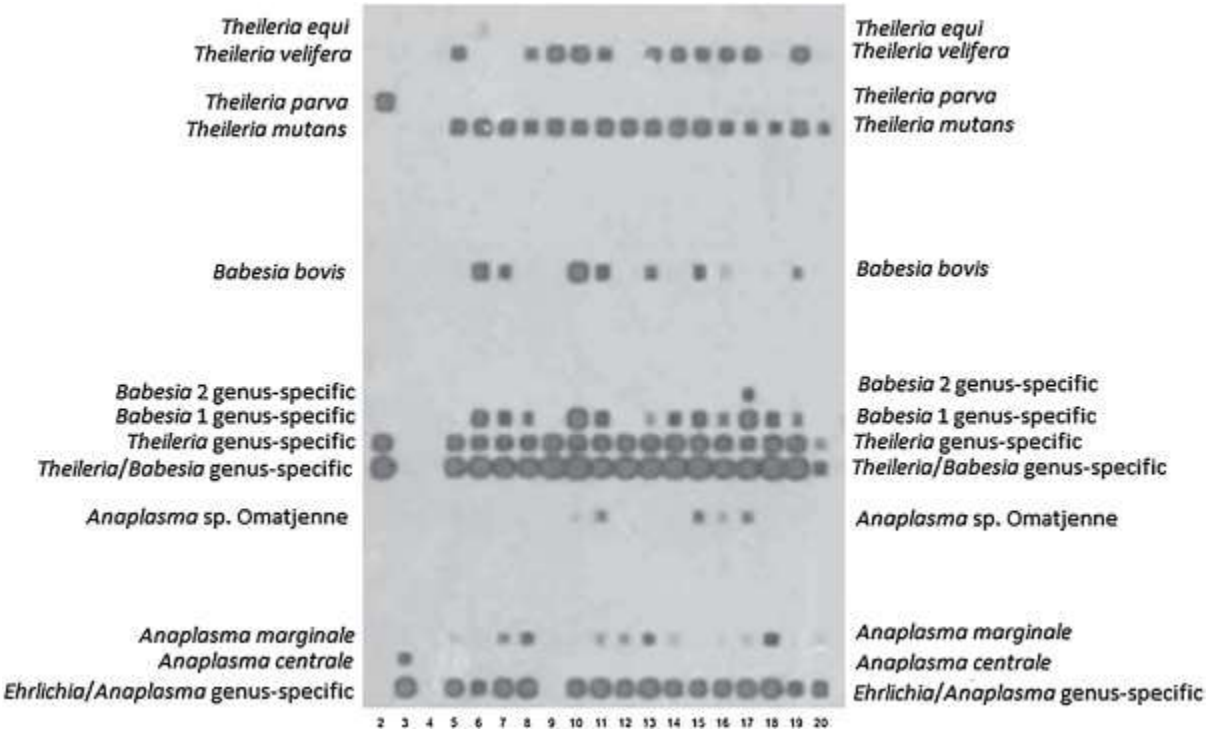

Fig. 2: Representative reverse line blot showing tick-borne haemoparasite infection status of 16 cattle sampled from five areas of Mungwi District, Zambia from April to July 2011. Genus/species-specific oligonucleotides were applied in horizontal rows, and PCR products from the test samples and controls in vertical lanes. Lanes 2 and 3, positive controls (*Theileria parva* and *Anaplasma centrale*, respectively); lane 4, negative control (no DNA template); lanes 5 to 20, PCR products from 16 cattle. The black spots on the X-ray film indicate a positive signal caused by hybridization of the PCR product to the probe immobilized on the membrane; the absence of black spots indicates negative signals.

Table 2: The occurrence of tick-borne haemoparasites in bovine blood samples from five localities in the Mungwi District, Zambia as determined by the RLB hybridization assay.

	Kapamba (n=50)	**Chifulo (n=102)**	**Chisanga (n=38)**	**Kowa (n=95)**	**Mungwi central (n=14)**	**TOTAL (n=299)**
SINGLE INFECTIONS						
Theileria equi	0	1 (0.9 %)	0	0	0	1 (0.3 %)
Theileria mutans	1 (2.0%)	8 (7.8%)	2 (5.3%)	0	3(21.4%)	14 (4.7%)
Theileria sp. (sable)	0	0	2 (5.3%)	0	0	2 (0.7%)
Theileria velifera	3 (6%)	7 (6.9%)	0	2 (2.1%)	0	12 (4%)
Babesia bovis	0	0	1 (2.6%)	0	0	1 (0.3%)
Ehrlichia canis	0	2 (1.9 %)	1 (2.6%)	0	0	3 (1%)
Single infections (SUBTOTAL)	***4 (8%)***	***18 (17.6%)***	***6 (15.8%)***	***2 (2.1%)***	***3 (21.4%)***	***33 (11%)***
MIXED INFECTIONS						
Theileria parva	1 (2.0%)	0	0	0	0	1 (0.4%)
Theileria equi	4 (8.0%)	0	1 (2.6%)	3 (3.1%)	0	8 (3.5%)
Theileria mutans	43 (86.0%)	26 (25.5%)	10 (26.0%)	73 (77.0%)	11 (78.6%)	163 (54.5%)
Theileria sp.(buffalo)	0	1 (0.9 %)	0	33 (35.0%)	0	34 (11.4%)
Theileria buffeli	0	0	1 (2.6%)	0	0	1 (0.4%)
Theileria sp. (sable)	4 (8.0%)	0	7 (18.4%)	0	0	11 (3.7%)
Theileria sp. (kudu)	0	0	0	20 (21.0%)	0	20 (6.7%)
Theileria velifera	45 (90.0%)	26 (25.5%)	8 (21.0%)	64 (67.4%)	11 (78.6%)	154 (51.5%)
Theileria taurotragi	0	5 (4.9%)	0	29 (30.5%)	0	34 (11.4%)
Babesia bovis	2 (4.0%)	8 (7.8%)	1 (2.6%)	10 (10.5%)	2 (14.3%)	23 (7.7%)
Babesia bigemina	0	0	0	10 (10.5%)	0	10 (3.3%)
Babesia caballi	0	16 (15.7%)	0	0	0	16 (5.3%)
Babesia sp. (sable)	0	38 (37.0%)	0	0	0	38 (12.7%)
Babesia gibsoni	0	38 (37.0%)	0	0	0	38 (12.7%)
Anaplasma bovis	1 (2.0%)	0	0	1 (1.0%)	0	2 (0.67%)
Anaplasma marginale	8 (16.0%)	38 (37.0%)	0	29 (30.5%)	2 (14.3%)	77 (25.7%)
Anaplasma centrale	0	0	0	3 (3.2%)	0	3 (1.0%)
Anaplasma sp. Omatjenne	4 (8.0%)	3 (2.9%)	0	14 (14.7%)	1 (7.0%)	22 (7.3%)
Ehrlichia canis	0	2 (1.96%)	0	32 (33.7%)	0	34 (11.4%)
Ehrlichia chaffeensis	0	0	0	12 (12.6%)	0	12 (4.0%)
Ehrlichia ruminantium	1 (2.0%)	0	0	0	0	1 (0.33%)
Mixed infections (SUBTOTAL)	***46 (92%)***	***66 (64.7%)***	***11 (28.9%)***	***92 (97%)***	***11 (78.6%)***	***226 (75.6%)***
Negative/below detection limit	0	12 (11.8%)	15 (39.5%)	1 (1.0%)	0	28 (9.4%)
***Theileria/Babesia* genus-specific only**	0	6 (5.9%)	0	0	0	6 (2.0%)
***Ehrlichia /Anaplasma* genus-specific only**	0	0	6 (15.8%)		0	6 (2.0%)

Fifteen percent of the mixed infections exhibited a combination of *Babesia* species and *A. marginale* and were from Chifulo (Table 2). A total of 28 of the samples screened (9.4%) were negative for haemoparasites and/or below the detection limit of the RLB test. Negative samples were from Chifulo, Chisanga and Kowa, while all samples from Kapamba and Mungwi central were positive. In 12 samples (4.0%), the PCR products failed to hybridize with any species-specific probes but hybridized only with either the *Ehrlichia*/*Anaplasma* (n = 6) or the *Theileria*/*Babesia* (n = 6) group-specific probes (Table 2).

Specific detection of *Babesia* and *Theileria* species

Nine *Theileria* species were detected, namely *T. parva*, *T. buffeli*, *T. equi*, *Theileria* sp. (sable), *Theileria* sp. (kudu), *Theileria* sp. (buffalo), *T. taurotragi*, *T. velifera* and *T. mutans* (Table 2,). The most prevalent *Theileria* species present were *T. mutans* (4.7% single infection; 54.5% mixed infections) and *T. velifera* (4.0% single infection; 51.5% mixed infections), and the infections were detected in all of the five sampled areas. Interestingly, only one sample from Kapamba tested positive for *T. parva*. Single *Theileria* species infections were detected in 0.7% of animals from Kowa, 1.0% from Mungwi central, 1.3% from Kapamba, 2.0% from Chisanga and 6.0% from Chifulo. The most common combination of mixed *Theileria* species infections which was detected in 22.0% of the samples was *T. mutans* and *T. velifera* reported from Kowa. From Kapamba, the most dominant mixed infection combination was *T. velifera* (90.0%) and *T. mutans* (86.0%). The most prevalent *Theileria* species in Chifulo were *T. velifera* (25.5%) and *T. mutans* (25.5%). *Theileria taurotragi* was detected as mixed infection in 4.9% of the samples from Chifulo. Samples from Chisanga tested positive for *T. mutans* (26.0%), *T. velifera* (21.0%) and *Theileria* sp. (sable) (18.6%). From Kowa, the most prevalent *Theileria* species were *T. mutans* (77.0%) and *T. velifera*

(67.4%). *Theileria* sp. (buffalo) and *T. taurotragi* were detected in 35.0% and 30.5% of the tested samples, respectively. The most prevalent *Theileria* species in Mungwi central were *T. mutans* (78.6%) and *T. velifera* (78.6%) (Table 2).

Five detected *Babesia* species were *B. bovis*, *B. bigemina*, *B. gibsoni*, *B. caballi* and *Babesia* sp. (sable) (Table 2). From all the sampled areas, a single *B. bovis* infection was detected in one of the sampled animals (0.3%) from Chisanga. Mixed *Babesia* species infections were detected in samples from Chifulo (33.8%) and Kowa (6.7%). The most common combination of mixed *Babesia* infections was detected in 33.0% of samples being *B. bovis*, *Babesia* sp. (sable), *B. gibsoni* and *B. caballi* and occurred in Chifulo. From Chifulo, the most prevalent *Babesia* species was *B. gibsoni* (37.0%) and *Babesia* sp. (sable) (37.0%). *Babesia bigemina* (10.5%) and *B. bovis* (10.5%) were detected in samples from Kowa. *Babesia bovis* was detected in samples from Kapamba (4.0%), Chisanga (7.8%) and Mungwi central (14.3%) (Table 2).

Specific detection of *Anaplasma* and *Ehrlichia* species

Four *Anaplasma* species and three *Ehrlichia* species were detected in the samples. The *Anaplasma* species were *Anaplasma bovis*, *A. centrale*, *A. marginale* and *Anaplasma* (formerly *Ehrlichia*) sp. Omatjenne (Table 2). There were no single *Anaplasma* species infections detected in the samples. Mixed *Anaplasma species* infections were detected in samples from Kowa (49.5%), Chifulo (40.0 %), Kapamba (26.0%) and Mungwi central (21.0%). *Anaplasma marginale* was detected in 25.7% of the sampled animals, *Anaplasma* sp. Omatjenne in 7.3%, *A. centrale* in 1.0% and *A. bovis* in 0.7% of the samples. No *Anaplasma* species infections were detected in Chisanga (Table 2).

Ehrlichia species detected were *E. ruminantium, E. canis* and *E. chaffeensis* (Table 2). Single *E. canis* infections were detected in samples from Chifulo (0.7%) and Chisanga (0.3%). Mixed *Ehrlichia* species infections were most prevalent in Kowa (34.0%) followed by Chifulo (2.0%) and Kapamba (2.0%). The most common combination of mixed *Ehrlichia* infections was *E. canis* and *E. chaffeensis* which were detected in 46.0% of samples from Kowa. Of all the sampled animals, only one sample from Kapamba (0.3%) tested positive for the presence of *E. ruminantium* (Table 2).

Association between co-infections of haemoparasites

The presence of different tick-transmitted pathogens within the same host concurrently, provides an opportunity for interaction that could generate a positive or negative outcome among pathogen populations. We determined the proportions of cattle that had single or mixed infections. In this context, mixed refers to the samples in which at least two haemoparasite species were detected. Out of the 259 cattle in which tick-borne pathogens were identified to the level of species, 226 (87.3%) had mixed infections and 33 (12.7 %) carried single infections. To determine whether interactions occur in a single host, the observed and expected frequencies of joint occurrence for *T. parva, A. marginale, B. bovis, B. bigemina, T. mutans, T. velifera* and *T. taurotragi* in cattle were compared. The results indicate that there were positive and negative associations among the 21 pairs of co-infections considered, seven of which were significant (Table 3). *Theileria mutans* was positively associated with *T. velifera* ($p<0.001$), *T. parva* (not significant, $p>0.05$), and *B. bovis* (not significant). There were also positive associations between *A. marginale* and *B. bovis* ($p=0.005$), and between *T. parva* and *A. marginale* (not significant).

Table 3: Observed and expected frequencies, and associations of co-infections for major tick-borne pathogens (*T. parva*, *A. marginale*, *B. bovis* and *B. bigemina*) and parasites of benign theileriosis (*T. mutans*, *T. velifera* and *T. taurotragi*) in 299 cattle from Mungwi District, Northern Province, Zambia included in a study carried out from July to September, 2010. A p-value of ≤0.05 was considered to be significant.

	Observed, expected frequency (*p*-value)						
	T. mutans	*T. velifera*	*T. parva*	*A. marginale*	*B. bovis*	*B. bigemina*	*T. taurotragi*
T. mutans		**138, 81.1**	**3, 1.7**	**40, 42.5**	**16,12.7**	**0, 5.5**	**0, 19.9**
		(<0.001)	(0.26)	(0.51)	(0.19)	(<0.001)	(0.001)
T. velifera			**0, 1.5**	**33, 37.9**	**15, 11.3**	**0, 4.9**	**0, 17.7**
			(0.25)	(0.23)	(0.13)	(0.002)	(<0.001)
T. parva				**2, 0.8**	**0, 0.2**	**0, 0.1**	**0, 0.4**
				(0.16)	(1.00)	(1.00)	(1.00)
A. marginale					**12, 5.9**	**0, 2.6**	**6, 9.3**
					(0.005)	(0.07)	(0.23)
B. bovis						**0, 0.8**	**2, 2.8**
						(1.00)	(1.00)
B. bigemina							**10, 1.2**
							(<0.001)

If the observed frequency was greater than expected, then the association is positive, and if it is less than expected, then the association is negative.

Tick identifications

A total of 5288 ticks were collected from 299 cattle from April to July 2011, and identified to species level. The tick species identified were *Amblyomma variegatum*, *Rhipicephalus decoloratus*, *Rhipicephalus microplus* and *Rhipicephalus appendiculatus* (Fig. 3). From Kapamba, the most prevalent tick species identified were *A. variegatum* (72.5%) and *R. microplus* (13.6%). *Amblyomma variegatum* (47.3%) and *R. decoloratus* (33.0%) were most prevalent in Chifulo. From Chisanga, *R. decoloratus* (43.3%) and *A. variegatum* (39.8%) were most prevalent. In Kowa, *A. variegatum* (40.4%) and *R. decoloratus* (36.3%) were most prevalent. *Amblyomma variegatum* (65.7%) was most prevalent in Mungwi central (Fig. 3).

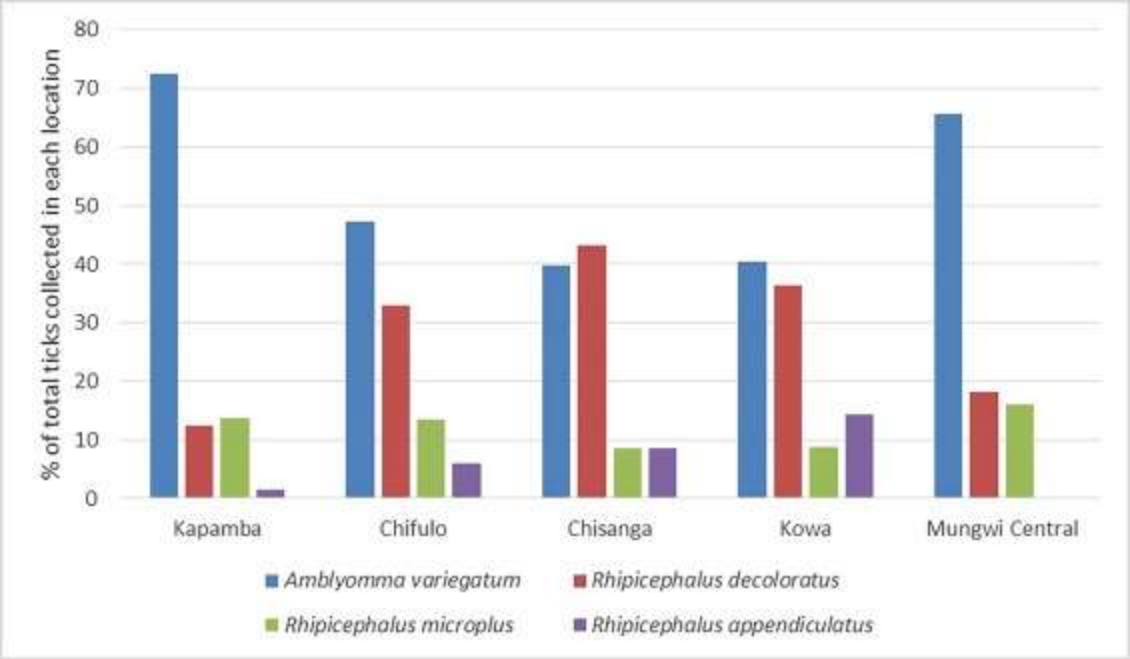

Fig. 3: Tick species identified from the cattle sampled from five areas of Mungwi district, Zambia from April to July 2011.

Risk factor analysis

We carried out univariate and multivariable analyses to determine the association between the potential risk factors and tick-borne transmitted infections. Sex of the cattle was not associated with any of the tick-borne infections considered in the univariate analysis. Multivariable analysis was carried out for tick-borne infections (*A. marginale*, *T. velifera*, *T. mutans*, *T. taurotragi* and *B. bigemina*) that had at least two variables that were significant ($p \leq 0.05$) from univariate analysis. There was a significant association between location and tick-borne pathogen status for *A. marginale* ($p<0.001$), *T. mutans* ($p=0.004$), *T. velifera* ($p=0.003$), *T. taurotragi* ($p=0.005$), but not for *B. bigemina* ($p=1.0$). Figure 4 shows the distribution of the major tick-transmitted haemoparasites (*T. parva*, *A. marginale*, *T. mutans*, *T. velifera*, *B. bovis* and *B. bigemina*) amongst cattle in the five locations. The highest prevalence for *A. marginale* was observed in Chisanga

(94.7%) followed by Kowa (30.4%). Kapamba and Mungwi central had higher prevalences for *T. mutans* (98% and 100%, respectively) and *T. velifera* (88% and 78.6%, respectively) compared to other locations (Fig. 4). The presence of *R. microplus* tick vectors on cattle was significantly associated with *B. bovis* (odds ratio, OR = 28.4, $p<0.001$) and *A. marginale* (OR=42.0, $p<0.001$) infections, while *A. variegatum* presence was significantly associated with *T. mutans* (OR=213.0, $p<0.001$) and *T. velifera* (OR=459.0, $p<0.001$) infections. *Rhipicephalus decoloratus* was significantly associated with *B. bigemina* (OR=21.6, $p=0.004$) and *A. marginale* (OR=28.5, $p<0.001$), while *R. appendiculatus* was significantly associated with *T. taurotragi* (OR=155.2, $p<0.001$). Age was considered in the multivariable analysis only for *T. mutans*. Cattle in the age categories of 13-24 months and >2 years were more likely (OR=2.2 and 1.6, respectively) to be infected with *T. mutans* as compared to those 4-12 months of age, although the association was not significant ($p>0.05$). The prevalences of *A. marginale*, *T. mutans*, *T. velifera* and *T. taurotragi* were high in Chisanga, Kapamba, Kowa and Mungwi central as illustrated in Table 2.

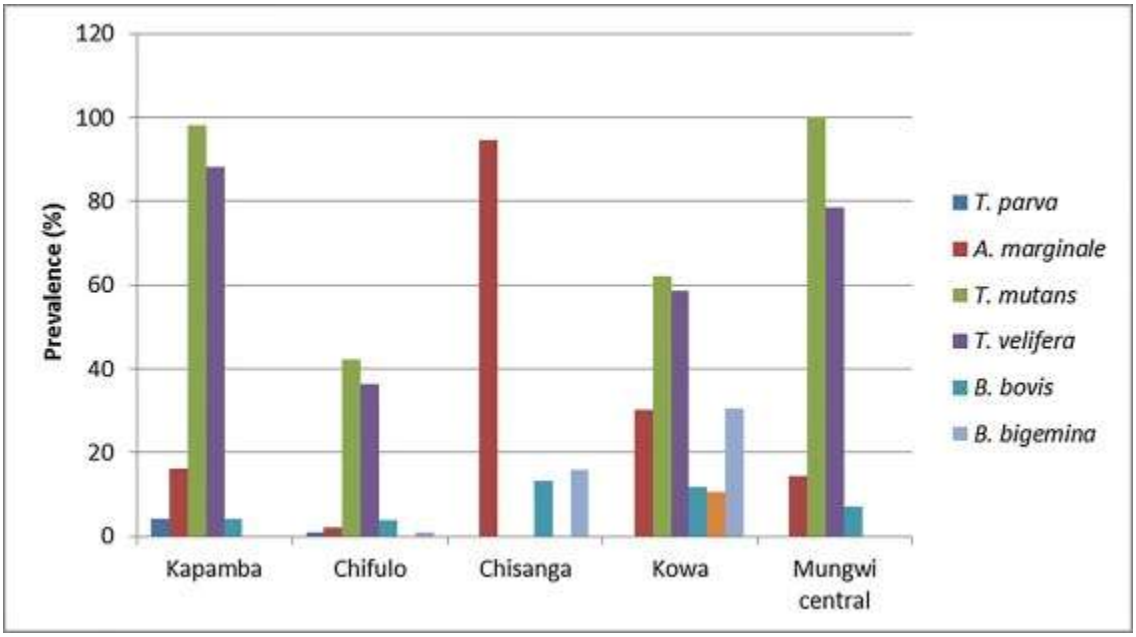

Fig. 4: The prevalence of the major tick-transmitted haemoparasites in cattle from Mungwi District, Northern Zambia included in a study carried out from July to Septembe 2010.

DISCUSSION

The most prevalent *Theileria* species detected in our study were the benign *T. mutans* (54.5%) and *T. velifera* (51.5%). *Amblyomma variegatum* was the most abundant tick species identified and could explain the positive association in the detection of *T. mutans* and *T. velifera* since these parasites are both transmitted by *A. variegatum* (Walker et al., 2003). Previous reports also indicated that *T. mutans* and *T. velifera* infections were significantly associated with the presence of the tick vector on cattle (De Vos and Roos, 1981). The high prevalence of *T. mutans* in our study is in agreement with the findings of Simuunza et al. (2011) who showed that *T. mutans* was the most prevalent tick-borne *Theileria* species in the Central, Lusaka and Eastern Provinces of Zambia. It is also in concordance with similar studies in Uganda (Byaruhanga et al., 2016) and Sudan (Salih et al., 2007). This would suggest that cattle in these parts of Africa are exposed to a high and continuous *T. mutans* challenge or that *T. mutans* is harbored at detectable levels for long periods post-infection. Alternatively, but less likely, cattle may be more susceptible to *T. mutans* infection than to other tick-borne pathogens (Simuunza et al., 2011).

Interestingly, only one sample from Kapamba tested positive for *T. parva*. This was an unexpected finding since ECF is thought to be present in the district of Mungwi in the Northern Province of Zambia and the tick vector, *R. appendiculatus*, was identified on animals from Kowa, Chisanga, Chifulo and Kapamba. In a sero-survey done during February and March of 2010 by the smallholder livestock investment project (SLIP), sponsored by the international food and agricultural development (IFAD), it was shown that 9.0% of cattle were seropositive for *T. parva* in the Mungwi District, Northern Province. Another tick and sero-survey conducted during February and March 2012 detected 29.0% *T. parva* sero-positive cattle in Mungwi District

(Fandamu and Sinyangwe 2012, personal communication) and the tick vector (*R. appendiculatus*) was present. We, therefore, expected to detect more *T. parva* positive samples. In this study, blood samples were collected from cattle from July to September 2010, a period when nymphs of *R. appendiculatus* are the most abundant life stage (Pegram et al., 1986; Walker et al., 2003). Low *T. parva* prevalence can be explained by low levels of infections in the preceding larvae stage of *R. appendiculatus. Theileria parva* survives the moult of *R. appendiculatus*; the parasite is acquired during the larval or nymphal feeding and transmitted in the next stage of nymphs (if acquired by larvae) or adults (if acquired by nymphs). After transmission of the parasite to a naïve animal, the ticks are free of infection (Walker et al., 2003). In the study area, farmers move with their animals from their homes to Chambeshi flood plains for agricultural activities (crop) during the wet season. Consequently, the lifecycle of the three-host tick species, including *R. appendiculatus,* and the activity of parasitism is interrupted. However, tick-transmitted agents generally persist for months or even years after the first attack, although in low numbers. The low prevalence of *T. parva* may also be attributed to a generally low infection rate in ticks and the low proportion of cattle infested with *R. appendiculatus* (only up to 14%), consequently low challenge for the cattle. Furthermore, the piroplasms of *T. parva* undergo only limited replication (Conrad et al., 1986), and fluctuations and loss of carrier state have been documented (Mans et al., 2015), which may lead to low detectable infection. Despite low prevalence of *T. parva,* cases of ECF occur in the study area. East Coast fever is acute and highly fatal for cattle, and most infected cattle in resource poor communal grazing areas die hence low occurrence of *T. parva,* given that the chance of timely diagnosis and treatment is slim. Previous serological studies in Mungwi District reported 9% to 29% seropositive animals, which is higher compared to the present study. Antibodies tend to persist for a long time in long-term carriers. Therefore, older animals can mislead the serological

tests explaining the differences herein detected. Another problem associated to serological tests is the cross-reactivity (sensitivity and specificity of the test) which can also explain the higher prevalence rates observed in the earlier sero-surveys. Serological tests detect previous exposure to infection; therefore, animals that survive ECF may have antibodies to *T. parva* but with very low parasitaemia to be detected by molecular methods. Nevertheless, a *T. parva* specific real-time PCR assay which is known to be more sensitive than the RLB (Sibeko et al., 2008) should be used in future studies to determine a more accurate *T. parva* prevalence in cattle in the Mungwi District, Northern Province, Zambia.

Despite the possible drawbacks, the RLB results in this study suggest that there are very low levels of *T. parva* infection in the cattle population in the Mungwi District. This, combined with the relatively low seroprevalence data previously reported by Fandamu and Sinyangwe (2012: unpublished observations), suggest that there is endemic instability of ECF in the Mungwi District. Makala et al. (2003) described the *Theileria* epidemiological situation in Zambia as "endemically unstable in parts of the Eastern and Northern Provinces" and ascribed it to the less favourable climatic conditions for *Rhipicephalus* ticks in this part of its range. These climatic conditions result in complex tick ecology, characterized by one or two tick generations a year and the occurrence of diapause, in contrast to a year round presence of ticks in Kenya, Tanzania and Rwanda (Makala et al., 2003).

Other *Theileria* species detected in our study included *T. buffeli* (n = 1), *Theileria* sp. (buffalo) (n = 34), *Theileria* sp. (sable) (n = 13), *Theileria* sp. (kudu) (n = 20) and *T. equi* (n = 9). Cattle are not common hosts, or not known to be hosts, of some of the *Theileria* species. We can only

speculate whether these are true findings due to incidental infections. An incidental infection could occur when a non-host is accidentally infected with a parasite through, for example, a tick bite. The parasite is usually eliminated by the incidental host, but can sometimes remain in the host for a short while, but it does not cause disease. *Theileria* sp. (sable)-like and *T. buffeli* sequences have been observed from cattle in South Africa (Mans et al., 2011). In another study, Muhanguzi et al. (2010b) demonstrated the detection of *T. buffeli* (12.6%), *Theileria* sp. (sable) (10.4%) and *Theileria* sp. (buffalo) (4.4%) in blood samples of cattle in Uganda using the RLB assay. *Theileria buffeli* has a cosmopolitan distribution and infects cattle, sheep, yaks and buffalo in Africa, Australia, New Zealand, Asia, Europe and the United States of America (reviewed by Sivakumar et al., 2014). A number of different species names have historically been assigned to *T. buffeli* including *T. buffeli*, *T. orientalis* and *T. sergenti*. However, both *T. buffeli* and *T. orientalis* continue to be used in literature depending on historical background of different authors (Mans et al., 2015). *Theileria buffeli*/*T. orientalis* has been found to exist in 11 distinct genotypes or perhaps species, namely *type* 1 (*chitose*), *type* 2 (*ikeda*), *type* 3 (*buffeli*), *types* 4-8, N1 *type*, N2 *type* and N3 *type* (reviewed by Sivakumar et al., 2014), with varying host specificity, clinical presentation and geographic distributions. Only *ikeda* and *chitose* are known to be pathogenic and cause considerable morbidity, production losses and/or mortality (Gebrekidan et al., 2016). Outbreaks of theileriosis caused by *T. buffeli* were reported in local Horro cattle in Ethiopia (Becerra et al., 1983). The known tick vectors for *T. buffeli* are *Haemaphysalis* spp. The species of *Haemaphysalis* ticks that transmit *T. buffeli* and have the ability to feed on cattle are distributed to a limited extent in Africa; found only in a few countries in North Africa (Walker et al., 2003). Therefore, detection of *T. buffeli* in this study means that ticks of another genus (or genera), and possibly even insects, transmit *T. buffeli* or there could be cross-reaction with another parasite in the species-specific

probe used. *Theileria* sp. (buffalo), which is associated with buffalo, is considered to be benign and not known to infect cattle (Mans et al., 2011). The vector for *Theileria* sp. (buffalo) is unknown. *Theileria* sp. (buffalo) 18S rRNA gene sequence is very similar to that of *T. parva* - therefore, misdiagnosis between the two species may occur; however, a real-time PCR assay can distinguish between the two species since only a *T. parva*-specific melting curve is generated in a test specific for *T. parva* (Sibeko et al., 2008). *Theileria* sp. (sable) and *Theileria* sp. (kudu) are parasites of antelopes (Nijhof et al., 2005). The vectors for *Theileria* sp. (sable) are *R. evertsi evertsi* and *R. appendiculatus*, and these have been reported in Zambia (Pegram et al., 1986). *Theileria* sp. (sable) was detected by the RLB hybridization from other clinically healthy animals, other than roan and sable antelope, such as African short-horn cattle (*Bos indicus*), African buffalo (*Syncerus caffer*), blue wildebeest (*Connochaetes taurinus*), klipspringer (*Oreotragus oreotragus*) and reedbuck (*Redunca arundinum*) from various regions in southern Africa and Tanzania; indicating a wide distribution of this parasite (Nijhof et al., 2005). Cattle, therefore, are likely to form a natural reservoir of *Theileria* sp. (sable). On the other hand, detection of *Theileria* sp. (sable) by the RLB hybridization assay can be due to cross-reactions with *T. velifera* (Mans et al., 2011). *Theileria equi* causes equine piroplasmosis in horses, donkeys and mules and is distributed in Australia, Asia, Europe, Africa, North and South America; transmitted by tick species of the genera *Amblyomma*, *Rhipicephalus*, *Hyalomma* and *Dermacentor* (Dantas-Torres and Otranto, 2017). Although *T. equi* is not known to infect cattle, *Hyalomma* spp. have been found in Zambia (Pegram et al., 1986); in the present study, we found *Amblyomma* and *Rhipicephalus* ticks. These ticks together with the presence of horses could contribute to the mechanical transmission of *T. equi* in cattle. A recent study on five Caribbean islands demonstrated the occurrence of *T. equi* in domestic ruminants - cattle, sheep and goats which may indicate an expansion of the host range of

the organism (Zhang et al., 2015). The findings of *Theileria* sp. (buffalo), *Theileria* sp. (sable), *Theileria* sp. (kudu) and *T. equi* in cattle suggests that these parasites might cause benign infections in other ungulate species, in this case domestic cattle. Further studies involving sequence analysis of the DNA of the tick-borne infections in cattle in the study area may provide more information about these uncommon infections, and help in redesigning species-species probes to reduce on cross-reactions or enable detection all variants. In addition, prospective studies involving the use of both molecular and serological techniques to study tick-borne haemoparasites could provide more epidemiological information. Whereas molecular diagnostic techniques detect current/active infection as well as carrier animals with low parasitaemias (Bekker et al., 2002; Gubbels et al., 1999; Sibeko et al., 2008), serological tests detect exposure to infection (and not necessarily current infection). Sampling of other animal species in the study area, and detection of infections in various tick species, may also add information to the transmission dynamics of the *Theileria* infections found in this study. The importance, magnitude and impact of these *Theileria* infections in cattle also requires further investigation.

As for *Babesia* species detected, *B. bovis* was present in 7.7% of the sampled animals and *B. bigemina* in 3.3% of the animals. We detected *B. bovis* in all of the five sampled areas with the highest detection in Mungwi central (14.3%) and Kowa (10.5%). *Rhipicephalus microplus*, the tick vector for *B. bovis* (Walker et al., 2003), was identified from animals from all of these areas and the presence of *R. microplus* was significantly associated with *B. bovis* infections. *Babesia bigemina* was only reported from Kowa (10.5%). The most abundant ticks identified from the sampled animals from Kowa were *R. decoloratus* (36.3%) and *R. microplus* (8.8%). The presence of *R. decoloratus* was significantly associated with *B. bigemina* infections, which may explain

why *B. bigemina* was only found in Kowa (10.5%), where *R. decoloratus* was the most abundant tick species. Our findings are in contrast to those of Jongejan et al. (1988) who reported that *B. bigemina* occurred throughout Zambia and that the infection caused by *B. bigemina* was more extensive than that caused by *B. bovis*, as *B. bigemina* has a wider vector range (Makala et al., 2003). Also, in another study by Iseki et al. (2007), it was found that the prevalence of *B. bigemina* infection (23.3%) in Zambia was substantially higher than that of *B. bovis* infection (14.4%) using a multiplex loop-mediated isothermal amplification (mLAMP) assay. However, our findings are in concordance with the results obtained by Simuunza et al. (2011). In their study, the prevalence of *B. bovis* detected in the Lusaka and Central provinces of Zambia in the wet and dry seasons was higher than that observed by Jongejan et al. (1988). The authors speculated that an increase in prevalence may indicate that *B. bovis* is becoming endemic in this part of the country, which could be due to uncontrolled movement of cattle that frequently occurs within Zambia. In the present study, *B. gibsoni* (for dogs), *B. caballi* (for horses), *Babesia* sp. (sable) (for antelopes) were also detected in the blood samples from cattle. Cattle are not known to be hosts of these species. Positive results for *B. gibsoni* in cattle may be attributed to mechanical transmission of the infections from dogs to cattle by the tick species found in this study. Notably, a high dog to cattle interaction has been demonstrated in Zambia; cattle are often herded by boys with dogs, which increases the likelihood of transmission by ticks (Banda et al., 2013). Nalubamba et al. (2015) demonstrated a high occurrence of *B. gibsoni* (59.7%) in natural dog populations in Zambia. *Babesia caballi* is a parasite of horses, donkeys and zebras and the main tick vectors in Africa are *Hyalomma* spp. and *Rhipicephalus* spp. The pathogen has, however, been detected in *A. variegatum* ticks collected from cattle in the Republic of Guinea (Tomassone et al., 2005). Possibly, the ticks collected from cattle in this study fed on *B. caballi*-infected equids and passed

on the infection to cattle. *Babesia* sp. (sable) was identified from a sable antelope (Oosthuizen et al., 2008), but the tick vector of this parasite remains unknown. The presence of *B. caballi* and *Babesia* sp. (sable) in cattle might lead to benign theileriosis in the animals.

Only one sample (from Kapamba) tested positive for the presence of *E. ruminantium* even though *A. variegatum* ticks were identified from 52.9% of the sampled animals from all study areas. Records of the Central Veterinary Research Institute (CVRI) for the period 1986–1997 revealed that heartwater occurred throughout Zambia (Makala et al., 2003; Mangani, 1997). The disease is believed to be responsible for numerous deaths occurring throughout the year, but especially during the rainy season from March to September. The disease is mainly seen in areas where regularly dipped animals are in close proximity to indigenous cattle with no acaricidal treatment and also where game is frequently seen in cattle-grazing areas. Heartwater also occurs in commercial farms when they have relaxed their normal tick control practices (Makala et al., 2003). The cattle sampled in our study were not regularly dipped and no game was spotted in cattle grazing areas. The presence of heartwater in the study area, therefore, can be explained by the biology of the pathogen and the tick vector. Ticks in the field in endemic areas exhibit low *E. ruminantium* infection levels, but present a highly virulent heartwater challenge (Allsopp, 2010). This may lead to a high case fatality rate among infected animals, and subsequently a low *E. ruminantium* infection prevalence in the cattle population. Using real-time PCR, *E. ruminantium* parasites were detected in the blood of experimentally infected animals only once the febrile stage of infection was reached (Allsopp et al., 2004; Steyn et al., 2008). This could be associated with the lifecycle of the pathogen. After an infected tick bite, the initial replication of the organism takes place in reticulo-endothelial cells and macrophages in the regional lymph nodes, after which

the organisms are disseminated via the blood stream to invade the endothelial cells of blood vessels in various organs and tissues, including the brain (Allsopp, 2010). It has been postulated that the febrile stage coincides with the movement of organisms from the lymph to the blood stream, which probably explains why *E. ruminantium* cannot be detected in the circulating blood of experimentally infected sheep before the febrile stage (having fever) (Steyn et al., 2008). The organism has been detected using the qPCR assay in field samples obtained from healthy cattle, which were therefore considered to be in a putative carrier state (Steyn et al., 2008). However, little is known about the location of *E. ruminantium* in carrier animals. If the organisms are not present in the circulating blood of carrier animals, this could lead to low detection of the pathogen in blood samples from carrier cattle. Another consideration is the seasonality and life cycle of *A. variegatum*, the tick vector of *E. ruminantium*. Like *R. appendiculatus*, *A. variegatum* is a three-host tick and undergoes one generation per year; adult activity occurs mainly in the rainy season (December to July). Being a three-host tick, the non-parasitic phase, which constitutes about 92% of the entire life cycle, is strongly affected by climate, vegetation and animal movements (Walker et al., 2003). Episodes of the dry season and migration of the cattle in Zambia can therefore delay or limit the propagation and transmission of *E. ruminantium* (Pegram et al., 1986).

The detection of *E. canis* and *E. chaffeensis* in the present study points to incidental infections in cattle and has implications for human health. *Ehrlichia canis* can cause illness in humans, dogs, and other canids are thought to be the reservoir host of the pathogen. While *E. canis* is mainly pathogenic for dogs, and the main tick vector is *R. sanguineus*, the pathogen may have a wider host range than currently recognized (Aguiar et al., 2013). Both *R. microplus* and *R. sanguineus* are able to infest dogs and cattle (Cabezas-Cruz et al., 2014). Therefore, dogs infected with *E.*

canis are a source of infection for *R. microplus* or *R. sanguineus* ticks that later infest cattle. Although *R. microplus* is a one-host tick species, the tick moves among cattle hosts during their parasitic lifetime (Chevillon et al., 2007), thereby increasing the chances of horizontal pathogen transmission among different hosts. The close interaction between dogs and cattle in the rural communities in Zambia (Banda et al., 2013) may increase the likelihood of transmission of *E. canis* to cattle. Furthermore, new species of cattle-related *Ehrlichia* spp. that are closely related and evolved from strains of *E. canis*, including those from South Africa, have recently been reported: *Ehrlichia* sp. UFMG-EV (*E. mineirensis*) that was isolated from Brazilian *R. microplus* and *Ehrlichia* sp. UFMT-BV that was found to be pathogenic for cattle in Brazil (Cabezas-Cruz et al., 2014). Elsewhere, *E. canis* was detected in blood samples of cattle (2.7%) in Uganda using RLB assay (Muhanguzi et al., 2010a). *Ehrlichia chaffeensis* causes human monocytic ehrlichiosis, an emerging zoonotic TBD. A wide range of mammals (goats, domestic and wild canids, lemurs) are reported to be naturally infected with *E. chaffeensis* (delos Santos et al., 2007). However, experimental infection in cattle with *E. chaffeensis* was demonstrated (delos Santos et al., 2007). There is need for further investigation of these *Ehrlichia* spp. in cattle through molecular, serological and epidemiological approaches.

Of the sampled cattle, 25.7% were positive for *A. marginale*; it was detected in all areas except Chisanga. In accordance with the transmission cycle of *A. marginale* (Walker et al., 2003), the infection was significantly associated with the presence of *R. microplus* and *R. decoloratus*. *Rhipicephalus microplus* and *R. decoloratus* are one-host ticks which spend about 25% of their lifecycle on the host (compared to 8% in three-host ticks) and undergo three to five generations per year (Pegram et al., 1986). The lifecycle and parasitism are therefore not as strongly influenced

by climate, vegetation and animal movements, and this may explain the relatively higher occurrence of *B. bigemina* and *B. bovis* than *T. parva* in the study area. Three samples (from Kowa) tested positive for the presence of *A. centrale*. To our knowledge, no vaccination using *A. centrale* is being conducted in the Mungwi District of Zambia. The presence of *A. centrale* is, therefore, probably due to natural infection, and could contribute to endemic stability of anaplasmosis in Zambia. Co-infection between *A. marginale* and *B. bovis* was positive and statistically significant. Both pathogens can be transmitted by *R. microplus*. The high prevalence of *A. marginale* in Chisanga (94.7%) compared to other locations may be explained by the observation that the highest proportion of *R. decoloratus* ticks (43.3%) was collected in this location (Fig. 3). Most cattle samples from Kapamba and Mungwi central were positive for *T. mutans* and *T. velifera*, which is consistent with the high proportions of *A. variegatum* ticks (Fig. 3) collected in these locations.

The major tick-borne haemoparasites detected in cattle in Mungwi District of Northern Zambia using the RLB hybridization assay were *T. mutans*, *T. velifera*, *A. marginale*, *B. bovis* and *B. bigemina*. The tick species identified confirmed the report by Pegram et al. (1986) that the most important ticks of cattle in Zambia are *R. appendiculatus*, *R. microplus*, *R. decoloratus* and *A. variegatum*. The results of our study also suggest that the cause of cattle mortalities in Mungwi during the winter outbreaks is mainly due to *A. marginale*, *B. bovis* and *B. bigemina* infections. This could be confirmed by the results of the RLB hybridization assay, clinical manifestation of the disease in the affected cattle (personal observation) and the tick species identified on the animals. It appears that in Mungwi, babesiosis due to *B. bovis* mostly infects cattle above one year of age. Calves appear to be less affected by *B. bovis* infection.

The relatively low prevalence of *T. parva, B. bigemina*, *B. bovis* and *E. ruminantium* found in our study could be indicative of the existence of a large pool of susceptible cattle resulting in a state of endemic instability and the occurrence of disease outbreaks. In contrast the high prevalence of *T. mutans, T. velifera* and *A. marginale* could indicate endemic stability. The high prevalence of *T. mutans* compared to the other pathogens was in agreement with similar studies in Uganda (Byaruhanga et al., 2016) and Sudan (Salih et al., 2007), indicating that cattle in these parts of Africa are exposed to a high and continuous challenge or that this species is harboured at detectable levels for long periods post-infection (Simuunza et al., 2011). Alternatively, cattle may be more susceptible to *T. mutans* (and *T. velifera*) infection than the other TBD pathogens investigated in this study.

Infection with multiple parasite species may increase or decrease the pathogenicity of the infections. This phenomenon is termed heterologous reactivity and is potentially an important determinant of both patterns of morbidity and mortality and of the impact of disease control measures (Woolhouse et al., 2015).

There is need for further epidemiological surveys in the Mungwi District, Northern Province, Zambia, using more specific and sensitive diagnostic tools or assays, to get a better understanding of the epidemiology of these tick-borne haemoparasites affecting cattle. The tick species that are likely to be involved in the transmission of *B. gibsoni*, *Babesia* sp. (sable), *T. equi*, *E. canis, E. chaffeensis* and *B. caballi* should be investigated. We conclude that integrated control policies should be developed to take account of multi-species pathogen communities that are commonly associated with clinical and sub-clinical TBD infections in Zambia.

AUTHOR STATEMENTS:

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Ethical statement

This study (V089/11) was approved by the Research Committee of the Faculty of Veterinary Science and the Animal Use and Care Committee of the University of Pretoria. Importation of dry blood spots on FTA filter paper was approved by Department of Agriculture, Forestry & Fisheries under Veterinary Import Permit number 13/1/1/30/0/8-124.

Conflict of interest

No conflict of interest.

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