Molecular detection and characterisation of protozoan and rickettsial pathogens in ticks from cattle in the pastoral area of Karamoja, Uganda

Charles Byaruhanga[a,b,*], Patience C. Akure[a], Donald M. Lubembe[a,c], Kgomotso Sibeko-Matjila[a], Milana Troskie[a], Marinda C. Oosthuizen[a], Hein Stoltsz[a]

[a] Vectors and Vector-Borne Diseases Research Programme, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa;

[b] National Agricultural Research Organisation, P.O. Box 259, Entebbe, Uganda.

[c] Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine and Surgery, Egerton University, P.O. Box 536, Egerton, 20115, Kenya.

* Corresponding author: cbyaruhanga27@yahoo.com (C. Byaruhanga).

Abstract

Ticks and tick-borne diseases (TBDs) significantly affect cattle production and the livelihoods of communities in pastoralist areas. Data on protozoan and rickettsial pathogens in ticks infesting cattle in Uganda is scanty; while it is an indicator of the likelihood of disease transmission and occurrence. A cross-sectional study was conducted amongst cattle in the Karamoja Region, northeastern Uganda, from July through September 2017, to determine the tick species diversity, identify protozoan and

rickettsial pathogens in the ticks, and characterise pathogenic species by sequence and phylogenetic analyses. About 50% of the ticks detected from each predilection site on each animal were collected from 100 purposively-selected cattle from 20 randomly-selected herds. Twelve tick species belonging to the genera *Amblyomma*, *Rhipicephalus* and *Hyalomma* were identified, the most abundant being *Amblyomma lepidum* (93.9%), followed by *Amblyomma variegatum* (2.0%) and *Rhipicephalus evertsi evertsi* (1.0%). Tick species that have not been reported in recent studies amongst cattle in Uganda were found, namely *Rhipicephalus pravus*, *Rhipicephalus praetextatus* and *Rhipicephalus turanicus*. The ticks were grouped into 40 pools, by species and location, and the reverse line blot (RLB) hybridisation assay was used to detect pathogens from the ticks. The most frequently detected tick-borne parasites were *Theileria mutans*, *Theileria velifera* and *Theileria parva*, each observed in 25% (10/40) of the tick pools. Tick-borne pathogens, namely *Babesia rossi*, *Babesia microti* and *Theileria* sp. (sable) that are not common to, or not known to infect, cattle were identified from ticks. The gene encoding *Ehrlichia ruminantium pCS20* region, the *Ehrlichia* and *Anaplasma* 16S rRNA gene, and *T*. *parva* p67 sporozoite antigen gene were amplified, cloned and sequenced. Seven novel *E*. *ruminantium pCS20* variants were identified, and these grouped into two separate clusters with sequences from other parts of Africa and Asia. The *T*. *parva* p67 sequences were of the allele type 1, and parasites possessing this allele type are commonly associated with East Coast fever in eastern Africa. Analysis of the *Ehrlichia* and *Anaplasma* 16S rRNA gene sequences showed that they were closely related to *Rickettsia africae* and to a new *Ehrlichia* species variant recently found in China. Our *R*. *africae* 16S rRNA sequences grouped with *R*. *africae* isolates from Nigeria, Egypt and Benin. The information on tick species diversity and pathogens in the various tick species provides an indicator of potential transmission amongst cattle populations, and to humans, and can be useful to estimate disease risk and in control strategies.

Keywords: *pCS20*; p67; reverse line blot; *Ehrlichia ruminantium*; *Theileria parva*; *Rickettsia africae*

Introduction

In Uganda, cattle are infested with several species of ixodid ticks (Acari: Ixodidae), which include *Rhipicephalus appendiculatus*, *Rhipicephalus evertsi evertsi*, *Rhipicephalus simus*, *Rhipicephalus decoloratus*, *Rhipicephalus pulchellus*, *Amblyomma variegatum*, *Amblyomma lepidum*, *Amblyomma gemma* and *Hyalomma truncatum* (Byaruhanga et al., 2015a; Kaiser et al., 1982; Magona et al., 2011; Nakayima et al., 2014; Rubaire-Akiiki et al., 2006; Tayebwa et al., 2018), as well as *Rhipicephalus compositus* and *Hyalomma rufipes* (Kaiser et al., 1982). Recently, *Rhipicephalus microplus* (Balinandi et al., 2020; Muhanguzi et al., 2020), *Rhipicephalus afranicus*, *Rhipicephalus sanguineus* sensu lato, *Amblyomma cohaerens* and *Amblyomma paulopunctatum* (Balinandi et al., 2020) were detected.

Rickettsial and protozoan parasites of the genera *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* are obligate intracellular microorganisms that multiply in the tick vectors and animal reservoirs, causing clinical infections (Dumler, 2005; Gubbels et al., 1999). The diseases caused lead to considerable economic losses attributed to death, decreased meat and milk production, decreased draught power and manure, and high cost of treatment and control (Byaruhanga et al., 2015b; Faburay et al., 2008; Kivaria, 2006; Ocaido et al., 2009). They are also a major impediment to the improvement of local breeds in endemic areas, through introduction or cross-breeding with highly productive but more susceptible cattle breeds from non-endemic regions (Faburay et al., 2007). Tick-borne protozoan parasites identified in previous studies from cattle in Uganda include *Theileria parva*, *Theileria mutans*,

Theileria velifera, *Theileria taurotragi*, *Theileria buffeli*, *Theileria* sp. (sable), *Theileria* sp. (buffalo), *Theileria bicornis*, *Babesia bigemina*, *Babesia bovis* and *Babesia vogeli* (Asiimwe et al., 2013; Byaruhanga et al., 2016; Muhanguzi et al., 2010b; Nakayima et al., 2014; Oura et al., 2011; Tayebwa et al., 2018), while rickettsial pathogens included *Ehrlichia ruminantium*, *Ehrlichia canis*, *Anaplasma marginale*, *Anaplasma centrale*, *Anaplasma bovis*, *Anaplasma phagocytophilum* and *Anaplasma* sp. (Omatjenne) (Asiimwe et al., 2013; Byaruhanga et al., 2016; Muhanguzi et al., 2010a; Oura et al., 2011; Tayebwa et al., 2018). The most pathogenic tick-borne pathogens detected in cattle in Uganda are *T*. *parva*, *B*. *bigemina*, *E*. *ruminantium* and *A*. *marginale* (Byaruhanga et al., 2016; Muhanguzi et al., 2010b; Tayebwa et al., 2018; Rubaire-Akiiki et al., 2006).

In Karamoja, livestock plays a vital role in the livelihoods of the communities, since they are a major source of income and are important for cultural purposes. The cattle in Karamoja Region are mainly of the short-horned East African Zebu breed, and are estimated to make up to 19.8% of the total national cattle population (14,368,000) (UBOS, 2017). Diseases of livestock are thus considered of importance amongst livestock keepers. Pastoralists traditionally travel with their cattle during the dry season in search of water and pastures for their animals. This movement, coupled with communal grazing, increases contact between cattle from different locations, and the likelihood of tick infestations and outbreaks of tick-borne diseases (TBDs) (USAID, 2017). As a result, TBDs were found to be the most important disease constraint to cattle production in the region, attributed to high morbidity, high mortality and control/treatment costs (Byaruhanga et al., 2015b).

Previous studies in Uganda have identified tick-borne pathogens in blood from cattle (Asiimwe et al., 2013; Byaruhanga et al., 2016; Muhanguzi et al., 2010a, b; Tayebwa et al., 2018), however, data on the protozoan and rickettsial pathogens in ticks infesting cattle is scanty (Nakayima et al., 2014). Therefore, there is a need to obtain information about the parasites circulating in the ticks as this would add to the current knowledge of the ecology and epidemiology of the tick-borne pathogens and diseases, and serve as an indicator for the likelihood of disease occurrence.

The present study, therefore, aimed to determine the tick species composition amongst cattle, as well as identify and characterise the various protozoan and rickettsial pathogens present in ticks collected from cattle in two districts of Karamoja Region in northeastern Uganda.

2. Materials and Methods

2.1 Ethics statement

Ethical approval was obtained from the Animal Ethics Committee of the University of Pretoria (V094-17). Approval was also provided by the Karamoja Livestock Development Forum (KLDF) (KLDF/P_Akure/2017) with regards to the technical and ethical aspects of conducting research in the Karamoja Region. Standard techniques were followed during the collection of ticks. Owners of the selected cattle herds were informed of the study and they provided informed verbal consent prior to the collection of ticks.

2.2 Study area

Karamoja Region is located in northeastern Uganda, and is bordered by South Sudan to the north and Kenya to the east (Figure 1). The region occupies almost 10% (27,511 km^2) of Uganda's land area, and

is divided into seven districts (as of June 2018): Kaabong, Kotido, Abim, Moroto, Napak, Amudat and Nakapiripirit (Cullis, 2018). The estimated human population is 1,059,300 (UBOS, 2017). The region is semi-arid, characterised by cycles of drought. Unlike most of Uganda, with two distinct rainy seasons, Karamoja has historically had a single rainy period from April through mid-September as it transitions into the dry season. Annual average rainfall ranges between 300 mm in the pastoral regions to 1200 mm in western areas of Abim and Nakapiripirit. Temperatures can be as high as 40°C, but the average is about 29°C in the afternoon and 17°C at night. Average annual temperatures range from 16°C in the highlands to 24°C in the rest of the region (Kara-Tunga Arts & Tours, 2019).

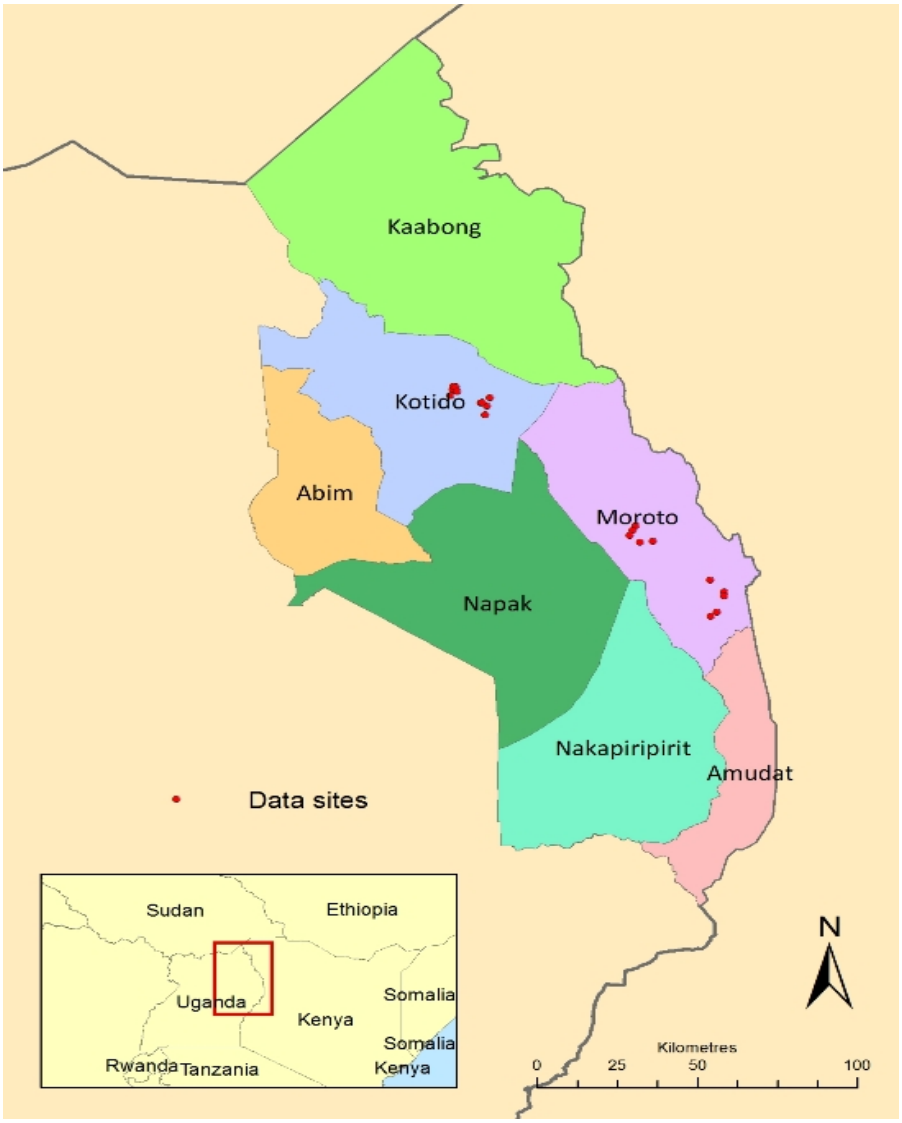

Figure 1. Map of Karamoja Region showing the sites (shown as red dots) where ticks were collected from cattle for the detection of haemoparasites. Inset is the map of Uganda showing the location of Karamoja Region and the neighbouring countries in the African continent.

2.3 Study design and tick collection

This cross-sectional study was conducted from July 2017 through September 2017. Twenty villages (manyattas) were randomly selected from two districts, Moroto and Kotido, which were purposively selected to represent the pastoral and agro-pastoral zones respectively. The selection of manyattas was done from a sampling frame generated on the basis of accessibility and information about cattle

keeping from the community leaders and extension workers. In each manyatta, five cattle that were highly infested with ticks were purposively selected. In total, 100 cattle were sampled for ticks in 20 villages. All sampled cattle were of the Short horned East African Zebu breed, and were apparently healthy by physical examination. Most cattle (69%, n=69) were three years of age or older, while a few (31%, n=31) were one to two years of age. Female cattle dominated (86%, n=86) compared to males (14%, n=14). The cattle were manually restrained in open areas by herdsmen and the researchers, followed by visual examination and palpation of the hair coat or skin around the common predilection sites (udder, tail switch, around anus, belly, perineum, neck, sternum, dewlap, axillae, ears, ventral surface of body, flanks) for ticks. About half of the detected ticks from each site were collected using blunt forceps or hands. The ticks were preserved in 70% ethanol prior to identification using a stereoscopic microscope, employing morphological keys described by Walker et al. (2013). Tick identification was done at the Parasitology laboratory of the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa.

2.4 DNA extraction

Adult and nymphal ticks were rinsed three times with sterile phosphate-buffered saline to remove residual ethanol, then air-dried and grouped in 40 pools (20 pools per district) of 2 to 10 ticks each, according to species and location. The ticks were placed into sterile tubes with MagNA Lyser Green Beads (Roche Diagnostics, Mannheim, Germany) and then homogenised. Genomic DNA was extracted from the tick pools using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The DNA was stored at -20°C until further analysis.

2.5 Reverse line blot (RLB) hybridisation assay

Two separate sets of polymerase chain reactions (PCRs) were performed; one for the amplification of the V4 hypervariable region of *Babesia* and *Theileria* 18S rRNA gene using primers RLB F2 and RLB R2 (S1 Table) (Nijhof et al., 2003, 2005), and the other set for amplification of the V1 hypervariable region of *Anaplasma* and *Ehrlichia* 16S rRNA gene using primers Ehr-F (Schouls et al., 1999) and Ehr-R (Bekker et al., 2002) (S1 Table). The PCR was conducted using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa) in a touchdown thermal cycling program described by Nijhof et al., (2005), followed by hybridisation of the PCR amplicons with five *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* genus-specifc probes and 38 species-specific probes on an RLB membrane (Bekker et al., 2002; Gubbels et al., 1999; Nijhof et al., 2003, 2005). The oligonucleotide probes included in this study are shown in S2 Table. DNA extracted from *B*. *bovis* and *A*. *centrale* live blood vaccines (Onderstepoort Biological Products [OBP], Pretoria, South Africa) were included as positive controls for the 18S rRNA and 16S rRNA amplification, respectively, while nuclease-free water was used as the negative control.

2.6 pCS20 Sol1^{TqM} quantitative real-time PCR (qPCR) for E. ruminantium

Due to lack of data on *E*. *ruminantium* sequence variation in Uganda, and high infestation of cattle with *Amblyomma* spp., the pathogen was selected for further study, first by detection and quantification using a more sensitive *pCS20* qPCR assay. All the 40 tick samples were analysed using a *pCS20* Sol1^{TqM} qPCR assay, using primers Sol1F [ACA AAT CTG GYC CAG ATC AC] and Sol1R [CAG CTT TCT GTT CAG CTA GT] and the probe Sol1 [6-FAM-ATC AAT TCA CAT GAA ACA TTA CATG CAA CTG G-QSY] as previously described (Cangi et al., 2017). The qPCR was performed on a StepOnePlus™ Real-Time PCR System

(Applied Biosystems, Life Technologies, Johannesburg, South Africa) using TaqMan® Universal PCR Master Mix (Applied Biosystems, Life Technologies, Johannesburg, South Africa). PCR-grade water was used as a negative control, while DNA extracted from *E. ruminantium* live blood vaccine strain from OBP, Pretoria, South Africa, was included to serve as a positive control.

2.7 Characterisation of tick-borne pathogens

2.7.1 PCR amplification of pCS20, p67 and 16S rRNA genes

All positive samples from the *E. ruminantium pCS20* qPCR were selected for amplification of the 279 bp fragment of the *pCS20* region of *E. ruminantium* in a semi-nested PCR previously described by Farougou et al. (2012), using primers Ehr *pCS20* F [Outer/Nested primer] [5'-ACT AGT AGA AAT TGC ACA ATC YAT-3'], Ehr *pCS20* R1 [Outer primer] [5'-RCT DGC WGC TTT YTG TTC AGC TAK-3'] and Ehr *pCS20* R2 [Nested primer] [5'-TGA TAA CTT GGW GCR RGD ART CCT T-3']. The variable region of the gene that encodes the p67 sporozoite protein (approximately 900 bp) was amplified from five *T. parva* RLB-positive tick pools using primers IL 613 [5'-ACA AAC ACA ATC CCA AGT TC-3'] and IL 792 [5'-CCT TTA CTA CGT TGG CG-3'] as described by Nene et al. (1996). A 498 bp fragment of the 16S rRNA gene of five samples that showed *Anaplasma*/*Ehrlichia* genus-specific signals, without signals with species-specific probes during RLB analysis, were amplified using primers Ehr-F (Schouls et al., 1999) and Ehr-R (S1 Table) (Bekker et al., 2002).

PCR was performed using Phusion™ Flash High-Fidelity PCR Master Mix (Thermo Scientific™, Randburg, South Africa). The positive control for the *pCS20* PCR was as indicated in section 2.6 above, while positive controls for the p67 and 16S rRNA PCRs were a *T. parva* positive sample (KNP102) from

African buffalo from South Africa (Sibeko et al., 2008) and DNA extracted from *A*. *centrale* live blood vaccine strain, respectively. PCR-grade water was used as negative control. PCR amplicons were analysed on 2.0% agarose gels stained with ethidium bromide.

2.7.2 Cloning and sequencing of PCR products

Amplicons from samples with good gel electrophoresis signals and of expected size (~279 bp for *E*. *ruminantium pCS20* [four samples]; ~900 bp for *T*. *parva* p67 [three samples], and ~ 498 bp for the 16S rRNA [five samples]) were purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany), cloned into the pJET vector (CloneJET® PCR Cloning Kit, Thermo Scientific™, Randburg, South Africa) and used to transform JM109 Competent Cells (Promega, Madison, USA). Up to 10 colonies per sample were screened by colony PCR using vector primers pJET 1.2F [5'-CGA CTC ACT ATA GGG AGA GCG GC-3'] and pJET 1.2R [5'-GAA GAA CAT CGA TTT TCC ATG GCA G-3'] as described in Byaruhanga et al. (2018). Subsequent plasmids with inserts of the correct size were sequenced at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa), with pJET 1.2F and pJET 1.2R primers.

2.7.3 Sequence and phylogenetic analyses

Sequences obtained from the *pCS20* region, p67 gene and 16S rRNA gene clones were subjected to Basic Local Alignment Search Tool [BLAST] (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with published sequences from GenBank employing the Multiple Alignment using Fast Fourier Transform (MAFFT) version 7.0 (Katoh and Standley, 2013). Sequence identities (% and number of nucleotides) were calculated by pairwise comparison using Molecular Evolutionary Genetics Analysis [MEGA] version 7.0 (Kumar et al., 2016). Before translation to predicted protein sequences, the p67 nucleotide

sequences were trimmed to 497 bp, removing a portion of the non-protein coding region (Nene et al., 1996). The p67 sequences were then classified by allele type based on the criteria proposed by Sibeko et al. (2010).

The jModelTest version 2.1.3 was used to select the best-fit nucleotide substitution models, TIM3 + G for the *pCS20* sequences and HKY for the 16S rRNA sequences, under the Akaike Information Criterion (AIC) as the model selection criterion (Darriba et al., 2012). Phylogenetic trees for the *pCS20* gene region and 16S rRNA gene were reconstructed using maximum likelihood (ML) as implemented in PhyML 3.1 (Guindon et al., 2010), parsimony in MEGA7 (Kumar et al., 2016) and Bayesian inference (BI) using MrBayes version 3.2 (Ronquist et al., 2012). The reliability for the internal branches for ML and parsimony methods was assessed using bootstrapping [1000 bootstrap replicates] (Felsenstein, 1985). Bayesian phylogenetic inference was achieved using Markov chain Monte Carlo (MCMC) analysis to calculate posterior probabilities for 1,000,000 generations, sampled every 1000 generations. Graphical representation and editing of the phylogenetic trees were performed with MEGA7 and Paint Tool for Windows 10.0. The topologies of the trees produced from BI, parsimony and ML analyses were compared.

2.7.4 Nucleotide sequence accession numbers

Sequences obtained in this study were deposited in GenBank database under the following accession numbers: ranging from **MK371026** to **MK371033** (*E. ruminantium pCS20*); from **MK673337** to **MK673339** (*T. parva* p67); and ranging from **MK656386** to **MK656389** (16S rRNA gene).

3. Results

3.1 Tick identification

A total of 4,897 adult ixodid ticks and 67 immatures (17 larvae and 50 nymphs) were collected from 100 cattle from the two districts of the Karamoja Region. From the adult ticks collected, 2,460 were females and 2,437 were males. All 50 nymphs were from the genus *Amblyomma*, while the larvae were not identified. About 80% of the female ticks were engorged. Three genera [*Amblyomma* (96.8%), *Rhipicephalus* (2.6%), *Hyalomma* (0.6%)] and 12 species of ticks were identified (Table 1). The most abundant tick species collected was *A*. *lepidum* (93.9%), followed by *A*. *variegatum* (2.0%). A few *R*. *appendiculatus* ticks, the vector for *T*. *parva,* were collected (0.1%), while the proportion of *R. decoloratus* ticks, the vector for *A*. *marginale* and *B*. *bigemina* was only 0.7%.

3.2 Detection of haemoparasites in ticks by RLB hybridisation

The number and percentage of tick pools found positive for various tick-borne pathogens are shown in Table 1. DNA of 10 species of tick-borne pathogens from the genera *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* were detected from the ticks. Of the 40 tick pools, 37 (93%) were positive for pathogens. Most tick pools (n=17) were infected with a single pathogen species, while 11 were infected with two pathogen species and two were infected with three pathogen species. No species-specific tick-borne pathogen DNA was detected in *R*. *appendiculatus* and *Rhipicephalus turanicus*. Some tick pools showed only genus-specific signals without the corresponding species-specific signal during RLB analysis, i.e., *Anaplasma*/*Ehrlichia* genus-specific in *Rhipicephalus* spp. (n=12), *Amblyomma* spp. (n=9) and *Hyalomma* spp. (n=5); *Babesia*/*Theileria* genus-specific in *Amblyomma* spp. (n=2); *Theileria* genus-

Table 1. Infection rates of ixodid ticks collected from cattle from Karamoja Region in Uganda with protozoal and rickettsial pathogens using reverse line blot hybridisation assay (from July through September 2017)

			Number tick pools positive for tick-borne infections									
Tick species	Ticks collected (%)	No. of tick pools examined	*Ehrlichia ruminantium*	*Anaplasma centrale*	*Babesia bigemina*	*Babesia microti*	*Babesia rossi*	*Theileria* sp. (sable)	*Theileria mutans*	*Theileria separata*	*Theileria velifera*	*Theileria parva*
R. appendiculatus	5 (0.1)	2	0	0	0	0	0	0	0	0	0	0
R. evertsi evertsi	51 (1.0)	4	0	0	0	1	1	1	2	1	1	0
R. pravus	13 (0.3)	2	0	0	0	1	0	0	0	0	0	1
R. praetextatus	4 (0.08)	2	0	0	0	0	0	0	0	0	0	1
R. pulchellus	18 (0.4)	4	0	1	0	0	0	0	2	0	2	1
R. turanicus	1 (0.02)	1	0	0	0	0	0	0	0	0	0	0
A. lepidum	4596 (93.9)	4	1	0	1	1	0	0	1	0	0	1
A. gemma	48 (0.9)	5	1	0	0	0	0	1	0	0	1	2
A. variegatum	98 (2.0)	4	1	0	0	1	0	1	1	0	1	1
R. decoloratus	33 (0.7)	5	0	0	0	0	0	0	4	0	2	2
H. truncatum	24 (0.5)	4	0	0	0	0	0	1	0	0	2	1
H. rufipes	6 (0.1)	3	0	0	0	0	0	1	0	0	1	0
		Total no. of positive pools (%)	3 (7.5)	1 (2.5)	1 (2.5)	4 (10.0)	1 (2.5)	5 (12.5)	10 (25.0)	1 (2.5)	10 (25.0)	10 (25.0)

Total number of tick pools examined = 40. *R*, *Rhipicephalus*; *A*, *Amblyomma*; *H*, *Hyalomma*

specific in *Amblyomma* spp. (n=2) and *Rhipicephalus* spp. (n=3); and *Babesia* genus-specific in *Rhipicephalus* spp. (n=6), *Amblyomma* spp. (n=3) and *Hyalomma* spp. (n=3).

Theileria mutans, *T*. *velifera* and *T*. *parva* were the most frequently detected pathogens, each detected in 10 of the 40 tick pools (25%). This was followed by *Theileria* sp. (sable) (5 pools) and *Babesia microti* (4 pools) [Table 1]. *Anaplasma centrale* was detected in only one pool of *R. pulchellus*, while *E. ruminantium* was detected in three pools, one from each of the tick species *A*. *gemma, A*. *lepidum* and *A*. *variegatum. Babesia bigemina* was detected in one pool of *A*. *lepidum.* Tick-borne pathogens, namely *Babesia rossi*, *B*. *microti* and *Theileria* sp. (sable) that are not common to, or not known to infect, cattle were identified from ticks in this study.

3.3 *E. ruminantium pCS20* Sol1^{TqM} qPCR results

Out of the 40 tick pools, 8 (20%) were positive for *E*. *ruminantium* using qPCR, with mean quantification cycle (C_q) of 32.04 (range 16.28 to 38.46). The positive pools were from *A*. *lepidum* (n=1), *A*. *gemma* (n=3), *A*. *variegatum* (n=3) and *H*. *truncatum* (n=1). Only three of these tick pools had been detected positive for *E*. *ruminantium* using RLB hybridisation assay (Table 1).

3.4 *E. ruminantium* amplicon and sequence analysis

Eight samples that were positive for *E*. *ruminantium* using qPCR were subjected to *pCS20* PCR, and amplicons of ~279 bp were obtained from four samples, one from each of the tick species *A*. *lepidum*, *A*. *gemma*, *A*. *variegatum* and *H*. *truncatum*. *Ehrlichia ruminantium* sequences (~279 bp) were identified by BLASTn homology search from 19 clones from the four samples. Some sequences showed

variation, even from within the same sample, while others were identical. Therefore, only eight representative sequences of each unique sequence were submitted to GenBank (Accession numbers: MK371026 to MK371033) and included for further analysis.

Sequence comparison demonstrated that the *E*. *ruminantium pCS20* sequences obtained herein varied from each other by 1 to 10 nucleotides. Seven sequences were novel, of which five (MK371026, MK371027, MK371028, MK371029, MK371030) were 98 to 99% identical (3 to 5 nucleotide difference, 95 to 100% query cover) with sequences identified from: *E*. *ruminantium* from sheep from South Africa (DQ631926) and Kenya (MG544305), cattle from Mozambique (KX373600), and the genome of *E*. *ruminantium* Welgevonden strain (CR925678). The other two novel *pCS20* variants identified herein (MK371032, MK371033) presented 98 to 99% identity (1 to 4 nucleotide differences, query cover 82 to 100%) with *E*. *ruminantium pCS20* sequences from goats from Sudan (MG383909), from *A*. *variegatum* (JQ039915) from Cameroon, and the *E*. *ruminantium* Sankat strain from Ghana (AY236065).

3.5 *Theileria parva* p67 sequence analysis

A previous study in Karamoja Region revealed high morbidity due to East Coast fever (ECF) (Byaruhanga et al., 2015b), and given a relatively high proportion of tick samples (25%) that were positive for *T*. *parva* using RLB in the present study, the gene encoding the *T*. *parva* p67 sporozoite protein was sequenced to identify the allele types and assess any variants. Single amplicons (~900 bp) were obtained from three of the five tick samples whose p67 genes were amplified. A total of 12 p67 sequences were obtained from the three samples, with four readable sequences from each sample. The alignment of the p67 predicted protein sequences together with published sequences is shown in

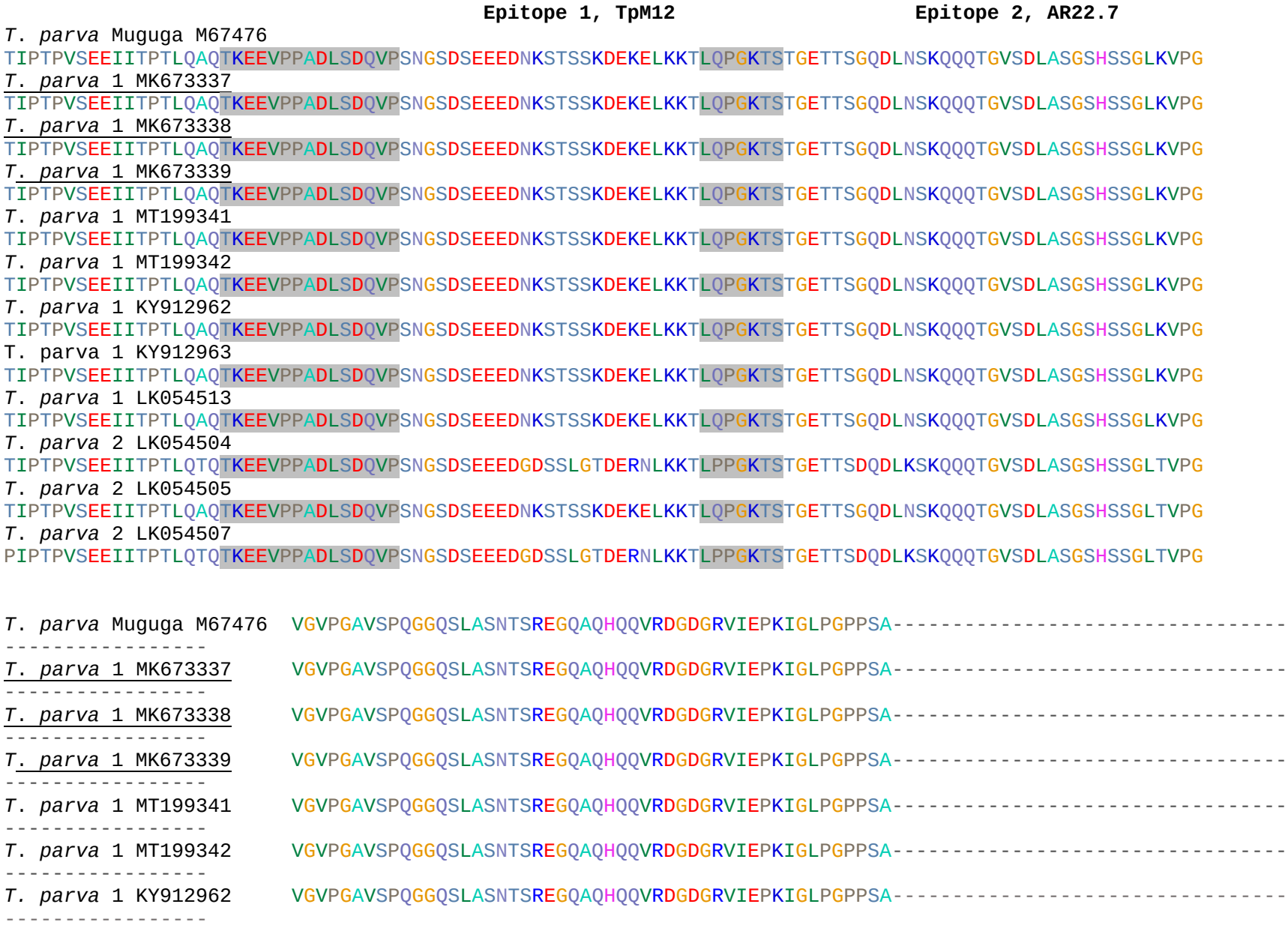
Epitope 1, TpM12
Epitope 2, AR22.7
T. parva Muguga M67476
T. parva 1 MK673337
T. parva 1 MK673338
T. parva 1 MK673339
T. parva 1 MT199341
T. parva 1 MT199342
T. parva 1 KY912962
T. parva 1 KY912963
T. parva 1 LK054513
T. parva 2 LK054504
T. parva 2 LK054505
T. parva 2 LK054507

Figure 2. Alignment of the predicted protein sequences of *Theileria parva* p67 allele types 1 and 2 in the ~497-bp region. Sequences obtained in this study, corresponding to allele type 1, are underlined. The accession number of each sequence is indicated next to the sequence name. Sequences from each sample were identical, and therefore only one representative sequence was included in the analysis. The B-cell epitopes, TKEEVPPADLSDQVP and LQPGKTS, are highlighted in grey.

T. parva 1 and *T. parva* 2 refer to allele types 1 and 2, respectively, as previously described (Nene et al., 1996; Sibeko et al., 2010).

Figure 2. The sequences obtained in this study are classified as p67 allele type 1 (Figure 2), based on the absence of a 129-bp insert (Nene et al., 1996; Sibeko et al., 2010), and in accordance with the ~900 bp single amplicons detected during PCR analysis. The predicted p67 protein sequences obtained in this study were identical to the cattle-derived Muguga isolate sequence (M67476) (Nene et al., 1992), and allele type 1 sequences detected in cattle-derived *T. parva* parasites from cattle from Karamoja (MT199341) and western (MT199342) regions in Uganda (Mukolwe et al., 2020), and in buffalo-derived *T*. *parva* parasites from buffalo (KY912963) and cattle (KY912962) from Kenya (Sitt et al., 2019). The TpM12 (TKEEVPPADLSDQVP) and AR22.7 (LQPGKTS) B-cell epitopes were identical to the reference sequences (Figure 2).

3.6 16S rRNA amplicon and sequence analysis

A relatively high proportion of samples (65%, 26/40) displayed *Anaplasma*/*Ehrlichia* genus-specific signals without species-specific signals during RLB analysis, and therefore the V1 hypervariable region of the 16S rRNA gene was amplified, cloned and sequenced from five randomly selected samples. This was to further investigate if this was due to presence of insufficient amplicons in the samples to give a species-specific signal, or occurrence of novel species or variants of known *Anaplasma*/*Ehrlichia* species, which could not be detected by the RLB assay. Four *Ehrlichia*/*Rickettsia* 16S rRNA gene sequences were obtained from three samples, and these were closely related to *Rickettsia africae* and *Ehrlichia chaffeensis*. Three of the sequences (GenBank accession nos. MK656386, MK656387, MK656388) from two samples from *A*. *gemma* were 98.7% identical with the 16S rRNA sequences of *R*. *africae* (JF949786), previously identified from *A*. *variegatum* from cattle from Nigeria, and were 99.8% identical with *Rickettsia* sp. (KP823595) from *Haemaphysalis elliptica* from a domestic dog from South

Africa. The fourth sequence (MK656389) (Figure 3) was 98.0% identical with *E*. *chaffeensis* type strain Arkansas from the United States of America and 98.9% identical with *Ehrlichia* sp. Tibet (AF414399) from *R*. *microplus* from China. The new *Ehrlichia* sp. sequence showed seven point mutations in the oligonucleotide probe region of *E*. *chaffeensis* (over a length of 24 bp), as shown in Figure 3. Other 16S rRNA sequences obtained correspond to distantly related bacteria, and these were not analysed.

Figure 3. Nucleotide alignment of the reverse line blot hybridisation (RLB) probe region of *Ehrlichia* sp. 16 rRNA gene sequence (underlined) determined in this study from cattle from Karamoja Region in Uganda with previously published *E. chaffeensis* and *Ehrlichia* sp. 16S rRNA gene sequences. The position of the *E*. *chaffeensis* RLB oligonucleotide probe is shown on the top of the alignment, followed by published sequences of *Ehrlichia* sp. and *E*. *chaffeensis*. The accession number of each sequence is indicated next to the species name. Nucleotide differences are shown in black letters on a white background.

3.7 Phylogenetic analysis

The topology of the phylogenetic trees generated, employing ML, MP and BI methods were similar; hence, only the ML trees are presented here (Figures 4 and 5). The newly-generated *E*. *ruminantium pCS20* nucleotide sequences (279 bp) grouped in two clusters (Figure 4), one cluster with previously reported isolates from cattle, ticks and sheep from Asia and all regions in Africa [clade 1] (82% bootstrap support, 0.7 posterior probability), and the second cluster with isolates from western, northern and southern Africa [clade 2] (84% bootstrap support, 0.74 posterior probability).

A maximum likelihood tree of the 16S rRNA sequences is shown in Figure 5. The newly-generated *R. africae* 16S rRNA sequences grouped with *R. africae* isolates from Nigeria, Egypt and Benin (clade 2) (Figure 5).

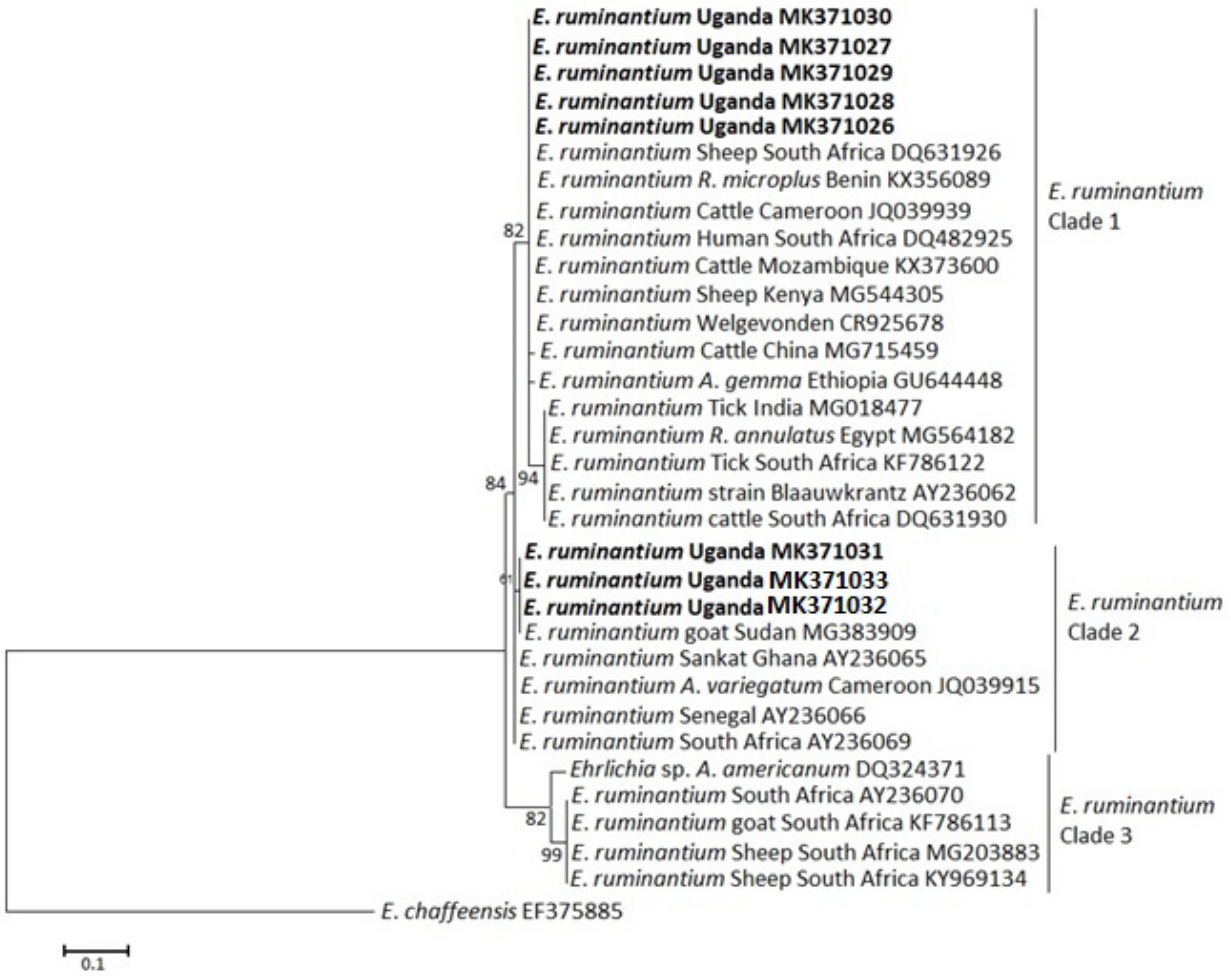

Figure 4. A maximum likelihood tree showing the phylogenetic relationship between the *Ehrlichia ruminantium pCS20* sequences identified in ticks that were collected from cattle in Karamoja Region in Uganda, with previously published sequences. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. Some sequences obtained in this study were identical to each other; therefore, only representatives of sequences are shown (in bold). The tree was rooted using the sequences of *Ehrlichia chaffeensis*. The accession number of each sequence is indicated next to the sequence name. Branch lengths are proportional to the estimated genetic distance (number of nucleotide substitutions per site over a length of 279 bp of the *pCS20* region) between the taxa.

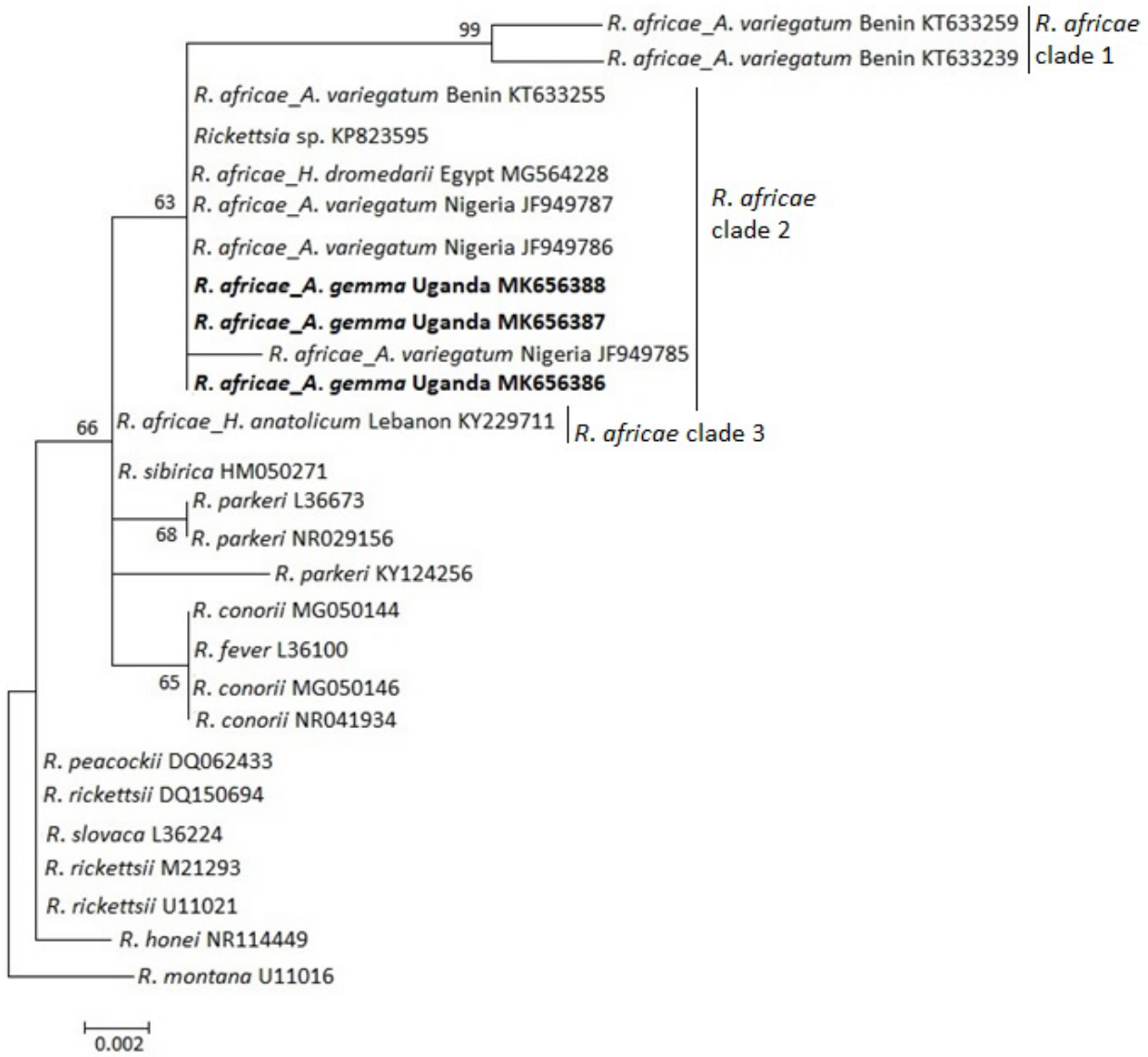

Figure 5. A maximum likelihood tree showing the phylogenetic relationship between the *Rickettsia africae* 16S rRNA sequences, identified in tick samples from cattle from Karamoja Region, Uganda (in bold) with reference sequences. The numbers at the internal nodes represent the percentage of 1000 replicates (bootstrap) for which the same branching patterns were obtained. The tree was rooted using 16S rRNA sequences of *Rickettsia montana*. The accession number of each sequence is indicated next to the sequence name. Branch lengths are proportional to the estimated genetic distance (number of nucleic acid substitutions per site over a length of 423 positions of the 16S rRNA gene) between the taxa.

4. Discussion

We found a higher tick species diversity amongst cattle in Karamoja than other parts of Uganda. Tick species *A. variegatum, R. appendiculatus, R*. *decoloratus* and *R. evertsi evertsi* were also reported in other parts of Uganda (Balinandi et al., 2020; Magona et al., 2011; Muhanguzi et al., 2020; Nakayima et al., 2014; Rubaire-Akiiki et al., 2006). Additionally, *A*. *lepidum*, *H*. *truncatum*, *A*. *gemma*, *R*. *pulchellus* (Byaruhanga et al., 2015a) and *H*. *rufipes* (Balinandi et al., 2020) were found on cattle in recent studies in Karamoja. *Rhipicephalus pravus*, *Rhipicephalus praetextatus* and *R. turanicus* are reported for the first time in Karamoja, and have not been reported in recent studies in Uganda. The finding of unique tick species in Karamoja can be explained by non-uniformity of Uganda's climate across regions; therefore, some tick species may not reproduce the whole year in some parts of the country. It is also possible that the newly reported ticks were spread by cattle from the neighbouring Turkana and West Pokot counties in Kenya, and Eastern Equatoria State in South Sudan, as a result of the transhumant herding system in Karamoja, coupled with communal grazing and sharing of water points.

Given their vectorial capacity, the presence of these tick species in Karamoja can increase the risk of transmission of tick-borne pathogens and incidence of diseases in livestock and human populations. *Rhipicephalus evertsi evertsi* transmits *A*. *marginale*, the cause of bovine anaplasmosis, and can also release toxins that cause paralysis in cattle and sheep (Walker et al., 2013). *Rhipicephalus pulchellus* transmits *T*. *taurotragi*, the cause of benign bovine theileriosis, and *R*. *conorii* that causes tick typhus in humans. The other tick species, *H*. *rufipes*, transmits *A*. *marginale* and *Babesia occultans* in cattle and *R*. *conorii* in humans (Walker et al., 2013), while

H. *truncatum* transmits *R*. *conorii* in humans and can release toxins that cause sweating sickness in cattle. Attachment of adult *H*. *truncatum* to the interdigital clefts and fetlocks can cause lameness, and the long mouth parts cause skin damage in cattle and sheep, which results in secondary bacterial infections and myiasis (Walker et al., 2013). Little is known about the association of *R*. *pravus* with disease; however, *R*. *praetextatus* can transmit *Rickettsia conorii* to humans. *Rhipicephalus praetextatus* also transmits the virus of Nairobi sheep disease, and the feeding ticks can cause toxicosis in cattle, resulting in paralysis (Walker et al., 2013). Although cattle are known hosts of *R*. *turanicus*, the tick often heavily infests sheep, therefore co-grazing with cattle can lead to spread from sheep to cattle. The vectorial role of *R*. *turanicus* for any pathogen in Africa is not known (Walker et al., 2013).

Eight of the 10 tick species collected in this study had comparable distribution between Moroto and Kotido Districts, which may imply wide distribution in Karamoja and the neighbouring countries. However, *R*. *turanicus* was only found in Kotido (one animal), while *R. pravus* was only found in Moroto (eight animals). This spatial difference can be explained by the difference in grazing dynamics between herds in the two districts and the herds from cross-border communities, which may be infested with unique tick species. In Kotido and Moroto, cattle often mix with herds from Turkana and West Pokot counties respectively.

The diversity of tick-borne pathogens observed in this study (10 species) is higher than that reported by Nakayima et al. (2014) in eastern Uganda, where only five species – *B*. *bovis*, *R*. *africae*, *Coxiella burnetti*, *A*. *bovis* and *A*. *phagocytophilum* – were detected, using species-

specific PCR and cytochrome C oxidase subunit 1 gene sequencing. Higher pathogen diversity in the present study could be associated with the relatively high tick species diversity (n=12), compared to only four species found by Nakayima et al. (2014). High tick species diversity may increase the risk of transmission and occurrence of various pathogen species.

Theileria mutans, *T*. *velifera* and *T*. *parva* were the most frequently detected tick-borne pathogens in the tick species, each found in 25% of the 40 tick pools examined. Apart from *A*. *lepidum* and *A*. *variegatum*, which are known vectors of *T*. *mutans* and *T*. *velifera*, DNA of the two organisms was also detected in *Rhipicephalus* and *Hyalomma* spp. *Theileria mutans* infection leads to mild clinical signs, however, pathogenic strains in eastern Africa cause severe anemia, icterus and sometimes death (Moll et al., 1986; Oura et al., 2004). Although *T*. *parva* was detected in tick species that are not its biological vectors, this may contribute to sustained circulation of the pathogen in the cattle population through the tick blood meal. Of the 10 samples found positive for *T*. *parva*, nine were from Moroto and only one was from Kotido. Our findings are in congruence with the higher *T*. *parva* seropositivity reported in Moroto (24.5%) than Kotido (4.8%) (Byaruhanga et al., 2015a), and this can be attributed to the variation in grazing dynamics and animal movements, which results in different rates of exposure to *T*. *parva* amongst the cattle. The distribution of other haemoparasites was comparable between the two districts.

Babesia microti was found in four tick pools (10%) from *R*. *evertsi evertsi*, *R*. *pravus*, *A*. *lepidum* and *A*. *variegatum*. The pathogen is a zoonotic intra-erythrocytic parasite, and is the most common species that causes human babesiosis, an emerging malaria-like illness. *Babesia*

microti is expanding into new areas and may be unfamiliar to clinicians in locations not primarily considered endemic (Westblade et al., 2017). *Babesia microti* is transmitted by *Ixodes scapularis* as the definitive host and mice as intermediate hosts. However, there are no reports of clinical cases in humans associated with *B*. *microti* in Uganda.

Babesia rossi was detected from one tick pool (2.5%) derived from *R. evertsi evertsi*. The pathogen is the main cause of babesiosis in dogs in sub-Saharan Africa, and *Haemaphysalis elliptica* (long considered to be *Ha*. *leachi*) is its only known vector (Penzhorn, 2011). Elsewhere in Uganda, *B*. *rossi* was identified in three dogs (2.9%) and in one tick pool derived from *Ha*. *elliptica* from around three conservation areas in the southwestern part of the country (Proboste et al., 2015). Although unlikely, it is possible that ticks that fed on dogs in the previous life cycle stage, may be infected when they feed on cattle in the adult stage. Indigenous African canids such as jackals and wild dogs also exist in the grazing areas in Karamoja, and these are known to harbour *B*. *rossi* without showing untoward effects (Penzhorn, 2011).

Theileria sp. (sable) was detected in five pools (12.5%) derived from *R*. *evertsi evertsi*, *A*. *gemma*, *A*. *variegatum*, *H*. *truncatum* and *H*. *rufipes*. The parasite was originally isolated from a sable antelope (Stoltsz and Dunsterville, 1992, cited by Nijhof et al., 2005), and mortalities associated with the parasite were reported in four species of African antelopes (Nijhof et al., 2005). *Theileria* sp. (sable) was also found in clinically healthy wild animals (African buffalo, Blesbok, Blue wildebeest, Klipspringer and Reedbuck) from various regions in South Africa and

Eswatini in southern Africa (Nijhof et al., 2005) and clinically healthy cattle from Tanzania (Nijhof et al., 2005), Zambia (Tembo et al., 2018), Kenya (Njiiri et al., 2015) and Uganda (Muhanguzi et al., 2010b).

The detection of pathogens from tick species that are not known vectors could be as a result of the presence of the pathogens in the blood meal, given that the ticks identified in this study were collected whilst attached and feeding on the animal host. There is also a possibility that male ticks of other species transfer the pathogens in their blood meals, as they move between hosts, especially if the interval between feeding is only a few hours. The detection of pathogens that are not common, or not known to infect cattle (*Theileria* sp. [sable], *B*. *microti* and *B*. *rossi*) can be attributed to incidental infections or due to cross-reactions with other pathogens during RLB analysis, and presence of variants of different pathogens amongst animals. The design of most of the current RLB probes has not taken into account all pathogen variants or closely related species (Pienaar et al., 2019), and this can lead to false detection of pathogens, due to similarity in RLB probes and the likelihood of cross-hybridisation between species (Pienaar et al., 2019). For example, the oligonucleotide probes for *Theileria* sp. (sable) and *T*. *velifera* currently used in the RLB assay differ in only three nucleotide positions (Mans et al., 2015), and therefore the reported occurrence of *Theileria* sp. (sable) from various studies (Nijhof et al., 2005; Tembo et al., 2018; Yusufmia et al., 2010) can be attributed to the similarity level that results in cross-hybridisation with *T*. *velifera*. Recent studies on *Theileria* sp. (sable) and *Theileria* sp. (sable-like) – using next generation sequencing (Mans et al., 2016) and qPCR (Pienaar et al., 2019), in which the parasites were not detected in cattle or buffalo – almost confirm that cross-hybridisation leads to erroneous detection of *Theileria* sp. (sable) (Pienaar et

al., 2019). There is therefore an urgent need to validate the RLB assay for some of the tick-borne pathogen species, including *Theileria* sp. (sable), *B*. *microti* and *B*. *rossi*. However, the findings may also suggest that cattle in the study area may form a natural reservoir of *Theileria* sp. (sable). This could then be transmitted by the potential tick vectors, *R*. *evertsi evertsi* and *R*. *appendiculatus* (Steyl et al., 2012), to roan antelope in the Karamoja rangelands, to cause theileriosis in these antelopes. Further studies are also required to further investigate and confirm the occurrence of uncommon pathogens circulating in Karamoja, and the extent and role of infections, such as *B*. *microti*, in the human population.

Theileria separata was found in one tick pool from *R*. *evertsi evertsi*, which is the right vector of the pathogen. The pathogen has been mainly identified in small or medium sized wild and domestic ruminants – causing ovine theileriosis in sheep and goats (Schnittger et al., 2003), and has been reported in several countries in southern and eastern Africa – transmitted by *Rhipicephalus* spp. (Morzaria, 1998).

B. *bigemina* was detected in only one pool; possibly because of the small number of *R*. *decoloratus* ticks (five pools from 33 ticks) analysed.

More than half (54%, 7/13) of the tick pools from *Amblyomma* spp. (*A*. *gemma*, *A*. *lepidum* and *A*. *variegatum*) were found positive for *E*. *ruminantium* using the species-specific *pCS20* qPCR assay. The high infestation of cattle with *E*. *ruminantium*-infected *Amblyomma* ticks, which are biological vectors of the pathogen, highlights the risk of heartwater disease for the susceptible

ruminant population in Karamoja. Therefore, one would expect a relatively high number of clinical cases of heartwater amongst cattle. Indeed, a recent participatory study with the pastoralists revealed an annual incidence score (relative to other diseases) of up to 10% for heartwater, and case fatality rates of up to 100% (Byaruhanga et al., 2015b), and the disease was ranked as one of the five most important diseases that affect cattle. On the other hand, if a good proportion of cattle are exposed, there is a likelihood of endemic stability, and thus few cases of heartwater. However, to maintain endemic stability, continuous exposure of the animals is required to maintain immunity. The period of exposure of *E*. *ruminantium* in cattle for immunity to develop is usually very short (about 6 weeks), and therefore animals that are not exposed remain susceptible, resulting in heartwater clinical cases.

Comparison of the *E*. *ruminantium pCS20* sequences showed that they are diverse. Phylogenetic analysis revealed two clusters of *E*. *ruminantium* strains from Karamoja, and each group was closely related to strains from other parts of Africa and Asia. The *E*. *ruminantium* sequence variation is in agreement with previous studies in South Africa (Ringo et al., 2018; Steyn et al., 2003). Recently, Lee et al. (2018) demonstrated three divergent *E*. *ruminantium* clusters based on the *pCS20* sequences from various countries and animal hosts. This *E*. *ruminantium* variation is attributed to its biological variability and the recombination that naturally occurs between different genotypes (Allsopp, 2010; Steyn et al., 2003). Therefore, new strains are continuously arising in the field. This can lead to wide variations in infectivity and pathogenicity of *E*. *ruminantium* in domestic ruminant populations in Karamoja, as has been demonstrated from other parts of the world (Allsopp et al., 2007).

Clustering of our *E*. *ruminantium* sequences is possibly in accordance with cross-border movement of cattle, which occurs in the dry season. Five variant sequences obtained from *A*. *gemma* from cattle from Tapac sub-county, which forms a border with Kenya in the northeast, grouped with sequences from sheep from Kenya (MG544305) and from *A*. *gemma* from Ethiopia (GU644448). The livestock keepers from Turkana and West Pokot counties in Kenya, and in extreme cases from Ethiopia, often cross into Uganda and share grazing areas with livestock in Karamoja, and this could explain the origin or admixture of the *E*. *ruminantium* isolates. Other three *E*. *ruminantium* sequences from *A*. *lepidum*, *A*. *variegatum* and *H*. *truncatum*, collected from Kotido in northern Karamoja, grouped with a sequence from a goat from Sudan, and this can be explained by cattle movements between the Toposa communities in South Sudan (bordering north) and Jie and Dodoth communities in Karamoja. Sequence variation of *E*. *ruminantium* observed in the present study, coupled with high infection rates of *Amblyomma* ticks, suggests that the strategy of introducing more productive exotic breeds of goats and cattle to Karamoja, as is with the Operation Wealth Creation (OWC), for cross-breeding or meat and milk production, could potentially result to increased heartwater mortalities. Therefore, the required disease control or prevention measures should be put in place.

Analysis of *T*. *parva* p67 sequences revealed the presence of allele type 1, which was identical to other published p67 sequences from *T*. *parva* isolates (Nene et al., 1996) and field parasites (Mukolwe et al., 2020). The predicted protein sequences of two p67 B-cell epitopes; TpM12 (TKEEVPPADLSDQVP) and AR22.7 (LQPGKTS), recognised by sporozoite-neutralising antibodies

(Nene et al., 1999), were identical to allele type 1 sequences from buffalo-derived *T*. *parva* parasites from eastern Africa (Mukolwe et al., 2020; Obara et al., 2015; Sitt et al., 2019). This further supports the hypothesis that there could be a subpopulation of *T*. *parva* parasites from the buffalo which infects cattle, and has adapted for cattle-to-cattle transmission in eastern Africa (reviewed in Norval et al., 1992; Nene et al., 1999). A recent study by Mukolwe et al. (2020) identified p67 allele type 1 from *T. parva* field parasites from cattle from Nakapiripirit district in Karamoja Region, which had 100% sequence identity to the Muguga isolate, other cattle-derived *T. parva* field parasites, and sequences from the current study. Therefore, it is unlikely that p67 sequences from the live vaccine components and cattle-derived *T. parva* field parasites are distinguishable. However, in comparison to the buffalo-derived *T. parva* field parasites, the contrary would be expected, as demonstrated with parasites from cattle and buffalo from South Africa (Mukolwe et al., 2020; Sibeko et al., 2010). The data about *T*. *parva* in this study adds to the knowledge of pathogen epidemiology, and may contribute to *T*. *parva* control efforts in cattle in Uganda using the current 'infection and treatment method' immunization protocol.

Sequences of *R*. *africae*, the causative agent of African tick-bite fever, were obtained from *A*. *gemma* pools. The pathogen is known to be transmitted mainly by *A*. *variegatum* ticks in western, central and eastern Africa, as well as in the eastern Caribbean and islands in the Indian Ocean (Socolovschi et al., 2009). In the present study, *R*. *africae* species-specific probe was not included on the RLB membrane, and this partly explains the observation of *Ehrlichia*/*Anaplasma* genus-specific signals without species-specific signals. Elsewhere in Uganda, *R*. *africae* was

identified from *A*. *variegatum* ticks collected from Zebu cattle in the districts of Kaberamaido and Dokolo (Lorusso et al., 2013) and from *A*. *variegatum* and *R*. *evertsi evertsi* in Soroti, Tororo and Amuria Districts (Nakayima et al., 2014) in eastern Uganda. Immature stages of *Amblyomma* spp. can feed on humans (Mediannikov et al., 2010), and the detection of *R*. *africae* in this study shows that people in cattle-keeping areas in Uganda are at risk of African tick-bite fever; therefore, health workers should also consider African tick-fever in the diagnosis of common illness.

This study provides information of tick-borne pathogen infection in various tick species in Karamoja Region, and serves as an indicator of potential levels and diversity of infections as well as possible transmission and disease risk amongst animal and human populations in the area and similar bioclimatic environments in the neighbouring countries of Kenya and South Sudan. This is the first study to characterise *E*. *ruminantium* in Uganda, and the first to determine tick-borne pathogen infection rates in the tick population in Karamoja, where almost 20% of the cattle population are reared.

A short coming of this study is that not all animals and tick predilection sites were examined systematically, which could have affected the number of ticks collected for some species. Moreover, tick collection was done only in the wet season, and not the whole year, and this may influence the tick species and relative abundance observed. Another limitation is that the occurrence of rickettsial and protozoan pathogens was not analysed against variables such as

breed, sex and age, because laboratory analysis was done on tick pools from groups of animals, rather than individual animals.

5. Conclusions

We determined the tick species composition amongst cattle, and detected and characterised protozoan and rickettsial pathogens in the tick species in Karamoja Region, in northeastern Uganda. The abundance of some tick species may increase the risk of infections in naïve animals or animals from non-endemic areas, but may be beneficial in maintaining endemic stability in resident animals. The findings of this study will add to our current knowledge of the ecology and epidemiology of the tick-borne pathogens in Uganda. The communities should be trained on basic diagnostic skills (e.g. common clinical signs and preparation of smears), for early diagnosis and treatment of TBDs, as well as strategic tick control to reduce tick burdens to only low level that can maintain endemic stability. Further investigation is needed on the tick-borne pathogen transmission dynamics and temporal risk for animals and humans, by including various animal species, and covering both wet and dry seasons.

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Conflict of interest

The authors declare that they have no competing interest.

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