











Original article



Ehrlichia ruminantium (Ehrlichiaeae) infection rates and genotyping in *Amblyomma* species from southern Africa

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ABSTRACT

Ticks are haematophagous ectoparasites of domestic and wild animals. With their vast geographical distribution and aptitude as vectors of a large variety of pathogens, they are ranked amongst the top two arthropod families of veterinary and medical concern. *Amblyomma*, the third largest genus in the Ixodidae, is important in southern Africa due to its vector competence for *Ehrlichia ruminantium* and other pathogens. *Ehrlichia ruminantium*, the causative agent of heartwater, a potentially lethal disease in ruminants, is classified as a notifiable disease by the World Organisation for Animal Health. *Amblyomma* species ticks were collected in five southern African countries from livestock and wildlife. They were morphologically identified to species level with taxonomic keys, and species identity was confirmed with molecular assays. Preliminary screening for *E. ruminantium* was conducted by targeting the pCS20 gene fragment. Genotyping of 39 *E. ruminantium* positives was obtained using Ampliseq technology. In total, 7,734 *Amblyomma* ticks were collected and identified as belonging to four species: *Amblyomma eburneum*, *Amblyomma hebraeum*, *Amblyomma pomposum* and *Amblyomma variegatum*. *Ehrlichia ruminantium* infection rates per country ranged from 7.1 % to 34.1 %. The genotyping analysis indicated the clustering of our sequences with strains Gardel, Welgevonden, Um Banein, Springbokfontein 4 and 2, Kwanyanga, and Blaauwkrans. The Ampliseq analysis was not effective in differentiating between strains found in southern Africa. This large study documents the genetic diversity and prevalence of *E. ruminantium* in ticks across southern Africa, highlighting implications for disease control and vaccine development.

1. Introduction

Ticks are haematophagous ectoparasites of companion, wild and livestock animals, occasionally found to parasitise humans as well (Walker and Olwage, 1987). With their vast geographical distribution and aptitude as vectors of a large variety of pathogens including

protozoa, bacteria, fungi, and viruses, they are ranked amongst the top two arthropod families of veterinary and medical concern (Walker *et al.*, 2003, Oliver Jr, 1989). *Amblyomma* is the third largest genus within the Ixodidae and has a significant impact in southern Africa due to its vector competence for *Ehrlichia ruminantium* (previously known as *Cowdria ruminantium*) (Walker, 1991).

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Ehrlichia ruminantium is a notifiable disease in the World Organisation for Animal Health (WOAH) database and causes heartwater in ruminants (Allsopp, 2010, World Organisation for Animal Health, 2021). The geographical spread of heartwater is wide, stretching over most of sub-Saharan Africa, Madagascar and the French West Indies in the Caribbean (Allsopp, 2010, Allsopp, 2015). The life cycle of *E. ruminantium* is biphasic, alternating between infectious and replicative forms. Infectious particles, termed dense core cells, are internalised by eukaryotic host cells. Inside the host cell, these dense core cells differentiate into the replicative form, known as reticulate cells, which replicate within a membrane-bound vacuole. Eventually, the reticulate cells transform back into the dense core cells, which are released from the host cell through extrusion, lysis, or other mechanisms yet to be identified. This cyclical process is essential for the pathogen's survival and propagation within its hosts (McClure et al., 2017).

Ehrlichia ruminantium is transmitted by at least 12 species of *Amblyomma* ticks and infects cattle, sheep, goats and 25 different species of wild ruminants (Allsopp, 2010). Though *E. ruminantium* can infect a wide host range, clinical disease is only observed and documented in domestic ruminants, whereas wild ruminants are considered reservoirs with asymptomatic infections (Pascucci et al., 2014). Among the 25 species of wild ruminants that can be infected with *E. ruminantium*, blesbuck (*Damaliscus pygargus phillipsi*), black wildebeest (*Connochaetes gnou*), African buffalo (*Syncerus caffer*), and eland (*Taurotragus oryx*) are considered to be the main reservoirs (Allsopp, 2010). *Ehrlichia ruminantium* has been identified in both dogs and humans, yet they are classified as incidental hosts (Totté et al., 1993, Allsopp and Allsopp, 2001).

Spickett (2013) calculated that the estimated economic impact of heartwater in southern Africa amounts to US\$ 44.4 million annually. In Zimbabwe, the estimated loss was around US\$ 5.6 million and was attributed to acaricide costs, milk loss and treatment costs (Mukhebi et al., 1999). Currently, there is only one commercially available "vaccine" for heartwater. It comprises merely of a cryopreserved preparation of infected sheep blood containing virulent infective *E. ruminantium* of the Ball 3 genotype (Du Plessis et al., 1989). Several challenges with this "vaccine" have been documented, including: the requirement of a cold chain before administration due to the rapid loss of infectivity of *E. ruminantium* within the blood after being thawed, the restricted availability of the live "vaccine" to endemic areas due to its infectious nature, and variable effectiveness of the immunity induced (Du Plessis et al., 1989, Allsopp and Allsopp, 2007). Another major challenge of the vaccine based on the Ball 3 isolate is that it is labour intensive, as vaccinated animals should be clinically monitored and are treated with tetracycline when a febrile response is detected. Numerous experimental vaccines such as attenuated (Faburay et al., 2007, Zweygarth et al., 2008, Latif et al., 2020), inactivated (Faburay et al., 2007, Adakal et al., 2010, Molepo et al., 2022), recombinant (Pretorius et al., 2010, Faburay et al., 2017) and DNA vaccines (Pretorius et al., 2007, Tshilwane et al., 2019) are in development. While some of these vaccines show promising trial results under laboratory conditions, many have failed during field trials.

Vaccine development requires information on the circulating strains in the designated area to ensure sufficient cross-protection between the genotypes. Characterisation using the small regulatory RNA (srRNA) gene has identified eight genotypes of *E. ruminantium* in southern Africa and Guadeloupe (Allsopp, 2010, Cangí et al., 2016). These genotypes are known to recombine, indicating a considerable possibility for the emergence of new genotypes (Allsopp and Allsopp, 2007). Southern Africa has been documented as the location of origin for this pathogen due to the presence of genetic variation in the isolates from this area. However, limited information on the prevalence and genotypes of circulating *E. ruminantium* has been documented in these countries, including: Angola, Botswana, Mozambique, Namibia, Zambia and Zimbabwe (Simuunza et al., 2011, Kubelová et al., 2012, Nakao et al., 2016, Cangí, 2017, Mandara, 2018, Tembo et al., 2018, Matos et al., 2019, Mnisi et al., 2022, Palomar et al., 2022, Dlamkile et al., 2023,

Kakono et al., 2003).

It is key to detect the majority of recombinants that are circulating to ensure that newly developed vaccines can provide cross-protection to all, or the majority, of the circulating variants. However, the recombination of the genotypes leads to inconsistencies during phylogenetic evaluation depending on the genes used for the analyses leading to incorrect genotype assumptions (Allsopp and Allsopp, 2007). Several genes have been utilised for the genetic characterisation of *E. ruminantium* field isolates such as 16S rRNA, pCS20, *rnc*, *ctaG*, *groESL*, *gltA*, *map1*, *sodB*, *ftsZ*, *lepA*, *lipA*, *lipB*, *secY*, *sucA* and *nuoB*. Historically, 16S rRNA and pCS20 were most commonly used to characterise *E. ruminantium*; however, low variation between sequences was documented. Multi-locus sequence typing (MLST) using several housekeeping genes has become the new gold standard (Adakal et al., 2009, Allsopp, 2010, Dlamkile et al., 2023, Pilet et al., 2012). The use of several housekeeping genes has proven capable of clearly discriminating between closely related strains, although there have been challenges with the amplification using numerous primers (Adakal et al., 2009, Pilet et al., 2012, Dlamkile et al., 2023). Amplification failure during MLST of any single gene results in the exclusion of that sample from further analyses, resulting in a reduced sample size.

AmpliSeq on the Ion platform is a massively parallel DNA sequencing method proven to be faster and more reliable than Illumina platforms (Plitnick et al., 2021). The high throughput method has been used for rapid detection of genetic variations in multiple human diseases and cancers (Millat et al., 2014a, Millat et al., 2014b, Plitnick et al., 2021, Burghel et al., 2015, Eastley et al., 2020), yet it has rarely been used to characterise genetic variation in arthropod-borne pathogens (Kattenberg et al., 2022). Therefore, this study focuses on the prevalence and genetic diversity of *E. ruminantium* within *Amblyomma* ticks across several southern African countries. By employing AmpliSeq gene amplification, we aim to enhance our understanding of *E. ruminantium* strains circulation, providing essential insights for the development of effective control measures against heartwater.

2. Materials and methods

2.1. Sample collection and identification

This study formed part of a larger project. Samples were collected from 2021 to 2022 as described in Smit et al. (2024). In brief, adult ticks of the genus *Amblyomma* were collected from cattle and goats in Angola, Zambia, and Zimbabwe, while collections were done from wildlife and cattle in Mozambique (Fig. 1). Unfortunately, Mozambique was the only country in which permission was obtained to collect from wildlife. Regarding livestock, visual inspections of animals were conducted prior to collections to identify animals infested with *Amblyomma* species ticks which were restrained to allow tick collections to be performed manually. Predilection sites such as the dewlap, perineum, axilla, udder, or interdigital spaces were targeted. Regarding wildlife, full tick collections were done from the hide of the hunted animals. These included African buffalo (*Syncerus caffer*), common eland (*Taurotragus oryx*), nyala (*Tragelaphus angasii*) and southern reedbuck (*Redunca arundinum*). Collections in South Africa were conducted by Dlamkile et al. (2023) from cattle from 2020 to 2022.

The ticks were preserved in 70 % ethanol and transported to the Department of Veterinary Tropical Diseases at the University of Pretoria, Onderstepoort, following the guidelines implemented by the Department of Agriculture, Land Reform and Rural Development (DALRRD). Adult ticks were morphologically identified to species level using identification keys compiled by Walker et al. (2003) and Voltzit and Keirans (2003). Morphological features were documented in Smit et al. (2024).

DNA extractions were performed on each individual tick using the Chelex 100 resin method as described in Smit et al. (2023), while molecular confirmation of tick identifications were conducted using 12S

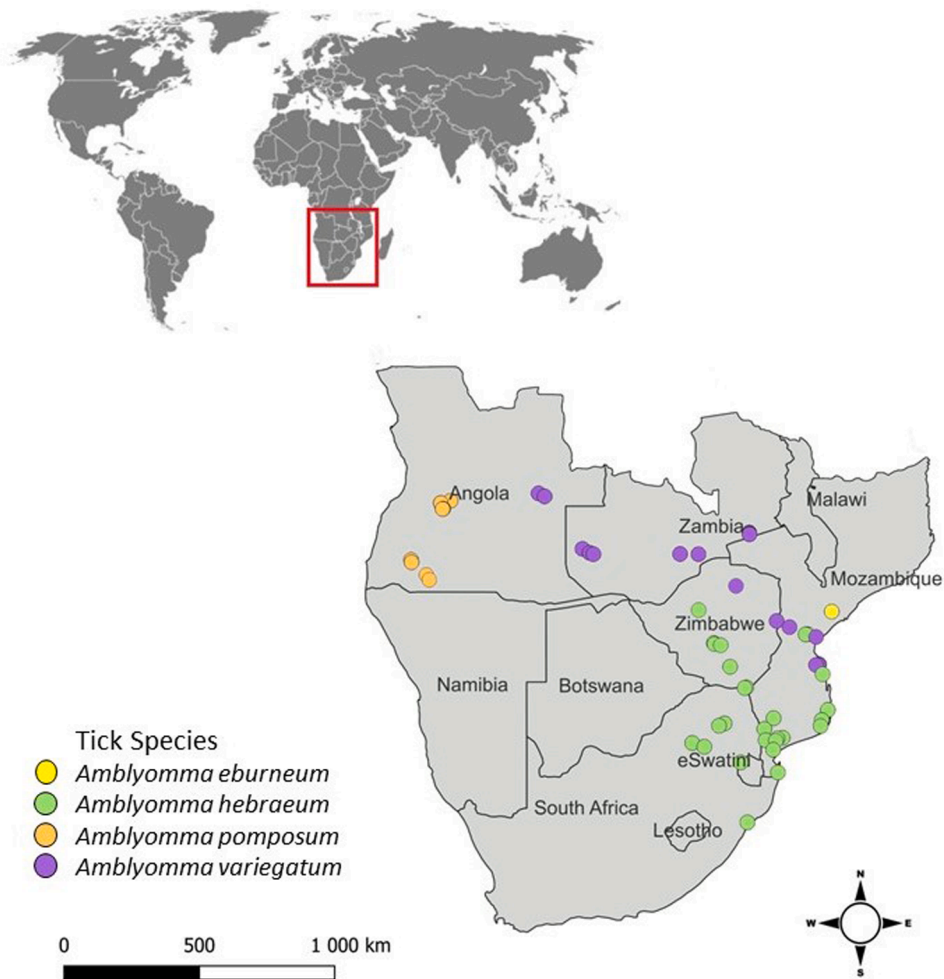


Fig. 1. Map of southern Africa, illustrating the collection points during the 2020-2022 sampling period of *Amblyomma* spp. The colour of the dot represents the species of *Amblyomma* tick collected at the sampling point. Figure was obtained from Smit et al. (2024).

rRNA (Beati and Keirans, 2001), 16S rRNA (Black and Piesman, 1994), cytochrome oxidase 1 (*coI*) (Folmer et al., 1994), and cytochrome b (*cytB*) (Simon et al., 1994) markers (Supplementary Data A: Table 1) as described in Smit et al. (2024).

2.2. Initial pathogen screening

Pathogen screening was conducted on individual *Amblyomma* ticks with a quantitative real-time PCR (qPCR) for *E. ruminantium* targeting the pCS20 gene fragment as described by Steyn et al. (2008). The 25 μ l reactions consisted of 12 μ l TaqMan Master Mix (1X final concentration) (ThermoFisher Scientific, United States), 0.625 μ l each of primers (Inqaba Biotech, South Africa) Sol1F and Sol1R (final concentration of 0.25 μ M per primer), 1.25 μ l of probe Sol1P (final concentration of 0.4 μ M) (Supplementary Data A: Table 1), 8.5 μ l double-distilled water and 2 μ l sample DNA. The qPCR was conducted using the StepOnePlus Real-Time PCR system (Applied Biosystems, United States) and cycling conditions comprised of an initial uracil-N-glycosylase incubation step at 50°C for 2 min followed by an AmpliTaq Gold pre-activation step at 95°C for 10 min. This was followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 55°C for 1 min. A positive control and no-template negative control were included for each set of reactions. The positive cut-off value was set at 38 CT.

2.3. Pathogen characterisation

A 100 positive *E. ruminantium* samples were selected for characterisation. Ten adult samples per species per country (if available) were selected at random with varying CT values below 38 (Supplementary Data B: Table 4). *Ehrlichia ruminantium* phylogenetic characterisation was conducted using Ampliseq Sequencing with IonTorrent technology targeting five genes: lipase A (*lipA*), lipase B (*lipB*), preprotein translocase membrane subunit Y (*secY*), succinyl-CoA ligase (*sucA*) and superoxide dismutase B (*sodB*) (Supplementary Data A: Table 2). The first checkpoint for the protocol was to have sufficient DNA for sequencing. Thus, DNA quantification for each sample was conducted on the Qubit 2.0 Fluorometer (ThermoFisher Scientific) using the Qubit double stranded DNA (dsDNA) High Sensitivity Assay (ThermoFisher Scientific) as per the manufacturer's instruction. Samples with a DNA concentration lower than 2 ng/ μ l did not pass the checkpoint and were excluded from further analyses (Supplementary Data B: Table 4). Library preparation was conducted as per manufacturer instructions using the Ion Ampliseq library kit 2.0 (ThermoFisher Scientific). In brief, a 12.5 μ l library master mix was prepared for each sample that passed the first checkpoint, by adding 5 μ l of 5X Ion Ampliseq HiFi mix with 25 ng of sample DNA and distilled water. The master mix was divided into two pools each containing 5 μ l, to which 5 μ l of either primer mix 1 or 2 was added (Supplementary Data A: Table 3). The libraries were then subjected to an enzyme activation step at 99°C for 2 min, and 24 cycles of denaturation at 99°C for 15 s, followed by hybridisation and

elongation at 60°C for 4 min using the VeritiPro Thermal Cycler (Applied Biosystems, United States). The two pools were combined and 2 µl of FuPa reagent was added to enable amplicon digestion. Incubation occurred at 50°C for 10 min, followed by 55°C for 10 min and ending with 60°C for 20 min using the VeritiPro Thermal Cycler.

A unique barcode was selected for each sample that passed the first checkpoint. Barcode ligation was performed as per manufacturer's instruction using the Ion Xpress™ Barcode Adapters Kits (ThermoFisher Scientific). In short, 4 µl switch solution was added to 2 µl of the adapted barcode previously mixed 1:1 with IonTorrent P1 adapter and 2 µl of DNA ligase to the pools containing FuPa reagent. The pools were incubated at 22°C for 30 min, followed by 68°C for 5 min and 72°C for 5 min using the VeritiPro Thermal Cycler.

The pools were subjected to magnetic bead purification using the Agencourt AMPure XP PCR purification kit (Beckman Coulter, United States) as per manufacturer's instructions. In short, 45 µl of AMPure XP beads was added to each pool. The pools were vortexed and incubated at room temperature for 5 min followed by a 3 min incubation at room temperature on a magnetic rack. The eluate was removed and discarded, after 150 µl of 70 % ethanol was added. The pools were slowly rotated while on the magnetic rack and the ethanol was removed and discarded. The ethanol wash was repeated once more. The pools were left on the rack and allowed to air dry for 5 min, after which 25 µl of low TE buffer was added. The pools were vortexed and incubated at room temperature for 5 min, followed by incubation on the magnetic rack for 3 min. The supernatant was recovered, and the beads discarded.

To ensure that the sequencing reaction produced good quality reads, library quantification was done for each pool using the Ion Universal Library Quantitation Kit (ThermoFisher Scientific) as per the manufacturer's instructions. If the pools passed the required 100 pM, they could proceed to the enrichment phase (Checkpoint 2 - **Supplementary Data B: Table 4**).

Both the emulsion PCR and the Ion Sphere Particles (ISPs) enrichment were performed as described in the Ion OneTouch manual with the Ion PGM Hi-Q View OT2 kit (ThermoFisher Scientific – discontinued). Before sequencing, a final quality control was conducted on the enriched ISPs using the IonSphere Quality Control kit (ThermoFisher Scientific – discontinued) (Checkpoint 3 - **Supplementary Data B: Table 4**). Sequencing was conducted as described in the Ion PGMTM Hi-Q View Sequencing manual using the Ion OneTouch 2 system (Life Technologies - United States)

2.4. Phylogenetic analysis

For each sample, raw reads were mapped the genome sequence of strain Gardel with “bwa-mem” (Li and Durbin, 2009), keeping only reads uniquely mapped with a quality above 60 (Phred score). Duplicates were filtered with samtools (Li et al., 2009). Variants were called using freebayes (Garrison and Marth, 2012), filtering variants with less than 10X coverage and a calling quality of 100. FASTA sequences were inferred from the variant-calling files using samtools.

The resulting sequences obtained for *lipA*, *lipB*, *secY*, *sucA* and *sodB* were concatenated on CLC main workbench version 23.0.4 (developed by CLC Bio, <http://www.clcbio.com>) and compared with those available in the GenBank database using BLAST (<https://www.ncbi.nlm.nih.gov/genbank/>) (**Supplementary Data C: Table 5**). *Ehrlichia canis* was used as the outgroup due to its close relation to *E. ruminantium* and all targeted regions of the same sample were available for comparison. Assembled sequences were aligned alongside reference sequences with the use of the online version of MAFFT version 7 (developed by <http://mafft.cbrc.jp/alignment/server/index.html>) with default parameters. The aligned matrix was manually viewed, edited, and truncated using MEGA 11. The best fit model was determined using the ModelFinder (Kalyaanamoorthy et al., 2017) option on the IQTREE platform. The maximum likelihood (ML) phylogenetic analysis of the *E. ruminantium* strains were conducted on IQTREE webserver (Nguyen

et al., 2015) using the TIM+F+I+G4 model, while using the UFBoot function (Hoang et al., 2018) with maximum bootstrap of a 1,000. The resulting tree was visualised and edited in iTOL version 6.8 (Letunic and Bork, 2021).

Haplotype analysis was conducted on each of the five targeted gene regions (*sucA*, *sodB*, *lipA*, *secY* and *lipB*), the concatenated matrix, and clade 4 individually using PopART version 7.1 (Bandelt et al., 1999) (**Supplementary Data D: Table 6**). For haplotype analyses of the individual genes, the 39 sequences obtained during this study were evaluated with no reference sequences. The concatenated alignment consisted of 62 sequences of which 39 were from this study while 23 were reference sequences from GenBank. Clade 4 was analysed individually to determine the differentiation capability of the IonAmpliseq method. Clade 4's alignment consisted out of 35 sequences obtained in this study with 16 reference sequences (a total of 51 sequences). A minimum spanning network (MSN) was conducted in PopART version 7.1 with an epsilon value of 0 for each gene, the concatenated alignment, and clade 4. Nucleotide diversity (π), number of segregating sites and simple Analysis of Molecular Variance (AMOVA) tests were conducted in PopART (Leigh et al., 2015) using default parameters for each alignment.

2.5. Statistical analysis

A Chi-square analysis was conducted in Microsoft Excel to evaluate the independence between infection rate and sex of tick species, as well as to evaluate the independence between infection rate and tick species. *Ehrlichia ruminantium* prevalence was calculated for each sex (male and female) by using the number of positives determined for the sex over the total number collected for the sex. For each country, prevalence was calculated by using the positives per species (males and females combined) over the total number of ticks collected in the country for that species. If the expected number was lower than five ticks, then the Fisher's exact test was used instead. Conservative p-value thresholds for acceptance of the null hypothesis were determined using the Bonferroni correction method and the total number of statistical tests conducted. Bonferroni correction resulted in a conservative p-value threshold for acceptance of the null hypothesis of $p < 0.0033$ (0.05/15). Upper and lower 95 % confidence intervals for prevalence were estimated using the binom.test function in R (R Core Team, 2013), which uses the Clopper-Pearson method for binomial data (Clopper and Pearson, 1934).

2.6. Ethical considerations

Approval to perform the project was received from the Research and Animal Ethics Committee at the University of Pretoria (REC 121-20). Additionally, approval was secured from the Department of Agriculture, Land Reform and Rural Development (DALRRD) in South Africa, in accordance with Section 20 of the Animal Diseases Act of 1984 (Act no. 35 of 84) (12/11/1/1 (1937SS)).

3. Results

In total 7,734 adult *Amblyomma* ticks were collected in southern Africa and were morphologically determined and molecularly corroborated as: *Amblyomma eburneum* (n = 208), *A. hebraeum* (n = 4,758), *Amblyomma pomposum* (n = 191), and *A. variegatum* (n = 2,577) (Smit et al., 2024). During the collections it was documented that *A. eburneum* was only collected from wildlife species and therefore restricted to Mozambique. *Amblyomma hebraeum* was restricted to the southern parts of Mozambique and Zimbabwe and was the only *Amblyomma* species collected in South Africa, while *A. pomposum* was restricted to central and western Angola. *Amblyomma variegatum* was the most widespread of the species and was collected from both wildlife and livestock.

Initial pathogen screening using the pSC20 gene indicated varying positivity rates for each tick species in each country. The overall

prevalence of *E. ruminantium* in Angola was 34.1 % (Table 1). The prevalence for *A. pomposum* was 13.6 %, whereas in female *A. pomposum* it was 50 % (albeit with a very small sample size, 2/4) and in males it was 12.8 % (24/187). There was no association between *E. ruminantium* infection rates and *A. pomposum* sex ($p = 0.082$). The prevalence in *A. variegatum* was 49.6 %, whereas in females it was 37.5 % (6/16) and in males it was 50.4 % (119/236). No association was observed between *E. ruminantium* prevalence and *A. variegatum* sex ($X^2_{(1, N = 252)} = 1.001, p = 0.317$). An association between *E. ruminantium* prevalence and the infected *Amblyomma* species was observed ($X^2_{(1, N = 443)} = 62.671, p < 0.001$).

The *E. ruminantium* prevalence in Mozambique was 7.1 % (Table 1), divided between the prevalence in ticks from livestock (8.0 %) and in ticks from wildlife (3.7 %). Both *A. hebraeum* and *A. variegatum* were collected from livestock. The prevalence of *E. ruminantium* in *A. hebraeum* was 8.9 %, whereas in females it was 4.9 % (46/932) and in males it was 10.5 % (242/2,304). There was an association between *E. ruminantium* infection rate and *A. hebraeum* sex ($X^2_{(1, N = 3,236)} = 25.372, p < 0.001$). The prevalence of *E. ruminantium* in *A. variegatum* from livestock was 4.7 %, whereas in females it was 1.5 % (3/194) and in males it was 5.6 % (41/733). There was no association between *E. ruminantium* infection rate and *A. variegatum* sex, after Bonferroni correction ($X^2_{(1, N = 927)} = 5.557, p = 0.018$), but a significant difference was observed between *E. ruminantium* prevalence in the two *Amblyomma* species from livestock ($X^2_{(1, N = 4,163)} = 16.937, p < 0.001$).

On wildlife in Mozambique, *A. eburneum* and *A. variegatum* were collected. The prevalence of *E. ruminantium* in *A. eburneum* was 7.7 %, whereas in females it was 2.0 % (1/49) and in males it was 9.4 % (15/159) (Table 1). No association was observed between *E. ruminantium* infection rate and *A. eburneum* sex ($p = 1.0$). The prevalence of *E. ruminantium* in *A. variegatum* from wildlife was 2.6 %, whereas in females it was 2.0 % (5/248) and in males it was 2.9 % (16/553). No

association was observed between the *E. ruminantium* prevalence and sex of *A. variegatum* ($X^2_{(1, N = 801)} = 0.516, p = 0.473$). However, the *E. ruminantium* prevalence in the two *Amblyomma* species from wildlife was significantly different ($X^2_{(1, N = 1,009)} = 12.018, p < 0.001$).

The overall prevalence of *A. hebraeum* for *E. ruminantium* in South Africa was 29.0 % (Table 1). Tick sexes were not documented in this country; thus, no comparison can be made.

The *E. ruminantium* prevalence in *A. variegatum* from Zambia was 16.0 % (Table 1), whereas it was 12.5 % in females (4/32) and 16.3 % in males (87/535). No association was observed between *E. ruminantium* prevalence and the sex of the *A. variegatum* ticks ($X^2_{(1, N = 567)} = 0.317, p = 0.573$).

The overall *E. ruminantium* prevalence in Zimbabwe was 12.2 % (Table 1). For *A. hebraeum*, the prevalence was 12.3 %, whereas in females it was 8.4 % (11/131) and in males it was 14.8 % (30/203). No association was observed between *E. ruminantium* prevalence and *A. hebraeum* sex ($X^2_{(1, N = 334)} = 3.011, p = 0.083$). For *A. variegatum*, the prevalence was 9.1 % (1/10), and the infected tick was a male. No difference in infection prevalence was observed between the two *Amblyomma* species from this country ($X^2_{(1, N = 345)} = 0.101, p = 0.751$).

Both *A. hebraeum* and *A. variegatum* ticks were collected from several different countries. The *E. ruminantium* prevalence in the two species were compared between the countries from which they were collected. There was a significant association between *E. ruminantium* prevalence and collection country for both *A. variegatum* ($X^2_{(4, N = 2,558)} = 9.488, p < 0.001$) and *A. hebraeum* ticks ($X^2_{(2, N = 3,933)} = 5.991, p < 0.001$).

From these confirmed positives, 100 were selected for further characterisation using Ampliseq Sequencing with IonTorrent technology. Quality control was performed on the 100 samples that were selected, of which only 42 samples had sufficient DNA of high quality and sufficiently enriched within ISPs. Of the 42 samples that passed the final checkpoint (Supplementary B: Table 4) and were subjected to

Table 1

Proportion and distribution of *Amblyomma* ticks collected in southern Africa and their *E. ruminantium* prevalence. Table displays information on each tick species, their hosts, country of origin, and prevalence information with confidence intervals for each sex separately and combined.

Collection country	Tick species	Host	Overall			Male			Female		
			No. collected	No. infected	Prevalence (%; 95 % CIs)	No. collected	No. infected	Prevalence (%; 95 % CIs)	No. collected	No. infected	Prevalence (%; 95 % CIs)
Angola	<i>A. pomposum</i>	Cattle/Goats	191	26	13,6 (9.1-19.3)	187	24	12,8 (8.4-18.5)	4	2	50 (6.8-93.2)
	<i>A. variegatum</i>	Cattle/Goats	252	125	49,6 (43.3-56)	236	119	50,4 (43.9-57)	16	6	37,5 (15.2-64.6)
Total			443	151	34,1 (29.7-38.7)						
Mozambique	<i>A. hebraeum</i>	Cattle/Goats	3,236	288	8,9 (7.9-9.9)	2,304	242	10,5 (9.3-11.8)	932	46	4,9 (3.6-6.5)
	<i>A. variegatum</i>	Cattle/Goats	927	44	4,7 (3.5-6.3)	733	41	5,6 (4-7.5)	194	3	1,5 (0.3-4.5)
	<i>A. eburneum</i>	Buffalo, Eland, Nyala, Reedbuck	208	16	7,7 (4.5-12.2)	159	15	9,4 (5.4-15.1)	49	1	2 (0.1-10.9)
	<i>A. variegatum</i>	Buffalo, Eland, Nyala, Reedbuck	801	21	2,6 (1.6-4)	553	16	2,9 (1.7-4.7)	248	5	2 (0.7-4.6)
Total			5,172	369	7,1 (6.5-7.9)						
South Africa	<i>A. hebraeum</i>	Cattle	686	199	29 (25.6-32.6)						
Zambia	<i>A. variegatum</i>	Cattle	567	91	16 (13.1-19.3)	535	87	16,3 (13.2-19.7)	32	4	12,5 (3.5-29)
Zimbabwe	<i>A. hebraeum</i>	Cattle	334	41	12,3 (9-16.3)	203	30	14,8 (10.2-20.4)	131	11	8,4 (4.3-14.5)
	<i>A. variegatum</i>	Cattle	11	1	9,1 (0.2-41.3)	10	1	10 (0.3-44.5)	1	0	0 (0-97.5)
Total			345	42	12,2 (8.9-16.1)						

sequencing, only 39 (92.9 %) provided useful sequence reads. Fragment lengths for each sample were the same size: *sucA* produced the largest fragment of 1,120 bp, while *lipA* was 420 bp in length and *secY* was 560 bp in length. Both *lipB* and *sodB* produced the shortest fragments of 280 bp each. The final alignment length after truncation was 2,385 bp.

The *sucA* alignment contained 1,120 bp of which 32 (2.9 %) positions were parsimony-informative sites. The nucleotide diversity was $\pi = 308,148$, while the simple AMOVA Φ_{ST} was calculated as -0.07 ($p = 0.799$). A total of 15 haplotypes were identified, of which nine were singletons (Supplementary D: Table 6). The *sodB* alignment contained 284 bp, of which 11 (3.9 %) positions were parsimony-informative sites. The nucleotide diversity was $\pi = 0.004$, while the simple AMOVA Φ_{ST} was calculated as 0.06 ($p = 0.235$). Eight haplotypes were identified, of which three were singletons (Supplementary D: Table 6). For the *lipA* gene, three samples (MSChi430, ZiMG20 and MSMun130) had complications with sequencing, resulting in truncated 3' ends. Nucleotide diversity was calculated for the *lipA* alignment including the sequences ($\pi = 0.003$) and excluding these sequences ($\pi = 0.002$). Ignoring the missing information, 13 (3.1 %) parsimony-informative sites were detected from the 424 bp alignment and the simple AMOVA Φ_{ST} was calculated as -0.07 ($p = 0.555$). A total of 10 haplotypes were identified, of which five were singletons. The *secY* alignment consisted of 561 bp, of which 11 (2.0 %) positions were parsimony-informative sites. The nucleotide diversity was estimated as $\pi = 0.001$, while the simple AMOVA Φ_{ST} was calculated as -0.108 ($p = 0.961$). Five haplotypes were identified, of which two were singletons (Supplementary D: Table 6). The *lipB* alignment consisted of 280 bp, of which six (2.1 %) were parsimony-informative sites. The nucleotide diversity was estimated as $\pi = 1.017e + 06$, while the simple AMOVA Φ_{ST} was calculated as -0.089 ($p = 0.761$). Four haplotypes were identified of which two were singletons

(Supplementary D: Table 6).

A total of 62 individual *E. ruminantium* sequences were analysed, resulting in the identification of 34 unique haplotypes (Fig. 2). Of the 2,385 bases of the concatenated matrix (*sucA*, *sodB*, *lipA*, *secY* and *lipB*), 327 sites (13.7 %) were variable, and the nucleotide diversity was $\pi = 0.011$. Of the 34 haplotypes, 21 were singletons. The simple AMOVA Φ_{ST} was calculated as 0.239 ($p = 0.128$). The 51 *E. ruminantium* sequences that clustered within clade 4 were analysed, resulting in 31 unique haplotypes (Fig. 3). Of the 31 haplotypes, 22 were singletons (Supplementary D: Table 6) and of the 2,385 bases, 32 (1.34 %) were parsimony-informative sites. The nucleotide diversity was estimated as $\pi = 135681$. The simple AMOVA Φ_{ST} was calculated as 0.147 ($p = 0.007$).

From the 100 *E. ruminantium* samples selected for Ion Torrent Ampliseq sequencing, only 39 provided useable sequence information for all five of the selected genes. The ML phylogenetic analysis of the concatenated *E. ruminantium* sequences depicted separation into four clades. The first clade (in blue - Fig. 4), includes the *E. ruminantium* strains Kumm2, Riverside and Omatjenne. These are all strains from South Africa but clearly separated from other southern African strains (bootstrap 100); thus, they are classified as forming a unique South African clade. Clade 2 (in yellow - Fig. 4), consisted of one *E. ruminantium* strain from an *A. pomposum* tick collected from livestock in Angola and three *E. ruminantium* strains from *A. variegatum* ticks collected from wildlife in Mozambique. This clade branched from the third clade (West African) although without bootstrap support, while none of these sequences clustered with other strains obtained from GenBank. The third clade (in green - Fig. 4), is the West African clade with four strains (Sankat 430, Kerr Seringe, Senegal virulent and Senegalp63). The fourth clade (in pink - Fig. 4), contains reference strains from southern and eastern Africa as well as Guadeloupe. The majority of

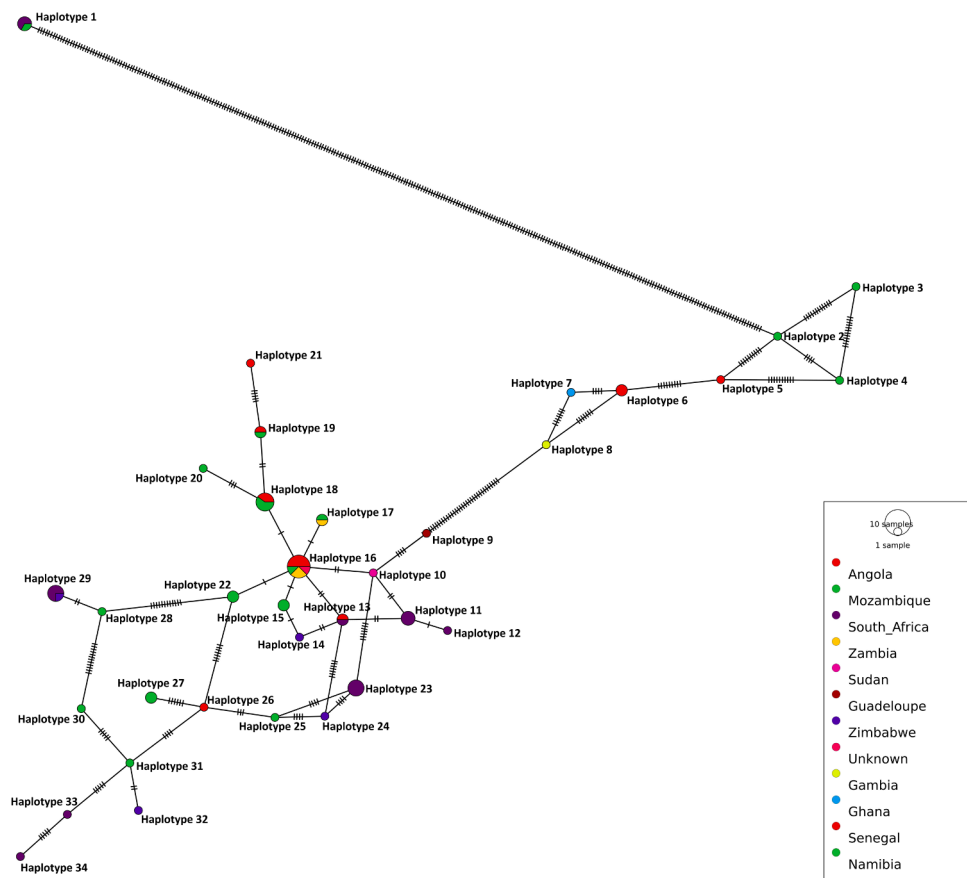


Fig. 2. Minimum spanning networks of the concatenated matrix constructed with PopART. Circle sizes reflect haplotype abundances. Colour represents country or origin. Hatch marks on each connection reflect the number of bases that are different between the haplotypes.

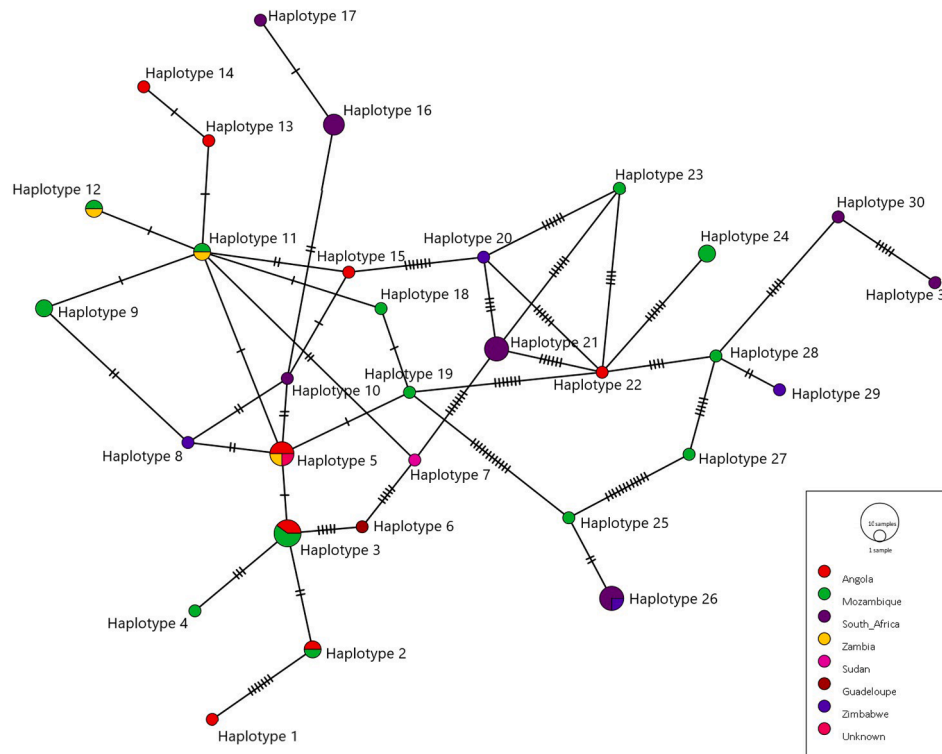


Fig. 3. Minimum spanning networks of the fourth clade (southern Africa and Guadeloupe) constructed with PopART. Circle sizes reflect haplotype abundances. Colour represents country or origin. Hatch marks on each connection reflect the number of bases that are different between the haplotypes.

the *E. ruminantium* strains obtained in this study clustered within this clade alongside reference strains such as Gardel, Welgevonden, Um Banein, Springbokfontein 4 and 2, Kwanyanga and Blaauwkrans. Clade 4 exhibited evidence of intraspecific population structure, with some branches displaying moderate or strong support. However, weak to no bootstrap support was observed for the majority of nodes. Moreover, no clustering was observed based on tick species, geographical spread, nor from the host from which the ticks were collected.

4. Discussion

Infection rates, tick density, and tick distribution patterns are crucial parameters to estimate the risk of transmission of tick-borne pathogens. This study aimed to determine the *E. ruminantium* prevalence and strains within *Amblyomma* species collected in several southern African countries.

In Angola, two *Amblyomma* species were identified, *A. pomposum* and *A. variegatum*. These two species form a parapatric boundary that is believed to be caused by substantive ecological differences in distribution landscapes, climate and varying vegetation types, although no studies have been conducted to elucidate the exact causes (Gomes, 1993), nor were any such major ecological differences observed during sample collections. *Amblyomma pomposum* is documented as the most abundant species in Angola, believed to be the primary vector of *E. ruminantium* in the country (Gomes, 1993, Sili et al., 2021). In contrast, both Kubelová et al. (2012) and Sili (2017) found no *E. ruminantium* infections in cattle in Angola, even though the main vectors were present and abundant. Kubelová et al. (2012) suggested that the absence of positives are indicative of endemic stability, while Sili (2017) argued that the probe used had a low detection rate. Sili et al. (2021) were the first to report on *E. ruminantium* prevalence in *A. pomposum* and found a 7 % positivity rate. In this study, we found that the *E. ruminantium* prevalence was 13.6 % (CI: 9.1-19.3 %) in *A. pomposum* and 49.6 % (CI: 43.3-56 %) in *A. variegatum*. Therefore, the

E. ruminantium prevalence in *A. pomposum* was approximately four times lower than in *A. variegatum*. Although *A. pomposum* had a lower positivity rate, it is more widely distributed across Angola, while *A. variegatum* was restricted to the Mexico province in eastern Angola. Consequently, even though *A. pomposum* is less likely to be infected with *E. ruminantium* than *A. variegatum*, it would still be considered the primary vector for *E. ruminantium* in Angola. Vector competency tests have been conducted on *A. pomposum* by Neitz (1947) and Serrano (1964); however, we cannot conclusively state whether *A. pomposum* is a more competent vector of the pathogen compared to *A. variegatum*. The *A. variegatum* collected in Angola had the highest overall *E. ruminantium* prevalence when compared to *Amblyomma* species from all other countries. This high prevalence could be attributed to a lack of vector control and vaccination against *E. ruminantium*, although no records are available on the cattle dipping and vaccination practices in the country.

In Mozambique, collections were made from cattle and wildlife; *A. hebraeum* and *A. variegatum* were collected from livestock. Bournez et al. (2018) documented an *E. ruminantium* prevalence ranging from 0.0 % to 26.7 %, whereas Matos et al. (2019) noted an *E. ruminantium* prevalence range of 5.0 % to 23.9 % in cattle blood. Herewith, we document a prevalence of 8.9 % (CI: 7.9-9.9 %) in *A. hebraeum* and 4.7 % (CI: 3.5-6.3 %) in *A. variegatum*, consistent with the aforementioned surveys. In *A. hebraeum*, males were statistically more likely to be infected as compared to the females. The higher infection rate in the males can be ascribed to their migratory habits; Stachurski (2006) documented that *A. hebraeum* males migrated from predilection sites and from hosts during the night. Males also tend to have longer lifespans as compared to females, and the migratory habits would allow them to acquire infections from multiple hosts as compared to females. This study also observed a significant difference between the infection rates between species, where *A. hebraeum* was twice as likely to be infected compared to *A. variegatum*. Mahan et al. (1995) demonstrated that *A. hebraeum* ticks were more susceptible under laboratory conditions to certain *E. ruminantium* strains (including Crystal Springs, Ball 3, Gardel,

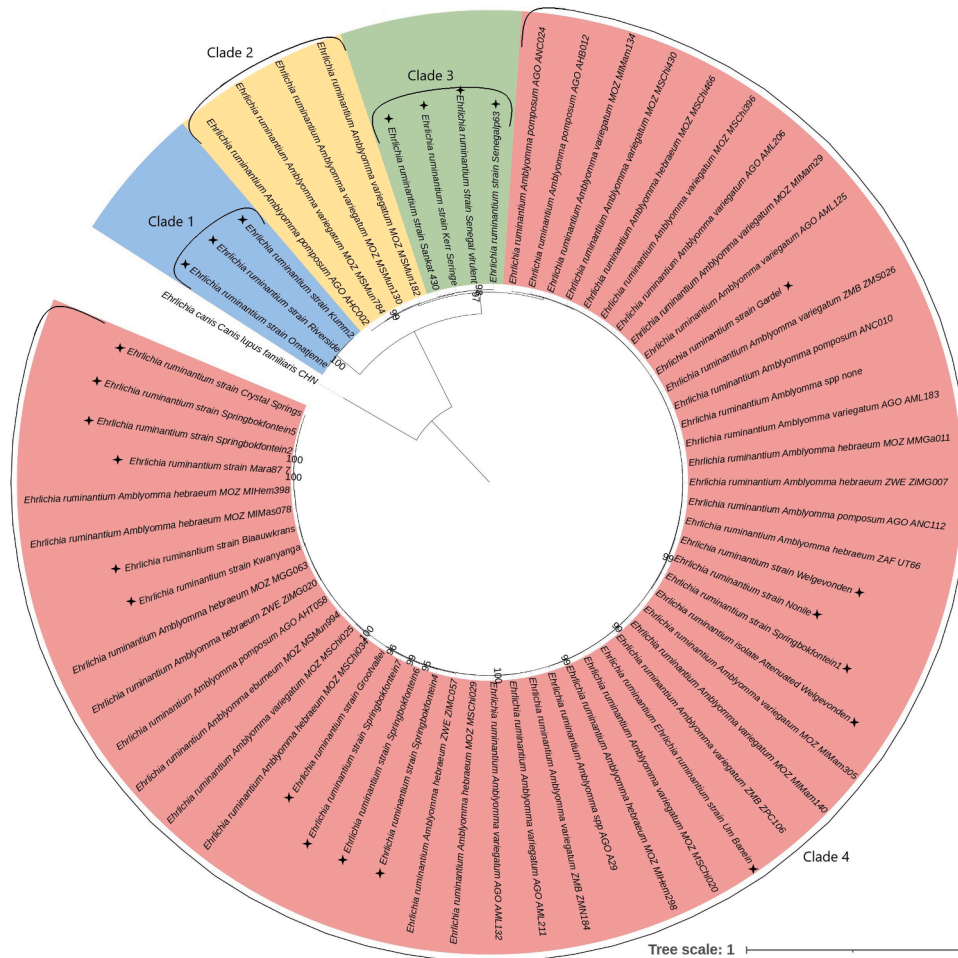


Fig. 4. Maximum likelihood analysis of the concatenated *Ehrlichia ruminantium* genes (*sucA*, *sodB*, *lipA*, *secY* and *lipB*). Bootstrap value is indicated at each branch node (bootstrap values under 95 were removed). Sample names contain the species name, vector name, country code and sample code. Blue indicates unique South Africa strains, yellow indicates unique Angola and Mozambique strains, green indicates west African strains, and pink indicates strains from southern and eastern Africa as well as Guadeloupe. Country of origin is indicated in the sample ID as: AGO – Angola, MOZ – Mozambique, ZAF – South Africa, ZMB – Zambia, and ZWE – Zimbabwe. Reference sequences obtained from GenBank are indicated with “♦”.

and Nigeria) relative to *A. variegatum*. The strains in this study closely resemble Gardel and this might explain why *A. hebraeum* had higher infection rates.

Amblyomma eburneum and *A. variegatum* were collected from wildlife in Mozambique. Information on *A. eburneum* is scarce; [Mwamuye et al. \(2017\)](#) merely noted that *A. eburneum* tested positive for *E. ruminantium* but did not provide any data on prevalence or infection rates. Both [André et al. \(2016\)](#) and [Oura et al. \(2011\)](#) screened African buffalo blood for *E. ruminantium* and noted a prevalence of 0.0 % in Mozambique and Uganda, respectively, while [Pascucci et al. \(2011\)](#) recorded a prevalence of 3.0 % in Namibia. In this study, and in [Smit et al. \(2023\)](#), we documented an *E. ruminantium* prevalence of 7.7 % (CI:4.5-12.2 %) in *A. eburneum* and 2.6 % (CI: 1.6-4 %) in *A. variegatum*. This is within the expected ranges when compared to other southern African countries. A significant difference was observed between the two species, where *A. eburneum* was three times more likely to be infected as compared to *A. variegatum*. However, no vector competency studies have been conducted on *A. eburneum*. We hypothesise that *A. eburneum* may be more susceptible to certain *E. ruminantium* strains as documented by [Mahan et al. \(1995\)](#) for *A. hebraeum*.

The *E. ruminantium* prevalence in the *Amblyomma* species collected from the wildlife was lower than in *Amblyomma* species collected from livestock in all countries. This contradicted our expectations, since wildlife, such as blesbuck, black wildebeest, African buffalo, and eland,

are documented as reservoirs for *E. ruminantium*. This difference in expected and observed *E. ruminantium* prevalence could be attributed to several factors. The wildlife sampled in this study was from a small sub-population of buffalo that inhabits the forest ecological niche within the Marromeu Complex of the Zambezi Delta. The buffalo occurs in low numbers in these forest niches; thus, the host availability for the *Amblyomma* species is low. *Ehrlichia ruminantium* is maintained trans-stadially; hence, the ability for the *Amblyomma* species ticks to transmit *E. ruminantium* between hosts could be reduced due to a low host density ([Peter et al., 2002](#)). Another contributing factor is the inefficient role of buffaloes as *E. ruminantium* hosts. [Peter et al. \(2002\)](#) speculated that the carrier status post-infection of buffalo with *E. ruminantium* is less than six months. The natural innate immunity to *E. ruminantium* in buffaloes was determined by [Gradwell et al. \(1976\)](#) when they observed no clinical signs of infection in naïve buffaloes that were infected with *E. ruminantium* in sheep blood. The short *E. ruminantium* carrier status of the buffalo alongside the low host density in the forest niches and the maintenance mechanism of *E. ruminantium* in the *Amblyomma* species may all have contributed to the lower *E. ruminantium* prevalence in the *Amblyomma* species from wildlife.

Several studies are available on the prevalence of *E. ruminantium* in *A. hebraeum* from South Africa. [Bryson et al. \(2002\)](#) documented infection rates ranging from 4.7 % to 25.0 %, while [Mnisi et al. \(2022\)](#) noted an infection rate of 12.3 %, [Jongejan et al. \(2020\)](#) documented 17.4 %, and

and Dlamkile et al. (2023) recorded a range from 19.0 % to 27.0 %. In this study we observed a prevalence of 29.0 % (CI: 25.6-32.6 %) in *A. hebraeum* from South Africa, which is at the upper end of the expected ranges for the country.

In Zambia, only *A. variegatum* was collected. Little information is available on the infection rates of *E. ruminantium* in Zambia. Tembo et al. (2018) screened cattle blood in Zambia and found a 0.3 % prevalence for *E. ruminantium*, whilst Simuunza et al. (2011) documented infections ranging from 5.4 % to 45.3 % depending on location and seasonality. With the high apparent *E. ruminantium* prevalence in cattle blood for Zambia, it is surprising that so little information is available on the prevalence of *E. ruminantium* in the vectors. In this study, we documented a prevalence of 16.0 % (CI: 13.1-19.3 %) in *A. variegatum* from Zambia, with no statistically significant differences between the sexes. It is likely that there are many cases of heartwater going unreported in this country due to an apparent lack of surveillance on vector distribution and infection rates. Though few studies are available for comparison, the described prevalence is within the expected ranges for southern African countries.

In this study, two *Amblyomma* species were documented in Zimbabwe, *A. hebraeum* in the southern parts and *A. variegatum* in the north-western areas. Several studies have been conducted on the prevalence of *E. ruminantium* in Zimbabwe. Norval et al. (1990) found infection rates of *E. ruminantium* in *A. hebraeum* ticks to range from 0.0 % to 44.9 % when using a release and control method of determining infectivity. Peter et al. (1999) conducted the first PCR-based detection of *E. ruminantium* in Zimbabwe from *A. hebraeum* and found a prevalence of 11.2 % in *A. hebraeum* from the lowveld and 10.2 % from *A. hebraeum* from the highveld. More recently, Mandara (2018) noted a prevalence of 15.7 % in *A. hebraeum* and 11.9 % in *A. variegatum*. In this study, we found that the *E. ruminantium* prevalence was 12.3 % (CI: 9-16.3 %) in *A. hebraeum* and 9.1 % (CI: 0.2-41.3 %) in *A. variegatum*. Our findings were well within the expected ranges for both *Amblyomma* species.

Both *A. hebraeum* and *A. variegatum* show significant variation in *E. ruminantium* prevalence depending on the country of collection, likely due to differing host densities and vector control strategies implemented in the respective countries. As of 2021, South Africa boasted the highest cattle population, estimated at approximately 12.2 million head, while Mozambique had the lowest, with only 2.22 million head (Food and Agriculture Organization of the United Nations, 2023). This discrepancy likely explains the lower prevalence of *E. ruminantium* in Mozambique and Zambia, which has an estimated cattle population of 3.82 million. In contrast, despite Zimbabwe having the second highest number of cattle at 5.35 million (Food and Agriculture Organization of the United Nations, 2023), it exhibits one of the lowest prevalences of the pathogen. The low prevalence of *E. ruminantium* in the *Amblyomma* species from Zimbabwe may result from rigorous government-led efforts in Zimbabwe to mitigate cattle mortalities from tick-borne diseases, especially theileriosis, primarily through intensive acaricide use (Nhokwara et al., 2023). Angola, with a substantial cattle population of 5.19 million, displays a higher prevalence of *E. ruminantium*, possibly due to less effective tick control measures compared to South Africa's robust acaricide dipping and vaccination programmes.

Surprisingly, *E. ruminantium* is rarely documented in cattle from the countries where the vector was collected, particularly Angola and Zambia. The underreporting could stem from a lack of awareness among farmers and veterinary officials about the pathogen and its associated disease. Many small stock farmers own resistant cattle breeds such as Zebu (*Bos indicus*) and attempt to control tick populations using acaricide treatment. These methods have proven useful in lowering *E. ruminantium* prevalence, but they are not entirely effective. Often, resistant breeds are less productive, and the complete removal of ticks could lead to the potential loss of herd immunity, resulting in sporadic outbreaks of heartwater (Allsopp, 2015). Vaccines would be an ideal solution for inducing herd immunity without exposure to infected ticks. However, high levels of variation observed in the *E. ruminantium*

sequences documented in several studies support the recombination of strains as suggested by Allsopp and Allsopp (2001). These recombination events could be the main factor hindering effective vaccine development (Cangi et al., 2016). While various vaccines are in development (Allsopp, 2015), none have shown efficacy in field studies, highlighting that novel approaches to address the impact of heartwater are needed.

It should be noted that the detection of *E. ruminantium* in ticks could result from their ingestion of infected host blood, therefore the proportion of ticks collected from *E. ruminantium*-infected animals could have affected these results. However, molecular detection of *E. ruminantium* in host blood is uncommon due to the transient nature of rickettsaemia, which is typically short-lived (Peter et al., 2002, Allsopp, 2010). It is also noted that *E. ruminantium* is transstadially transmitted; therefore, an infection could be acquired during the larval or nymphal stage from other animals and detected in the adult. Therefore, we believe that the direct contribution of host blood to the positivity rate in ticks is negligible.

Haplotype analysis of the fourth clade indicated that certain haplotypes were specific to a certain *Amblyomma* tick species but were not restricted to the country of collection. For instance, haplotypes 3, 5 and 11 (of clade 4) were restricted to *A. variegatum* but originated from countries including Angola, Mozambique, and Zambia. It is well known that *A. variegatum* and *A. hebraeum* are efficient vectors of *E. ruminantium* (Petney et al., 1987). The majority of the haplotypes were identified to occur in either of these species, further highlighting the potential treat of outbreaks with the presence of these widespread species in many regions of southern Africa.

Based on the ML analysis of the 39 *E. ruminantium* samples obtained in this study, our data fell into two clades. One clade consisted of four sequences obtained in this study; three from *A. variegatum* ticks collected from wildlife in Mozambique and one from an *A. pomposum* tick collected from cattle in Angola. These sequences did not cluster with any strains obtained from GenBank, although the West Africa strains formed the nearest neighbour, albeit with poor bootstrap support. As mentioned, wildlife is documented as tick hosts in the southern Africa region and several wild hosts constitute reservoirs for *E. ruminantium* (Allsopp, 2010). The *A. variegatum* vectors for this *E. ruminantium* clade were collected from wildlife, mainly African buffalo, while the *A. pomposum* tick was collected from cattle. Cangi et al. (2016) noted that no records are available on the influence of wildlife, cattle, and small ruminant movements on the genetic diversity of *E. ruminantium*. They hypothesised that the movement of cattle dating back more than 6, 500 years ago and the more recent translocations facilitated the genetic diversity of *E. ruminantium*, allowing for recombination to occur (Ajmone-Marsan et al., 2010). *Ehrlichia ruminantium* was present in Africa before the cattle migration, circulating in the wildlife populations. Thus, with the introduction of cattle that were naïve to the bacterium, new host populations were unlocked. They documented that the cattle moved from the north-eastern part of the continent to West and East Africa (Payne, 1964, Epstein, 1971, Maillard et al., 1993). These translocations may possibly explain the clustering of *E. ruminantium* sequences from Angola and Mozambique and their apparent close relationship to strains from West Africa.

The major clade contains most of the samples, which were found to cluster with the southern and eastern African and Guadeloupe strains obtained from GenBank such as Gardel, Welgevonden, Um Banein, Springbokfontein 4 and 2, Kwanyanga, and Blaauwkrans. However, we could only compare our results to type strains that are available on GenBank, the majority of which are from South Africa. Sequence information from other countries is severely lacking presently. The sequences showed great variation, with the 51 sequences in this clade clustered into 31 unique haplotypes. The AMOVA indicated that there was a significant distribution of genetic diversity within this clade. The clustering observed was not linked with *Amblyomma* species vectors, nor the geographical location from which the ticks were collected. Weak bootstrap values were also observed for the majority of the nodes. This

clade highlights the extensive genetic variation within the pathogen and the potential for significant regional differences in vaccine response due to this diversity.

While evaluating the concatenated matrix, with all clades represented, no significant variation was observed. This non-significant variation in the concatenated matrix is due to the large number of nucleotide differences between the clades, skewing the overall AMOVA analyses and should not be used as an accurate measure of variation. However, despite this weak node support, the simple AMOVA indicated that the sequence variation was significant for the fourth clade alone. The current MLST genes used comprised *lipA*, *lipB*, *sodB*, *secY* and *sucA*, which are housekeeping genes and are clustered closely on the *E. ruminantium* genome (Collins et al., 2005, Adakal et al., 2009). Difficulty in amplification of several of the markers has been reported by Dlamkile et al. (2023) and Nakao et al. (2011), and could be seen in this study where only 39 out of 42 sequences exhibited successful amplification for all five of the markers. We cannot exclude the effects of DNA quality on the successful outcomes of the amplifications, as DNA extracted using the Chelex method still contains impurities, which are known to hinder amplification (Singh et al., 2018). The majority of the samples (96/100) passed the initial quantity checkpoint, indicating that sufficient DNA was present in the samples; however, they were excluded in the subsequent checkpoints. We can speculate that the impurities within the extracted DNA could have hindered succeeding amplification and ligation steps. This resulted in only 42/100 samples that could be sequenced. Therefore, we suggest using “pure” DNA, extracted in a manner that reduces the impurities in the final elution. These challenges in amplification warrant investigations into the development of new markers with stronger differentiating capabilities and better amplification success. These new markers could potentially target virulence genes, or polymorphic genes evolving rapidly in the process of pathogen-host-vector interactions.

5. Conclusion

Pathogen diversity studies, such as this one, provide essential information to guide vaccine design, as well as research into new polymorphic genes for either phenotypic or genetic characterisation. This study demonstrated the variability in the *E. ruminantium* strains found in southern Africa. We have documented the prevalence of *E. ruminantium* in four *Amblyomma* species collected from five countries. The majority of these *E. ruminantium* samples clustered with strains from east and southern Africa and the Guadeloupe clade. In contrast, four samples clustered in their own clade, seemingly related to West African strains. Notably, there is a limited differentiation within the east and southern African strains, with no significant separation associated with the *Amblyomma* species vector, nor the geographical source of the sequences. This highlights the need for the development of new markers with enhanced differentiating capabilities, focusing on virulence genes or other polymorphic genes. Enhanced markers could radically improve our ability to genotype *E. ruminantium* strains, facilitating more precise and effective vaccine formulations. This study thus not only maps the current landscape of *E. ruminantium* diversity but also sets a clear directive for future research aimed at mitigating the impact of heartwater disease through improved diagnostic and vaccine technologies.

CRediT authorship contribution statement

Andeliza Smit: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Fernando C. Mulandane:** Writing – review & editing, Investigation, Data curation. **Stephane H. Wójcik:** Writing – review & editing, Investigation, Formal analysis. **Choolwe Malabwa:** Writing – review & editing, Investigation, Formal analysis. **Gourgelia Sili:** Writing – review & editing, Investigation, Formal analysis. **Stephen Mandara:** Writing – review & editing, Investigation, Formal analysis.

Kaïssa Plaisir Pineau: Writing – review & editing, Investigation, Formal analysis, Data curation. **Sylvie Lecollinet:** Writing – review & editing, Validation, Supervision, Investigation, Formal analysis, Data curation. **Antoni Exbrayat:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Valérie Rodrigues:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Hannah Rose Vineer:** Writing – review & editing, Investigation, Formal analysis. **Zinathi Dlamkile:** Writing – review & editing, Investigation, Formal analysis. **Wilhelm H. Stoltz:** Writing – review & editing, Supervision, Methodology. **Damien F. Meyer:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Darshana Morar-Leather:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Benjamin L. Makepeace:** Writing – review & editing, Supervision, Methodology. **Luis Neves:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tbd.2025.102462](https://doi.org/10.1016/j.tbd.2025.102462).

Data availability

Data will be made available on request.

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