Veterinary Parasitology 330 (2024) 110240

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

p67 gene alleles sequence analysis reveals *Theileria parva* parasites associated with East Coast fever and Corridor disease in buffalo from Zambia

Chimvwele N. Choopa [a,b], Walter Muleya [c], Paul Fandamu [d], Lubembe D. Mukolwe [e], Kgomotso P. Sibeko-Matjila [a,*]

[a] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, Pretoria 0110, South Africa
[b] Central Veterinary Research Institute, Department of Veterinary Services, Ministry of Fisheries and Livestock, Lusaka 10101, Zambia
[c] Department of Biomedical Sciences, School of Veterinary Medicine, University of Zambia, Lusaka 10101, Zambia
[d] Department of Veterinary Services, Ministry of Fisheries and Livestock, Lusaka 50060, Zambia
[e] Department of Veterinary Pathology, Microbiology & Parasitology, Faculty of Veterinary Medicine and Surgery, Egerton University, Egerton, Kenya

ARTICLE INFO

Keywords:
Theileria parva
Theileriosis
East Coast fever
Corridor disease
Buffalo
p67
Buffalo-derived *T. parva*

ABSTRACT

Theileriosis caused by *Theileria parva* infections is responsible for high cattle mortalities in Zambia. Although infected buffalo are a risk to cattle, the characterization of *T. parva* parasites occurring in this host in Zambia has not been reported. Furthermore, considering the advances in the development of a p67 subunit vaccine, the knowledge of p67 genetic and antigenic diversity in both cattle and buffalo associated *T. parva* is crucial. Therefore, blood samples from buffalo (n=43) from Central, Eastern and Southern provinces, and cattle (n=834) from Central, Copperbelt, Eastern, Lusaka, and Southern provinces, were tested for *T. parva* infection and the parasites characterized by sequencing the gene encoding the p67 antigen. About 76.7 % of buffalo and 19.3 % of cattle samples were PCR positive for *T. parva*. Three of the four known p67 allele types (1, 2 and 3) were identified in parasites from buffalo, of which two (allele types 2 and 3) are associated with *T. parva* parasites responsible for Corridor disease. Only allele type 1, associated with East Coast fever, was identified from cattle samples, consistent with previous reports from Zambia. Phylogenetic analysis revealed segregation between allele type 1 sequences from cattle and buffalo samples as they grouped separately within the same sub-clade. The high occurrence of *T. parva* infection in buffalo samples investigated demonstrates the risk of Corridor disease infection, or even outbreaks, should naïve cattle co-graze with infected buffalo in the presence of the tick vector. In view of a subunit vaccine, the antigenic diversity in buffalo associated *T. parva* should be considered to ensure broad protection. The current disease control measures in Zambia may require re-evaluation to ensure that cattle are protected against buffalo-derived *T. parva* infections. Parasite stocks used in 'infection and treatment' immunization in Zambia, have not been evaluated for protection against buffalo-derived *T. parva* parasites currently circulating in the buffalo population.

1. Introduction

Theileria parva, a protozoan parasite transmitted by the brown ear tick, *Rhipicephalus appendiculatus,* causes theileriosis in cattle (Blouin and Stoltsz, 1989; De Vos, 1981; Waladde et al., 1993). The African buffalo (*Syncerus caffer*) is the natural reservoir of various cattle disease-causing pathogens, including *T. parva* and a carrier of several other *Theileria* species that infect cattle, such as the relatively benign *T. mutans, T. velifera* and *T. taurotragi* (Norval et al., 1992; Samish et al., 2004). Bovine theileriosis is widespread in eastern, central and southern Africa, affecting 12 countries including Zambia (Lawrence et al., 1992). The first case of theileriosis in Zambia was reported in 1922, in the Nakonde area of the now Muchinga Province, while Chipata in Eastern Province diagnosed its first case in 1947 (Mangani and Chizyuka, 1985). The Hufwa area of Monze district in Southern Province, experienced a malignant form of theileriosis in 1977/78 (Mangani and Chizyuka, 1985). In the Eastern Province of Zambia, fatalities due to *T. parva* infections have been reported to be as high as 60 %, although, this could

* Corresponding author.
E-mail address: kgomotso.sibeko@up.ac.za (K.P. Sibeko-Matjila).

https://doi.org/10.1016/j.vetpar.2024.110240
Received 13 February 2024; Received in revised form 21 June 2024; Accepted 21 June 2024
Available online 24 June 2024

be an underestimate due to underreporting resulting from the assumption that most of the deaths in cattle are of natural causes (Billiouw et al., 2002). Nonetheless, approximately 1.4 million cattle, of the total 4.7 million cattle in Zambia, are at risk of the disease (Mulenga et al., 2018; The 2022 livestock survey report, 2022). Furthermore, the African buffalo is among the major wildlife that attracts tourists to the country, even though it has a large effect on the livestock industry due to it being a reservoir for various pathogens that infect livestock (Munang'andu et al., 2006).

Infections with *T. parva* manifest in three disease syndromes in cattle, namely East Coast fever (ECF), January disease and Corridor disease (Perry et al., 1991; Uilenberg et al., 1982), with the first two caused by cattle-derived *T. parva* and the latter by buffalo-derived *T. parva*. Parasites classified as cattle-derived *T. parva* are associated with, or adapted to cattle as the host of origin, while buffalo-derived *T. parva* parasites are associated with origin of infection from contact with infected buffalo (Medley et al., 1993). In addition, infection with buffalo-derived parasites does not result in carrier state as the infected animals die before the parasite develops to the tick infective stage or recover and clear the infection within a few months (Mbizeni et al., 2013; Norval et al., 1992; Uilenberg, 1999). Furthermore, from the antigenic perspective, cattle-derived *T. parva* parasites are homologous while buffalo-derived parasites have greater antigenic diversity (Grootenhuis, 1989; Maritim et al., 1992). Consequently, live vaccines, mostly based on cattle-derived parasite stocks, fail to provide protection against the latter.

The gene encoding the sporozoite antigen p67 has been characterized and four allele types (types 1, 2, 3 and 4) that distinguish cattle- and buffalo-derived *T. parva* parasites identified (Nene et al., 1996; Sibeko et al., 2010). p67 allele type 1 is generally associated with cattle-derived *T. parva* (Nyagwange et al., 2018), although the allele has also been detected in buffalo-derived parasites (Sibeko et al., 2010). However, recent reports have identified sequence variations within the p67 allele type 1 that differentiate *T. parva* strains derived from cattle and buffalo (Mukolwe et al., 2020). p67 allele types 2, 3 and 4 are exclusively associated with buffalo-derived *T. parva* strains (Nene et al., 1999; Sibeko et al., 2010; Sitt et al., 2019).

In Zambia, the disease caused by *T. parva* infections is usually referred to as ECF or Corridor disease (Nambota, 1991) although there are no confirmed reports of the latter in cattle. There are 10 provinces in Zambia (Fig. 1). In the past, ECF was confined to Eastern, Northern, Muchinga and Southern Provinces. However, the disease has now spread to former ECF-free provinces like Central and Copperbelt provinces (Nambota et al., 1997).

Zambia takes a multifaceted approach to the control of theileriosis, involving a combination of vector control using acaricides, cattle movement control, chemotherapy and immunization by the infection and treatment method (ITM). In southern African countries, other than South Africa where immunization is not allowed, immunization by ITM employs a single *T. parva* stock, while countries in eastern Africa use a cocktail which comprises of three *T. parva* stocks, namely Muguga, Kiambu 5 and Serengeti-transformed (Morzaria et al., 2000), known as the Muguga cocktail. Thus, two individual *T. parva* stocks are used in Zambia for immunization against *T. parva*, with Chitongo used in the Southern Province, and Katete in the Eastern Province (Makala et al., 2003). The two vaccine stocks are from unimodal and bimodal regions respectively (Billiouw et al., 2002; Mulumba et al., 2001); these are regions where the tick developmental stages occur once or twice per year, respectively. Chitongo and Katete vaccine stocks only provide protection against homologous challenge (Uilenberg, 1999). The Department of Veterinary Services in the Ministry of Fisheries and Livestock extended the ITM to some districts of Central, Copperbelt and Lusaka provinces of the country in 2018. To date, ITM is the only means used to immunize cattle against *T. parva* infections (Bastos et al., 2019; Patel et al., 2019) and there are no parasite stocks that provide protection against the buffalo-derived *T. parva* parasites.

Theileria parva parasites from cattle in Zambia have been characterized by use of monoclonal antibody (MAb) profiles, comparing isolates obtained from the Food and Agricultural Organization (FAO) (1982–1986) and Belgian Animal Disease Control Project (ASVEZA) (1985–1989) with those collected between 1994 and 1995 (Nambota

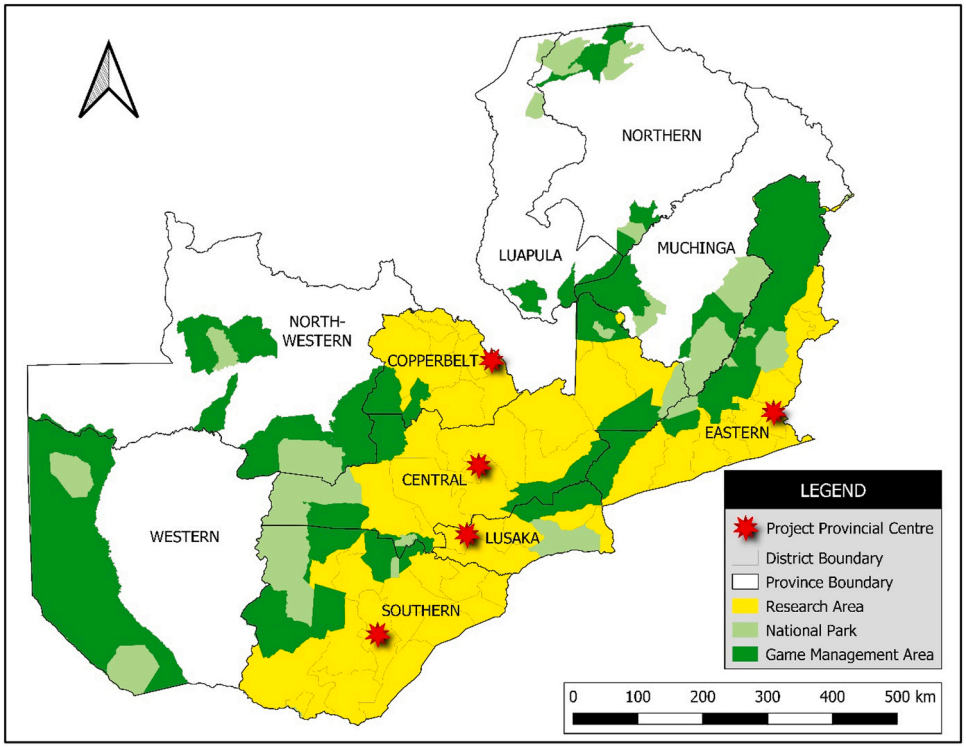


Fig. 1. Map of Zambia showing locations of the various provinces that formed part of the research sites. (Map provided by the Ministry of Fisheries and Livestock, Zambia).

et al., 1997). The latter isolates were obtained following the introduction of ITM using the Muguga cocktail and showed similar MAb profiles to the immunizing stocks, which were different from those of the old stocks, highlighting the introduction of antigenic diversity by foreign vaccine stocks. The Zambian local ITM vaccine stocks, Chitongo and Katete, and a limited number of other cattle-derived *T. parva* isolates from Zambia, have also been characterized using genes encoding antigen genes polymorphic immunodominant molecule (PIM), p104 and p150 by PCR-restriction fragment length polymorphism (RFLP) (Geysen et al., 1999). Results from this study corroborated the MAb study, with the genetic data generally showing a homogenous, clonal population structure, with the exception of one isolate, Zam 5. Subsequently, selected *T. parva* isolates, including Zam5, Chitongo and Katete, were characterized using the gene encoding the p67 antigen, confirming Zam 5 to be a buffalo-derived isolate and the two vaccine stocks were profiled as cattle-derived isolates with p67 allele type 1 associated with ECF (Mukolwe et al., 2020; Sibeko et al., 2010). Notably, previous characterization studies were mostly performed in experimentally infected cattle and *in vitro* cultures.

To date, data on the genetic diversity of *T. parva* parasites from cattle field samples, especially from former ECF-free provinces, and buffalo in Zambia remain scanty. The first analysis of *T. parva* parasites from the African buffalo in Zambia was reported over a decade ago, done using serological methods on samples collected between 1996 and 2005 from the wildlife-livestock interface localities (Munang'andu et al., 2009). This study therefore endeavored to perform molecular characterization of cattle- and buffalo-derived *T. parva* parasites using the p67 antigen gene. The study also investigated the possible presence of buffalo-derived *T. parva* parasites in cattle, including those from former ECF-free provinces.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Research and Animal Ethics Committees of the Faculty of Veterinary Science, University of Pretoria (REC Certificate # REC258–19). Additional approvals for collection of blood samples from cattle in Zambia, and import permits were obtained from the Ministry of Fisheries and Livestock, Zambia, and the Department of Agriculture, Land Reform and Rural Development, South Africa, respectively. Approval for the use of archived blood samples from buffalo was also obtained from the same authorities.

2.2. Sample collection and DNA extraction

Blood samples used in this study were collected from 834 crossbred cattle, two years or older, from Central (n=190), Copperbelt (n=148), Eastern (n=198), Lusaka (n=126) and Southern (n=172) provinces of Zambia (Table 1). The sample collection was done between 2018 and 2021, at no particular time of the year. Whole blood (6 ml) was collected in EDTA vacutainers (Becton Dickinson, Oxford, UK) from the jugular vein of cattle. Forty-three buffalo blood samples were obtained from the Central Veterinary Research Institute sample repository, previously collected from game ranches in Central Province (n=14), as well as national parks in Eastern (n=20) and Southern (n=9) provinces (Table 1). DNA was extracted from blood samples using the PureLink® Genomic DNA Kit (Invitrogen, Waltham MA, USA), according to the manufacturer's protocol. The DNA was eluted in 100 µl of the elution buffer and stored at 4 °C and −20 °C for short- and long-term storage respectively.

2.3. Screening for Theileria parva infection

Detection of *T. parva* genomic DNA in cattle and buffalo samples was done using a p104 gene-based nested PCR assay (Skilton et al., 2002). A total reaction volume of 12.5 µl consisted of 1.25 µl 10X DreamTaq buffer, 0.0625 µl of 5 U/µl DreamTaq DNA polymerase (ThermoFisher Scientific™, Waltham MA, USA), 0.25 µl of 2 mM dNTPs, 0.25 µl of 10 µM of each forward and reverse primer, 3 µl DNA template and 7.44 µl RNase-free water. The amplification conditions were as previously described (Skilton et al., 2002). The amplified products were analyzed by gel electrophoresis on 2 % GelRed pre-stained agarose gel.

Table 1
Provincial and district information of the origin of cattle and buffalo samples.

Province	District	Cattle samples	Buffalo samples
Central	Chisamba	20	0
	Chibombo	121	9
	Kabwe	20	0
	Kapiri Mposhi	27	0
	Mumbwa	2	0
Copperbelt	Masaiti	12	0
	Mpongwe	136	0
Lusaka	Chongwe	121	0
	Chilanga	5	0
Eastern	Chipata	53	0
	Katete	13	0
	Petauke	26	0
	Sinda	106	0
	Lumezi	0	20
Southern	Choma	15	0
	Gwembe	20	0
	Monze	129	0
	Namwala	8	0
	Itezhi-Tezhi	0	14
TOTAL		**834**	**43**

2.4. PCR amplification of the p67-encoding gene

A nested PCR targeting the central variable region of the p67 antigen gene was performed using the outer primers IL 246 (5'-CGACACTGAACGATGCAAATA-3') and IL 247 (5'-GAGTTATTGTTAGTGGACGAT-3') for amplification of a 2.2 kbp DNA fragment, and the inner primer set IL 613 (5'-ACAAACACAATCCCAAGTTC-3') and IL 792 (5'-CCTTTACTACGTTGGCG-3') to amplify the variable internal gene fragment with the size range of 800–1100 bp (Nene et al., 1996). The PCR reaction mixture consisted of 1.25 µl 10X DreamTaq buffer (ThermoFisher Scientific™, Waltham MA, USA), 0.25 µl of 2 mM dNTPs, 0.25 µl of 10 µM of each forward and reverse primer, 0.0625 µl of 5 U/µl DreamTaq DNA polymerase, and 3 µl DNA template. Sterile PCR-grade water was added to make up a final volume of 12.5 µl. The same reaction mixture was used for the secondary PCR, with 1 µl of the amplicon from the primary PCR used as a DNA template. The thermal amplification profiles for both PCR assays were as previously described (Nene et al., 1996). PCR products were assessed by gel electrophoresis on a 2 % GelRed pre-stained agarose gel.

2.5. Sanger sequencing

The p67 PCR products obtained from cattle samples were purified directly from the PCR reaction mix using the PureLink PCR Purification Kit (ThermoFisher Scientific™, Waltham MA, USA) according to the manufacturer's protocol. Due to mixed infections with parasites possessing different p67 alleles, PCR products from buffalo samples were purified from bands cut out from agarose gel using sterile scalpel blades. Sixty-eight amplicons, including 46 from cattle and 22 from buffalo samples, were subjected to bi-directional DNA sequencing using Sanger sequencing, conducted either at LGC Genomics GmbH (Germany) using the ABI 3730XL DNA Sequencer (Applied Biosystems) or University of Zambia, School of Veterinary Medicine using the ABI 3500 Genetic Analyzer (Applied Biosystems, Waltham MA, USA).

2.6. Sequence data analysis

The quality of p67 sequences was assessed using Trev from the Staden package (Staden et al., 2000) and were confirmed by sequence similarity search using the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/geo/query/blast.html). The assembly of forward and reverse sequences, as well as sequence editing were performed using the Staden package 2.0.0b11–2016 (Staden, 1996). Generated consensus sequences were translated to predicted protein sequences and multiple alignments of both nucleotide and protein sequences were prepared in the CLC Main Workbench 21.0.3 (Qiagen, Hilden, Germany) using the Needleman–Wunsch algorithm, for comparison with reference sequences of different p67 allele types from the GenBank (Supplementary material: Table S1) and to prepare sequences for phylogenetic analysis.

2.7. Phylogenetic analysis

The maximum likelihood phylogenetic analysis was conducted in MEGA 11 (Tamura et al., 2021). To determine the best evolutionary model for our dataset, a model test was performed using MLModelTest from MEGA 11. The phylogenetic analysis was then performed using the HKY+G model, as determined by MLModelTest. A total of 39 sequences were analysed, including 17 generated from the current study and 22 reference sequences representing p67 allele types 1, 2, 3 and 4 (Supplementary material: Table S1). A total of 617 nucleotide positions in the p67 variable region were considered in the analysis with 1000 bootstrap replicates as confidence interval used during the construction of the phylogenetic tree.

3. Results

3.1. PCR detection of Theileria parva infection

Theileria parva DNA was detected in 22.1 % (161/834 cattle and 33/43 buffalo) of samples investigated (Fig. 2). The highest percent of infections in cattle was detected in Lusaka Province (27.8 %) while the lowest was from Copperbelt Province (11.5 %). Buffalo from various provinces had a nearly comparable percentage of infected samples, with 80.0 % (16/20), 77.8 % (7/9) and 71.4 % (10/14) in Eastern, Southern and Central provinces respectively.

3.2. p67 gene characterization of Theileria parva isolates

3.2.1. p67 amplicon size discrimination

Out of the 194 *T. parva* positive DNA samples, the central variable region of p67 gene was successfully amplified in 68 (35.1 %) samples, due to low sensitivity of the conventional PCR assay used, especially evident in low infection samples. Amplified samples consisted of 46 amplicons from cattle samples and 22 from buffalo (Fig. 2). Following agarose gel electrophoresis, same size amplicons (900 bp) were observed in all cattle samples, and multiple size amplicons including 800 bp (n=1), 900 bp (n=12) and 1100 bp (n=19), were obtained from buffalo samples.

3.2.2. Identification of p67 allele types

A total of 78 p67 sequences were obtained from the 68 samples that generated p67 amplicons. More sequences, in comparison with the analyzed sample number, were obtained due to buffalo samples that had multiple bands. Sequence analysis revealed three of the four known p67 allele types, including allele type 1 from 900 bp amplicons (n=46 from cattle; n=12 from buffalo), type 2 from 1100 bp amplicons (n=1 from buffalo) and type 3 from 800 bp amplicons (n=19, only from buffalo) (Fig. 3). Only p67 allele type 1 was detected in both cattle and buffalo sequences, and was the only allele detected from cattle samples. The only p67 allele type 2 sequence obtained was detected in buffalo from Southern Province.

3.2.3. Phylogenetic relationship of p67 antigen gene sequences from Theileria parva parasites from cattle and buffalo

The phylogenetic analysis of p67 antigen gene sequences revealed two major clades, A and B (Fig. 4). Clade A consists of allele types 1 and 2 with their variants, while clade B consists of allele types 3 and 4. There was further segregation of allele type 1 sequences within sub-clade 1, with sequences from buffalo samples grouping together in sub-group 1.1, and sequences from cattle samples in sub-group 1.2 (Fig. 4). The sequence clustering showed no geographical association. Only one allele type 2 sequence was obtained from buffalo samples in this study, and it grouped together with allele type 2 reference sequences in sub-clade 2.

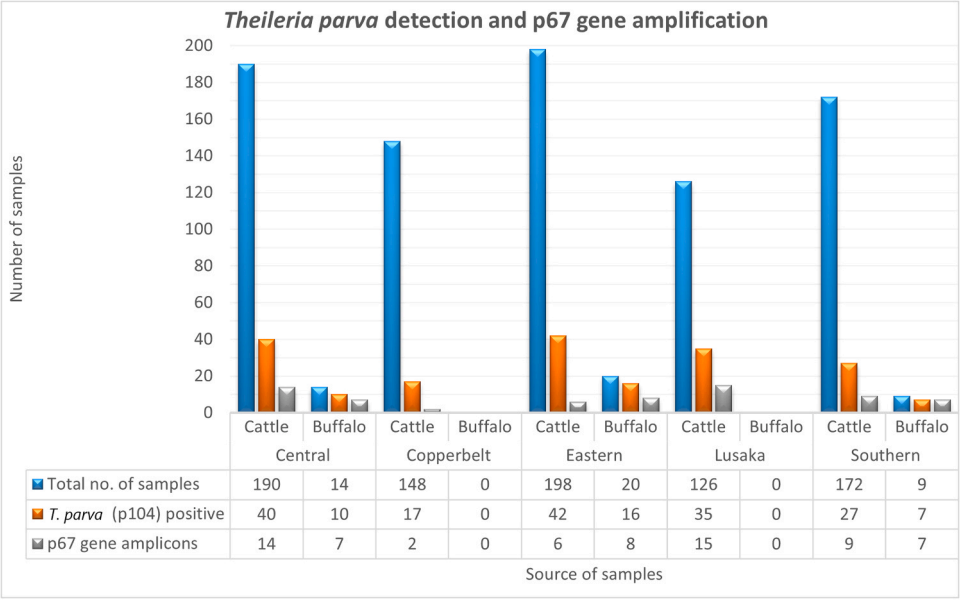
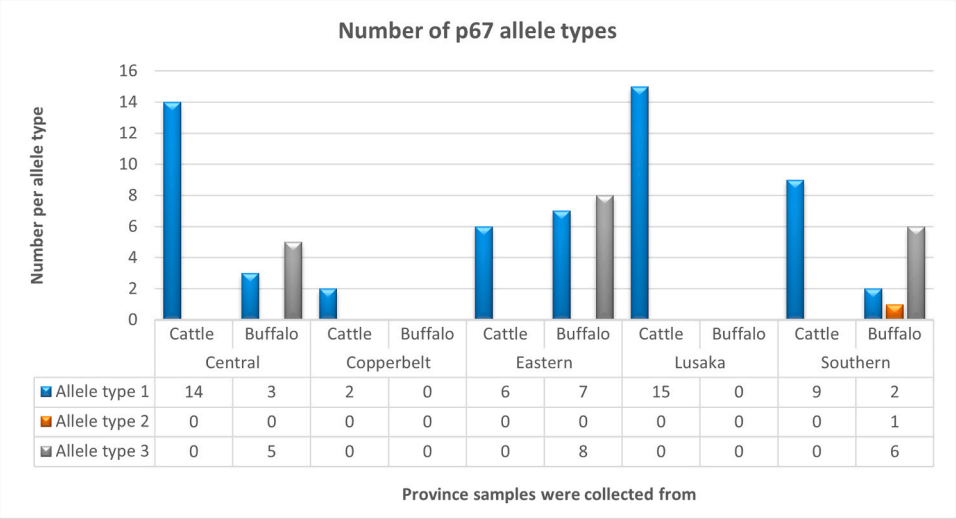


Fig. 2. Detection of *T. parva* and amplification of the central variable region of the p67 antigen gene in blood samples collected from cattle and buffalo, from five provinces in Zambia.



	Central		Copperbelt		Eastern		Lusaka		Southern	
	Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo
Allele type 1	14	3	2	0	6	7	15	0	9	2
Allele type 2	0	0	0	0	0	0	0	0	0	1
Allele type 3	0	5	0	0	0	8	0	0	0	6

Fig., 3. p67 allele types identified from DNA sequence analysis of amplicons obtained from cattle and buffalo samples from five provinces in Zambia.

3.3. p67 epitope sequence variations

The two B-cell epitopes recognized by murine monoclonal antibodies, TpM12 and AR22.7, which are found on the p67 predicted protein sequences (Nene et al., 1999; Obara et al., 2015) were used in the analysis of the sequence variations that may affect the antigenic nature of p67. From the analysis of the sequences in the two p67 epitope regions, it was noted that there were no amino acid substitutions in all p67 allele type 1 sequences from cattle samples for both epitopes, while there was only one substitution in the B-cell epitope, TpM12, from sequences from buffalo samples (Table 2). The amino acid substitution (TKEEVPPADLSDQV**L**) detected in the latter is consistent across all the three provinces (Central, Eastern and Southern) where buffalo samples were collected and it was similar to the one previously reported for the reference sequence from buffalo from Mozambique (accession number MT199345), except for an additional substitution of E for K (T**E**EEVPPADLSDQV**L)** (Table 2).

The sequence analysis of the two epitopes in allele type 2 did not show any sequence variations when compared to reference sequences (Table 3). TpM12 epitope region in allele type 3 sequences from buffalo from Zambia showed amino acid substitutions consistent with those from the reference sequences from buffalo (Table 3). However, there were two substitutions exclusive to sequences from Zambia, **P**KEEVPP**PSDSE**— and TKEEVPP**TSDSE**—, detected from the same allele type. No allele type 4 sequences were detected in the current study.

4. Discussion

The characterization of the gene encoding the p67 antigen has been used to evaluate the genetic diversity of *T. parva* parasites in various studies of which the locus has allowed distinction of ECF and Corridor disease causative agents (Mukolwe et al., 2020; Nene et al., 1999; Sibeko et al., 2010). Molecular characterization of *T. parva* using various loci, other than p67, has been extensively performed for parasites from cattle in Zambia (Muleya et al., 2012, 2022; Nambota et al., 1997). Hence, this study marks the initial effort to characterize parasites from buffalo and cattle from Zambia, based on the p67 antigen gene allelic diversity. Given that the African buffalo serves as a reservoir host for *T. parva*, it is essential to characterize parasites circulating in buffalo, especially with the increasing risk posed by combined game and livestock farming ventured into by farmers in some areas of Zambia. Transmission of buffalo-derived parasites to naïve cattle will be devastating to the country that is yet to identify ITM vaccine stocks that can protect against these parasites.

The PCR amplification of the *T. parva* p104 gene showed a high occurrence of infection in investigated buffalo samples, ranging between 72 % and 80 %. Two of the three provinces where buffalo samples were collected recorded the highest occurrence of infection, Eastern (80 %) and Southern (78 %). The high occurrence of infections in buffalo is a potential risk of infection to cattle should the two hosts co-graze in the presence of the tick vector, leading to an outbreak of Corridor disease. Notably, buffalo-derived parasite infections were not detected in cattle samples that were investigated. However, infection with these parasites has been reported over 20 years ago in Zambia (Geysen et al., 1999), from a cattle sample, Zam5, which was later reported to possess the buffalo associated p67 allele type 3 (Sibeko et al., 2010). The sample was collected from a naturally infected bovine in the Southern Province (Geysen et al., 1999). Most cattle farmers in Southern Province practise transhumance farming, thus they move their cattle to the Kafue flats which is an area that borders the Kafue National Park, a home to a large number of game animals including the African buffalo. Hence, the findings from our study become challenging to explain as cattle samples from Eastern and Southern provinces were collected in areas where interaction of cattle and buffalo is possible since there are no fences that separate the national parks from the cattle keeping communities. One possible explanation for this result could be, since Corridor disease is a self-limiting disease (Latif et al., 2001; Young et al., 1986), cattle that are infected with these parasites die and those that recover clear the infection (Choopa, 2015; Mbizeni et al., 2013). The latter may be a likely scenario if cattle in these areas are not constantly challenged by buffalo-derived *T. parva*-infected ticks. Therefore, with a 'once off' tick transmission, the infection cannot be established at high levels, thus remains below the limit of detection, especially when characterization is by conventional PCR-based tools whose sensitivity depends on high amounts of the target nucleic acid material. It is also possible that, with the high rate of deforestation in Zambia to open up land in the country, wild animal populations may move further away from areas that are gradually being inhabited by humans, thus reducing the likelihood of interaction with domesticated animals. On the contrary and consistent with the absence of buffalo-derived infection in cattle from Central Province, buffalo in game ranches from this province are fenced off therefore reducing the possibility of sharing common grazing with cattle.

Three of the four known p67 alleles including allele types 1, 2 and 3, from amplicons generated from buffalo samples, were confirmed by DNA sequencing. The three allele types were detected in samples from all three provinces where the buffalo originated. Sequences associated

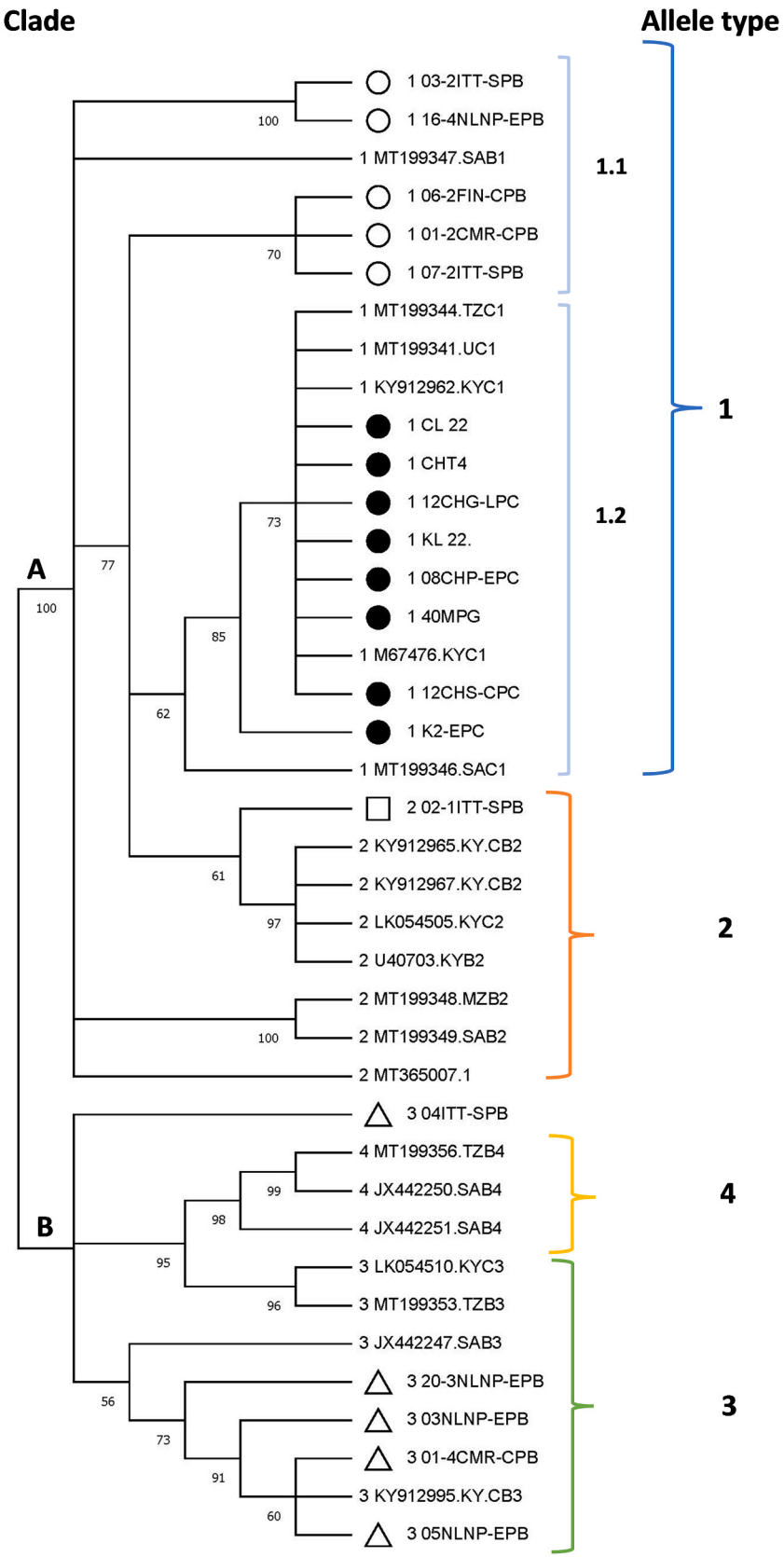


Fig. 4. The maximum likelihood phylogenetic tree showing the phylogenetic relationship of *T. parva* parasites based on the representative p67 gene sequences (n=17) in reference to published sequences (n=22) from parasites from eastern and southern Africa. Three of the four known p67 allele types were detected from *T. parva* parasites from cattle and buffalo from five provinces (Central, Copperbelt, Eastern, Lusaka and Southern) in Zambia. The sequences marked with circles, triangles, squares highlight sequences from Zambia, obtained from the current study. Sequences from buffalo have hollow shapes while shapes indicating sequences from cattle are filled.

Table 2
Epitope polymorphisms within predicted p67 amino acid sequences of allele types 1 from buffalo and cattle samples.

Seq. ID	**Species**	Geographic origin	Accession Number (references)	p67 epitope		Allele type
				TpM12	**AR22.7**	
Muguga	Cattle	**Kenya**	**M67476.1** (Nene et al., 1996)	**TKEEVPPADLSDQVP**	**LQPGKTS**	1
05CHG2	Cattle	Lusaka Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
CHT4	Cattle	Southern Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
K2	Cattle	Eastern province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
11KBW	cattle	Central Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
SERAMPG	Cattle	Copperbelt Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
Chitongo	Cattle	Southern Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
Katete	Cattle	Eastern Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
ILRI-1	Cattle	**Kenya**	**KY912962.1** (Sitt et al., 2019)	**TKEEVPPADLSDQVP**	**LQPGKTS**	1
ILRI-2	Buffalo	**Kenya**	**KY912963.1** (Sitt et al., 2019)	**TKEEVPPADLSDQVP**	**LQPGKTS**	1
02–1FIN	Buffalo	Central Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	1
01–2CMR	Buffalo	Central Province	Present study	TKEEVPPADLSDQV**L**	LQPGKTS	1
06–2FIN	Buffalo	Central Province	Present study	TKEEVPPADLSDQV**L**	LQPGKTS	1
07–2ITT	Buffalo	Southern Province	Present study	TKEEVPPADLSDQV**L**	LQPGKTS	1
FU-N17	Cattle	**Kenya**	**MT365007** (Mwamuye et al., 2020)	TKEEVPPADLSDQV**L**	L**P**PGKTS	1
Moz_Buf_5c	Buffalo	**Mozambique**	**MT199345.1** (Mukolwe et al., 2020)	**T**EEEVPPADLSDQV**L**	LQPGKTS	1

Information on reference sequences is highlighted in bold font.
Amino acid substitutions in the B-cell epitopes TpM12 (TKEEVPPADLSDQVP) and AR22.7 (LQPGKTS) sequences are bold and underlined.

Table 3
Polymorphisms within the epitope regions of predicted p67 amino acid sequences of allele types 2, 3 and 4 from buffalo samples.

Sequence ID	**Species**	Geographic origin	Accession Number (references)	p67 epitope		Allele type
				TpM12	**AR22.7**	
Moz_Buf_10	Buffalo	**Mozambique**	**MT199348.1** (Mukolwe et al., 2020)	**TKEEVPPADLSDQVP**	**LQPGKTS**	2
02–1ITT	Buffalo	Southern Province	Present study	TKEEVPPADLSDQVP	LQPGKTS	2
ILRI-4	Buffalo/Cattle	**Kenya**	**KY912965.1** (Sitt et al., 2019)	**TKEEVPPADLSDQVP**	**LQPGKTS**	2
ILRI-6	Buffalo/Cattle	**Kenya**	**KY912967.1** (Sitt et al., 2019)	**TKEEVPPADLSDQVP**	**LQPGKTS**	2
Hlu3	Buffalo	**South Africa**	**AF079176** (Nene et al., 1996)	**TKEEVPPADLSDQVP**	**LQPGKTS**	2
KNP102–9	Buffalo	**South Africa**	**JX442247.1** (Sibeko et al., 2010)	**TKEEIPPKSDSE—**	-	3
01–4CMR	Buffalo	Central Province	Present study	TKEEVPP**KSDSE**—	-	3
03ITT-SPB	Buffalo	Southern Province	Present study	**P**KEEVPP**PSDSE**—	-	3
03NLNP	Buffalo	Eastern Province	Present study	TKEEVPP**KSDSE**—	-	3
04ITT	Buffalo	Southern Province	Present study	TKEEVPP**TSDSE**—	-	3
05NLNP	Buffalo	Eastern Province	Present study	TKEEVPP**KSDSE**—	-	3
18NLNP	Buffalo	Eastern Province	Present study	TKEEVPP**KSDSE**—	-	3
TZ_TC3	Buffalo	**Tanzania**	**MT199353.1** (Mukolwe et al., 2020)	**TKEEVPPASSSD—**	-	3
ILRI-34	Buffalo/Cattle	**Kenya**	**KY912995.1** (Sitt et al., 2019)	**TKEEVPPASSSD—**	-	3
Moz_Buf_9	Buffalo	**Mozambique**	**MT199351.1** (Mukolwe et al., 2020)	**TKEEIPPKSDSE—**	-	3
TZ_TA1	Buffalo	**Tanzania**	**MT199356.1** (Mukolwe et al., 2020)	**TKEEVPPASGSD—**	-	4
KNP102–26	Buffalo	**Tanzania**	**JX442250.1** (Sibeko et al., 2010)	**TNEEVPPADLSD—**	-	4
KNPW8–48	Buffalo	**South Africa**	**JX442251.1** Sibeko et al., 2010)	**TKEEVPPASSSD—**	-	4

Information on reference sequences is highlighted in bold font.
Amino acid substitutions in the B-cell epitopes TpM12 (TKEEVPPADLSDQVP) in allele type 2, 3 and 4 sequences, and AR22.7 (LQPGKTS) in allele type 2 sequences are highlighted in bold and underlined.

with allele type 1 were also detected from cattle samples, and this was the only allele type that was found in these samples. p67 allele type 1 is associated with cattle-derived *T. parva* parasites responsible for ECF (Nene et al., 1996; Sibeko et al., 2010), and has been previously detected in the two Zambian vaccine strains, Chitongo and Katete (Mukolwe et al., 2020; Sibeko et al., 2010). Consistently, the phylogenetic analysis of p67 gene sequences from cattle field samples collected from the five provinces of Zambia showed a close relationship with reference sequences from the two vaccine strains, Chitongo (Genebank accession OR760218) and Katete (Genebank accession OR760219) (Mukolwe et al., 2020), and the ECF strain from Kenya, *T. parva* Muguga (Genebank accession M67476). It is not clear though if the detected parasites in the current study are from the vaccine stocks used in Zambia or they are all field strains. Therefore, a multi-loci analysis is needed to differentiate vaccine strains from field strains so that the true epidemiology of theileriosis in Zambia can be accurately determined, especially among non-clinical cases. Nonetheless, the detection of a homogenous *T. parva* population possessing the p67 allele type 1 has also been reported in other countries such as Burundi (Atuhaire et al., 2021) and Kenya (Mukolwe et al., 2020). Two p67 B cell epitope regions, TpM12 and AR22.7, are recognized by murine monoclonal antibodies (MAbs) that neutralize sporozoite infectivity (Nene et al., 1999). Their analysis from allele type 1 sequences indicated limited sequence diversity of the p67 gene in cattle-derived *T. parva* parasites, a finding that is consistent with previous reports (Mukolwe et al., 2020; Nene et al., 1996; Obara et al., 2015). On the contrary, sequences from the buffalo-derived *T. parva* parasites in Zambia exhibited high sequence diversity, especially in two of the three p67 allele types (1 and 3) detected. Considering that p67 is a candidate gene for the development of a subunit vaccine (Lacasta et al., 2018; Musoke et al., 2005; Nene et al., 1992), a careful consideration of antigenic diversity from buffalo-derived parasite isolates is necessary, especially in areas where there is a risk of transmission of buffalo-derived parasites into cattle.

Amplicons of the size associated with allele type 2 (1100 bp) were obtained from eight buffalo samples from Central (n=4), Eastern (n=1) and Southern (n=3) provinces; however, only one amplicon produced a good sequence. Although allele type 2 is generally widely distributed among buffalo-derived *T. parva* parasites, it appears to occur in low levels in the investigated samples; hence, efforts to sequence DNA from parasites possessing this allele type were not successful. The majority of

buffalo-derived parasites possessed p67 allele type 3 (n=19), and this allele was detected from all provinces where buffalo samples were obtained, suggesting the wide distribution of these parasites in the buffalo populations and without regional segregation. Allele type 4 was not detected in buffalo samples from this study; however, it may be that parasites possessing this allele type could be present at low levels in the buffalo population in Zambia. Previous studies where parasites possessing this allele type have been reported, suggest low occurrence compared to other allele types (Mukolwe et al., 2020; Sibeko et al., 2010). Since this was the first study with regards to the analysis of p67 gene in *T. parva* parasites from buffalo in Zambia, and the number of samples investigated was limited, thus more samples should be examined to ascertain the occurrence of parasites possessing allele type 4 in the Zambian buffalo population.

Theileria parva-positive cattle were detected in all five provinces in this study inclusive of the three provinces where ITM was recently introduced; Lusaka (27.8 %) with the highest occurrence of *T. parva* infection, followed by Central (21.1 %), and Copperbelt with the least number of *T. parva*-positive cattle samples (11.5 %). Historical records suggest that the spread of *T. parva* in Zambia started from Muchinga to Eastern Province and from Southern Province through Lusaka and Central provinces, then to the Copperbelt (Mangani and Chizyuka, 1985; Nambota et al., 1994). Copperbelt, which had the lowest occurrence of *T. parva* infected cattle is the furthest from the Southern Province. The varied geographic location, in relation to the Southern Province, could explain the high occurrence of positive cattle in Lusaka and the lowest occurrence in Copperbelt provinces. Furthermore, farmers in the southern region of Zambia, have in the recent past been migrating to the north, towards Lusaka, Central and Copperbelt provinces, in search of pastures for their cattle and better rainfall pattern for cropping (Cliggett, 2000). It is likely that infections were higher in Lusaka than Southern Province since there may be more naïve cattle where the disease was introduced later on as opposed to the region where the disease has been endemic for a longer period.

Considering the detection of buffalo-derived *T. parva* parasites in buffalo from the study areas in Zambia, the assertion that both ECF and Corridor disease exist in Zambia (Nambota et al., 1994) cannot be ignored. Moreover, buffalo are not restricted by any physical boundaries from interacting with cattle in most parts of Zambia. Therefore, the risk of Corridor disease outbreaks occurring at the wildlife-livestock interface remains. Some commercial farmers in Zambia have gone into game ranching and the most common game animals kept are buffalo, which are sourced from the major game parks. Some of these farms that have buffalo also keep cattle; therefore, the interaction between the two is possible and creates a situation that is likely to favor the transmission of *T. parva* parasites from buffalo to cattle. Nevertheless, in the recent history, there have not been reports of Corridor disease outbreaks in Zambia, likely because theileriosis outbreaks in Zambia are generally attributed to ECF without any characterization of the parasites responsible. Another plausible reason is that, since Corridor disease is self-limiting, animals may succumb to the disease without the knowledge of veterinary authorities due to poor reporting of disease cases that periodically lead to mortalities. This is very common with emerging and small-scale farming communities. Dipping for tick control by acaricides and widespread use of antibiotics by livestock owners could also be a contributing factor to the absence of Corridor disease cases in this country. Strict measures should be implemented to ensure that cattle do not co-graze with buffalo to avoid transmission from ticks that are already infected with buffalo-derived *T. parva* parasites.

5. Conclusion

This study revealed that *T. parva* parasites with p67 allele types associated with Corridor disease are present in the buffalo population within Zambian national parks and game ranches. Although infection with buffalo-derived *T. parva* parasites was not detected in cattle, the risk of infection exists due to occasional contact between buffalo and cattle. Consequently, implementing control measures to prevent Corridor disease outbreaks is crucial. It is not known if the current ITM interventions used in Zambia do provide protection against buffalo-derived *T. parva* parasites. Further, additional studies with a larger sample of buffalo are needed to confirm the absence of *T. parva* parasites with p67 allele type 4 and to gain more insight into the diversity of *T. parva* parasites occurring in the Zambian buffalo population.

Institutional Review Board Statement

The study protocol was approved by the Research (reference number: REC258–149) and Animal Ethics (Committees of the University of Pretoria (project AEC certificate number: V015–19) and the Ministry of Agriculture, Zambia.

Funding

This research was funded by National Research Foundation (South Africa) under Support for Rated and Unrated Researchers (SRUG190408428060). The funder had no role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

CRediT authorship contribution statement

Chimvwele Namantala Choopa: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Lubembe Donald Mukolwe:** Writing – review & editing, Methodology, Data curation. **Kgomotso Penelope Sibeko-Matjila:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization. **Paul Fandamu:** Writing – review & editing, Conceptualization. **Walter Muleya:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kgomotso P. Sibeko-Matjila reports financial support was provided by National Research Foundation, South Africa. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Acknowledgements

Authors are thankful to the field staff of the Department of Veterinary Services under Ministry of Fisheries and Livestock in Zambia for the cooperation during collection of samples from cattle and the Central Veterinary Research Institute for providing buffalo blood samples from its repository. Gratitude is also extended to the University of Pretoria, University of Zambia and Central Veterinary Research Institute for the use of their laboratories as well as support provided by staff at these laboratories. The authors further appreciate the International Atomic Energy Agency for supporting sequencing of DNA samples.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110240.

References

Atuhaire, D.K., Muleya, W., Mbao, V., Niyongabo, J., Nyabongo, L., Nsanganiyumwami, D., Salt, J., Namangala, B., Musoke, A.J., 2021. Molecular characterization and population genetics of *Theileria parva* in Burundi's unvaccinated cattle: towards the introduction of East Coast fever vaccine. PLoS One 16, 27.

Bastos, R.G., Franceschi, V., Tebaldi, G., Connelley, T., Morrison, Ivan, Knowles, W., Donofrio, D.P., Fry, G., L.M, 2019. Molecular and antigenic properties of mammalian cell-expressed *Theileria parva* antigen Tp9. Front Immunol. 10, 1–13.

Billiouw, M., Vercruysse, J., Marcotty, T., Speybroeck, N., Chaka, G., Berkvens, D., 2002. *Theileria parva* epidemics: a case study in eastern Zambia. Vet. Parasitol. 107, 51–63.

Blouin, E.F., Stoltsz, W.H., 1989. Comparative infection rates of *Theileria parva* lawrencei in salivary glands of *Rhipicephelus appendiculatus* and *Rhipicephalus zambenziensis*. Onderstepoort J. Vet. Res 56, 211–213.

Choopa, C.N., 2015. Diagnosis of tick-borne disease in cattle in Bushbuckridge, Mpumalanga, South Africa and identification of *Theileria parva* carriers. Masters dissertation, University of Pretoria.

De Vos, A.J., 1981. *Rhipicephalus appendiculatus*: cause and vector of diseases in Africa. J. S Afr. Vet. Assoc. 52, 315–322.

Geysen, D., Bishop, R., Skilton, R., Dolan, T.T., Morzaria, S., 1999. Molecular epidemiology of *Theileria parva* in the field. Trop. Med Int Health 4, 21–27.

Grootenhuis, J.G., 1989. The role of wildlife in the epidemiology of cattle Theileriosis., in: Dolan, T.T. (Eds), Proceedings of a workshop on East Coast Fever immunisation, 20–22 September 1988, Lilongwe, pp. 129–136.

Lacasta, A., Mwalimu, S., Kibwana, E., Saya, R., Awino, E., Njoroge, T., Poole, J., Ndiwa, N., Pelle, R., Nene, V., Steinaa, L., 2018. Immune parameters to p67C antigen adjuvanted with ISA206VG correlate with protection against East Coast fever. Vaccine 36 (11), 1389–1397.

Latif, A.A., Hove, T., Kanhai, G.K., Masaka, G.K., S., 2001. Laboratory and field investigations into the *Theileria parva* carrier-state in cattle in Zimbabwe. Onderstepoort J. Vet. Res 68, 203–208.

Lawrence, J.A., Perry, B.D., Williamson, M.S., 1992. East Coast fever, 2nd edition. In: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock, vol. 1. Oxford University Press, Cape Town, pp. 448–467, 2nd edition.

Cliggett, Lisa, 2000. Social components of migration experiences from Southern Province, Zambia. Hum. Organ. 59, 125–135.

Makala, L., Mangani, P., Fujisaki, K., Nagasawa, H., 2003. The current status of major tick borne diseases in Zambia. Vet. Res 34, 27–45.

Mangani, P.M.C., Chizyuka, G.H.B., 1985. Theileriosis in Zambia; in: Irvin A.D. (Eds.); Immunization against theileriosis in Zambia, Proceedings of a Joint Workshop. ILRAD, Nairobi, pp. 41–44.

Maritim, A.C., Young, A.S., Lesan, A.C., Ndungu, S.G., Stagg, D.A., Ngumi, P.N., Nguni, P. N., Ngumi, P.N., 1992. Transformation of *Theileria parva* derived from African buffalo (*Syncerus caffer*) by tick passage in cattle and its use in infection and treatment immunization. Vet. Parasitol. 43, 1–14.

Mbizeni, S., Potgieter, F.T., Troskie, C., Mans, B.J., Penzhorn, B.L., Latif, A.A., 2013. Field and laboratory studies on Corridor disease (*Theileria parva* infection) in cattle population at the livestock/game interface of uPhongolo-Mkuze area, South Africa. Ticks Tick. Borne Dis. 4, 227–234.

Medley, G.F., Perry, B.D., Young, A.S., 1993. Preliminary analysis of the transmission dynamics of *Theileria parva* in eastern Africa. Parasitology 106, 251–264.

Morzaria, S., Nene, V., Bishop, R., Musoke, A., 2000. Vaccines against *Theileria parva*. Ann. N. Y Acad. Sci. 916, 464–473.

Mukolwe, L.D., Odongo, D.O., Byaruhanga, C., Snyman, L.P., Sibeko-Matjila, K.P., 2020. Analysis of p67 allelic sequences reveals a subtype of allele type 1 unique to buffaloderived *Theileria parva* parasites from southern Africa. PLoS One 15, 1–17.

Mulenga, A., Lupindu, A.M., Mlangwa, J.E.D., 2018. Participation and its determinants in East Coast Fever immunization by small- scale cattle keepers in Mazabuka district of Zambia. Tanzan. Vet. Assoc. Proc. 36, 109–119.

Muleya, W., Atuhaire, D.K., Mupila, Z., Mbao, V., Mayembe, P., Kalenga, S., Fandamu, P., Namangala, B., Salt, J., Musoke, A.J., 2022. Sequence diversity of Tp1 and Tp2 antigens and population genetic analysis of *Theileria parva* in unvaccinated cattle in Zambia's Chongwe and Chisamba districts. Pathogens 11, 1–23.

Muleya, W., Namangala, B., Simuunza, M., Nakao, R., Inoue, N., Kimura, T., Ito, K., Sugimoto, C., Sawa, H., 2012. Population genetic analysis and sub-structuring of *Theileria parva* in the northern and eastern parts of Zambia. Parasit. Vectors 5, 255.

Mulumba, M., Speybroeck, N., Berkvens, D.L., Geysen, D.M., Brandt, J.R.A., 2001. Transmission of *Theileria parva* in the traditional farming sector in the southern province of Zambia during 1997-1998. Trop. Anim. Health Prod. 33, 117–125.

Munang'andu, H.M., Siamudaala, V., Matandiko, W., Mulumba, M., Nambota, A., Munyeme, M., Mutoloki, S., Nonga, H., 2009. Detection of *Theileria parva* antibodies in the African buffalo (*Syncerus caffer*) in the livestock-wildlife interface areas of Zambia. Vet. Parasitol. 166.

Munang'andu, H.M., Siamudaala, V.M., Nambota, A., Bwalya, J.M., Munyeme, M., Mweene, A.S., Takada, A., Kida, H., 2006. Disease constraints for utilization of the African buffalo (*Syncerus caffer*) on game ranches in Zambia. Jpn J. Vet. Res 54, 3–13.

Musoke, A., Rowlands, J., Nene, V., Nyanjui, J., Katende, J., Spooner, P., Mwaura, S., Odongo, D., Nkonge, C., Mbogo, S., Bishop, R., Morzaria, S., 2005. Subunit vaccine based on the p67 major surface protein of *Theileria parva* sporozoites reduces severity of infection derived from field tick challenge. Vaccine 23, 3084–3095.

Nambota, A., 1991. Immunisation against Theileriosis in the Southern Province of Zambia. In Dolan T.T. (Eds) Proceedings of a workshop on East Coast Fever Immunisation, Lilongwe, Malawi, 20–22 September 1988 pp. 87–89.

Nambota, A., Samui, K.L., Sugimoto, C., Kakuta, T., Onuma, M., 1994. Theileriosis in Zambia: Etiology, epidemiology and control measures. Jpn J. Vet. Res 42, 1–18.

Nambota, A.M., Lovelace, C.E.A., Chitambo, H., Kakuda, T., Sugimoto, C., Onuma, M., 1997. Characterization of some *Theileria parva* stocks from Zambia using monoclonal antibodies. J. Vet. Med Sci. 59, 1–4.

Nene, V., Iams, K.P., Gobright, E., Musoke, A.J., 1992. Characterisation of the gene encoding a candidate vaccine antigen of *Theileria parva* sporozoites. Mol. Biochem Parasitol. 51, 17–27.

Nene, V., Gobright, E., Bishop, R., Morzaria, S., Musoke, A., 1999. Linear peptide specificity of bovine antibody responses to p67 of *Theileria parva* and sequence diversity of sporozoite-neutralizing epitopes: implications for a vaccine. Infect. Immun. 67, 1261–1266.

Nene, V., Musoke, A., Gobright, E., Morzaria, S., 1996. Conservation of the sporozoite p67 vaccine antigen in cattle-derived *Theileria parva* stocks with different cross-Immunity Profiles. Infect. Immun. 64 (6), 2056–2061.

Norval, R.A.I., Perry, B.D., Young, A.S., 1992. The epidemiology of theileriosis in Africa. Academic Press, pp. 77–82.

Nyagwange, J., Tijhaar, E., Ternette, N., Mobegi, F., Tretina, K., Silva, J.C., Pelle, R., Nene, V., 2018. Characterization of the *Theileria parva* sporozoite proteome. Int J. Parasitol. 48, 265–273.

Obara, I., Ulrike, S., Musoke, T., Spooner, P.R., Jabbar, A., Odongo, D., 2015. Molecular evolution of a central region containing B cell epitopes in the gene encoding the p67 sporozoite antigen within a field population of *Theileria parva*. Parasitol. Res 114, 1729–1739.

Patel, E., Mwaura, S., Di Giulio, G., Cook, E.A.J., Lynen, G., Toye, P., 2019. Infection and treatment method (ITM) vaccine against East Coast fever: reducing the number of doses per straw for use in small holder dairy herds by thawing, diluting and refreezing already packaged vaccine. BMC Vet. Res 15, 1–6.

Perry, B.D., Kruska, R., Lessard, P., Norval, R.A.I., Kundert, K., 1991. Estimating the distribution and abundance of *Rhipicephalus appendiculatus* in Africa. Prev. Vet. Med 11, 261–268.

Rodger, Staden, 1996. The staden sequence analysis package. Mol. Biotechnol. 5, 233–241.

Samish, M., Ginsber, H., Glazer, I., 2004. Biological control of ticks. Parasitology 129, S389–S403.

Sibeko, K.P., Geysen, D., Oosthuizen, M.C., Matthee, C.A., Troskie, M., Potgieter, F.T., Coetzer, J.A.W., Collins, N.E., 2010. Four p67 alleles identified in South African *Theileria parva* field samples. Vet. Parasitol. 167, 244–254.

Sitt, T., Henson, S., Morrison, W.I., Toye, P., 2019. Similar levels of diversity in the gene encoding the p67 sporozoite surface protein of *Theileria parva* are observed in blood samples from buffalo and cattle naturally infected from buffalo. Vet. Parasitol. 269, 21–27.

Skilton, R.A., Bishop, R.P., Katende, J.M., Mwaura, S., Morzaria, S.P., 2002. The persistence of *Theileria parva* infection in cattle immunized using two stocks which differ in their ability to induce a carrier state: analysis using a novel blood spot PCR assay. Parasitology 124, 265–276.

Staden, R., Beal, K.F., Bonfield, J.K., 2000. The Staden Package, 1998. Bioinformatics Methods and Protocols. Humana Press, New Jersey, pp. 115–130.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38, 3022–3027.

The livestock survey report, 2022, Lusaka, Zambia.

Uilenberg, G., 1999. Immunization against diseases caused by *Theileria parva*: a review. Trop. Med Int Health 4 (9). A12-A20.

Uilenberg, G., Perié, N.M., Lawrence, J.A., de Vos, A.J., Paling, R.W., Spanjer, A.A.M., 1982. Causal agents of bovine theileriosis in southern Africa. Trop. Anim. Health Prod. 14, 127–140.

Waladde, S.M., Young, A.S., Ochieng, S.A., Mwaura, S.N., Kima, F.N., 1993. Transmission of *Theileria parva* to cattle by *Rhipicephalus appendiculatus* adults fed as nymphae in vitro on infected blood through an artificial membrane. Parasitology 107 (3), 249–256.

Young, A.S., Leitch, B.L., Newson, R.M., Cunningham, M.P., 1986. Maintenance of *Theileria parva* infections in an endemic area of Kenya. Parasitology 93, 9–18.