Veterinary Parasitology 269 (2019) 21–27

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Research paper

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

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ARTICLE INFO

Keywords:
Theileria parva
Buffalo
Cattle
p67
Diversity
East Coast fever
Corridor disease

ABSTRACT

Theileria parva is a tick-transmitted, apicomplexan protozoan found in buffalo (*Syncerus caffer*) and cattle in eastern, central and southern Africa. The parasite causes a fatal, lymphoproliferative disease in susceptible cattle. Previous studies have shown that the parasites in buffalo comprise a more heterogeneous population than those in cattle, which has led to the concept that the population of parasites circulating in cattle represents a restricted subpopulation of those in buffalo. The present study was undertaken to identify if and where this restriction may occur in cattle naturally infected with parasites from buffalo, by sequencing the *T. parva* p67 antigen gene from eight buffalo and 12 acutely infected cattle from the same endemic site in Kenya. From 103 sequences, we detected 44 different alleles. Nine alleles were found in both cattle and buffalo, and 17 and 18 found only in the cattle and buffalo populations respectively. Nucleotide and amino acid sequence analyses revealed a similar level of diversity of parasites in both hosts. Principal coordinates and phylogenetic tree analyses did not reveal any clustering associated with the host animals, and the number and degree of mixed *T. parva* infections was similar in the respective populations. The results suggest that any restriction in the ability of *T. parva* from buffalo to survive and be transmitted from cattle occurs after entry into and initial transformation of bovine lymphocytes.

1. Introduction

Theileria parva is a tick-transmitted, apicomplexan protozoan found in African buffalo (*Syncerus caffer*) and cattle in eastern, central and southern Africa (Norval et al., 1992), where it causes considerable economic losses (Mukhebi et al., 1992). The infective sporozoite stage of the parasite quickly enters host lymphocytes and induces them to proliferate, with the parasite dividing at the same time as the host cell (Hulliger et al., 1964; Morrison et al., 1981). The parasite causes few if any clinical signs in buffalo, whereas in cattle there are two similar but distinct forms of the disease: East Coast fever and Corridor disease caused by cattle- and buffalo-derived parasites, respectively (Irvin and Mwamachi, 1983; Neitz et al., 1955). Both forms of the disease result in high levels of mortality (> 90%) in susceptible cattle. A notable difference between the two disease types is that cattle infected with cattle-derived *T. parva* show high levels of parasitosis involving both the intralymphocytic schizont stage and the blood-borne piroplasm stage of the parasite (Irvin and Mwamachi, 1983), while cattle infected with buffalo-derived *T. parva* show low levels of schizonts in peripheral lymphoid tissues and only occasional parasitaemia (Neitz et al., 1955; Jura and Losos, 1980). Consequently, infections with buffalo-derived parasites in cattle are transmitted poorly or not at all by ticks.

Previous studies of the sequence diversity among *T. parva* antigens have revealed much greater diversity among buffalo-derived parasites than those from cattle (Pelle et al., 2011). The less heterogeneous nature of the cattle-maintained parasites, together with the shorter evolutionary history of the parasite with cattle compared to buffalo, have given rise to the theory that the parasites maintained in cattle represent a subpopulation of *T. parva* that have become adapted to tick transmission among cattle (Pelle et al., 2011). In line with this, it has been shown that passage of buffalo-derived parasites through the tick-cattle cycle results in the eventual emergence of a parasite population exhibiting the characteristics of cattle-maintained *T. parva* (Barnett and Brocklesby, 1966).

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https://doi.org/10.1016/j.vetpar.2019.04.006
Received 9 December 2018; Received in revised form 8 April 2019; Accepted 11 April 2019

Table 1
T. parva p67 reference sequences used in the sequence alignments, PCoA and phylogenetic tree analyses.

Gene Accession No.	Protein Accession No.	Allele type	Strain	Host	Country of origin	Reference
NW_876245	XP_763305	1	Muguga	Cattle	Kenya	Gardner et al., 2005
U40703	AAB06703	2	Stab. 3081	Buffalo	Kenya	Nene et al., 1996
JX442244	AFU34358	3	Marl_49	Buffalo	South Africa	Sibeko et al., 2010
JX442250	AFU34364	4	KNP102-26	Buffalo	South Africa	Sibeko et al., 2010

One possible explanation for the less heterogeneous nature of the cattle-maintained *T. parva* and the lower parasitoses observed in cattle infected with buffalo-derived parasites is that only a subset of *T. parva* enter and transform cattle lymphocytes. To examine this, sequence analysis of the *T. parva* p67 gene and protein was used to compare the diversity of parasites in the blood of cattle soon after disease development to that in buffalos grazing the same general area of the Ol Pejeta Conservancy in the Laikipia region of Kenya.

2. Materials and methods

2.1. Sample collection

The cattle comprised 12 *Bos indicus* (Boran) castrated males from ILRI's Kapiti ranch and were used as unvaccinated, control animals in a field trial undertaken in June/July 2013 on Ol Pejeta conservancy, Kenya, to assess the effectiveness of a live sporozoite vaccine against *T. parva*. Details of the field trial, including the location of the field site, have been provided previously (Sitt et al., 2015). As described in the previous article, serological results confirmed that the cattle had not been exposed to *T. parva* prior to the study. They were introduced into an area on the Ol Pejeta conservancy known to be grazed by buffalo but not by cattle, ensuring that the cattle were exposed only to buffalo-derived parasites. The area of the conservancy in which the study site was located is separated from the cattle-grazing area of Ol Pejeta by the Ewaso Nyiro river. Cattle blood samples were routinely taken by jugular venepuncture on alternate days. The samples analyzed in the current study were those taken on the nearest day prior to death or treatment, or, from the surviving animals, on days 20–23 after introduction to the study site. The clinical signs presented by the animals and the post mortem and histological observations of those that died were detailed in Sitt et al. (2015) and were typical of animals undergoing acute *T. parva* infection. No samples from vaccinated calves were used for the p67 analysis due to the possibility of amplifying genotypes derived from the vaccine. Blood samples were also obtained by jugular venepuncture from eight buffalo on Ol Pejeta after sedation by veterinary staff of the Kenya Wildlife Service in January 2012. At the time of sampling, the buffalo were located within 13 kms of the field site, although it should be noted that buffalo are free to move within the conservancy. All procedures were approved by ILRI's Institutional Animal Care and Use Committee (IACUC approvals 2011–11 and 2013–03). Sampling of buffalo was approved by the Kenya Wildlife Service (KWS/BRM/5001).

2.2. DNA extraction, p67 PCR, cloning and sequencing

The diversity within parasite populations was assessed through sequence analysis of the p67 gene of *T. parva,* which encodes a polymorphic sporozoite surface protein involved in the entry of the parasite into host lymphocytes (Musoke et al., 1984). The procedures for the DNA extractions, nested p67 PCR and cloning into plasmids have been described previously (Sitt et al., 2015). Six clones were selected from each sample and between two and all six clones per animal resulted in readable sequence.

2.3. Sequence analysis

Sequence analysis, including quality control of the chromatograms, sequence editing, alignments and predicted protein translations were conducted using Geneious version 7.1.4 created by Biomatters Ltd. (http://www.geneious.com). Prior to translation of nucleotide sequences to predicted protein sequences, the 29bp intron sequence was removed. Nucleotide and protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to identify sequences. Sequences were grouped according to the classification proposed by Sibeko et al. (2010) who defined four p67 allele types based on the presence of two indels of 129bp (absent in type 1) and 174 bp (absent in types 3 and 4). We aligned sequences within each group using Clustal Omega. (https://www.ebi.ac.uk/Tools/msa/clustalo/) as shown in Supp. Data 1-3. Details of the group-specific reference sequences used in the alignments can be found in Table 1. The p67 sequences are available in GenBank with accession numbers KY912962- KY913006. Each accession number represents a unique sequence, except KY912964 which is homologous to but much shorter than KY912965. Additional information about the sequences and accession numbers can be found in Supp. Table S1.

To estimate protein diversity, the type 2 and type 3 sequences were classified as cattle (all sequences derived from cattle) or buffalo (all sequences derived from buffalo) sequences. Diversity was not calculated for Type 1 because of the low sequence count. The average diversity within each type was calculated on the amino acid sequences by comparing the number of amino acid substitutions per site from averaging over all sequence pairs within each group. The Poisson correction model was implemented and pairwise deletion was applied to sequence gaps. 500 bootstrap replications were conducted to calculate the standard error (S.E.). All calculations were done in MEGA7. Student's paired, two-tailed *t*-test was used to assess the significance of differences in diversity between allele groups.

2.4. Principal coordinates analysis and phylogenetic tree development

Pairwise distance matrix was computed in R (version 3.4.4) from a multiple sequence alignment of all the samples together with a representative reference sequence for each type using the dist.alignment function in the seqinr package. Principal coordinates analysis (PCoA) was performed on the distance matrix using the cmdscale function in the vegan package. Short sequences (KY912978, KY912964, KY912969) were excluded from PCoA as they were uninformative.

Maximum likelihood phylogeny was inferred from sequence alignment of all samples together with a representative reference sequence for each type using PhyML (V3.1; Guindon et al., 2010) under a general time reversal model. 100 bootstrap replicates were calculated. The tree was visualised in FigTree v1.4.3.

3. Results

The p67 DNA sequences and predicted protein sequences and alignments are shown in Supp. Data 1–3, grouped according to allele type (Sibeko et al., 2010).

Table 2
Sequence and allele information from animals in the study population.

Animal	Number of sequences[1]	Number of alleles[2]	Alleles detected in individual animals[3]		
Cattle			Type 1	Type 2	Type 3
2104	6	1	2962		
2124[S]	6	3			**2979**, **2980**, 2989
2150	5	5		**2964**[4], **2967**	2978, **2983**, **2997**
2158	6	6		**2965**[4], **2970**, 2973	**2979**, **2987**, 3004
2166[S]	5	3		**2965**	**2995**, **2997**
2227[S,P]	5	5			**2979**, 2990, **2995**, 2996, 3003
2253	6	4		**2965**, 2966, 2968, 2969	
2310	6	5			**2979**, 2984, **2995**, **2997**, 2998
2325[P]	6	5		**2967**, 2974	**2979**, 2988, **2997**
2352	2	2		2975	**2995**
2381	6	2			**2979**, 2985
2384[T]	6	2			**2995**, **2997**
Total no. sequences / alleles	65	43	1	13	29
No. distinct alleles[5]	n/a	26	1	9	16
Buffalo					
301	6	3			**2979**, 2982, 3006
302	3	3			2991, 2994, 3001
303	4	4		2972	2992, **2995**, 3002
304	3	3		**2970**	**2979**, **2995**
305	6	4			**2979**, **2980**, **2997**, 2999
306	6	6	2963		2977, 2981, 2986, **2987**, 3000
307	4	4		**2967**, 2971	**2979**, **2983**
308	6	6		**2965**, 2976	**2983**, 2993, 3000, 3005
Total no. sequences / alleles	38	33	1	6	26
No. distinct alleles[5]	n/a	27	1	6	20

[1] Number of readable sequences obtained from the six clones isolated from each animal.
[2] Number of different alleles present in the sequences from each animal.
[3] Alleles found in each animal, identified by last four digits of accession number. Alleles in bold type were found in both cattle and buffalo.
[4] The sequence of 2964 is homologous to, but shorter than 2965, so these are considered to be the same allele based on the available sequences. These sequences are identical to a reference.
[5] Alleles occurring more than once are included only once in these totals.
[S] These animals survived without treatment.
[P] Piroplasms were detected in these animals.
[T] The animal survived after treatment for *T. parva* infection.

3.1. Similar numbers of distinct p67 alleles in cattle and buffalo samples

As shown in Table 2, 103 p67 sequences were obtained from the 12 cattle and eight buffalo blood samples, with at least two readable sequences being obtained from each sample. The buffalo-derived samples yielded slightly fewer sequences, with an average of 4.75 sequences per sample compared with 5.42 sequences per cattle-derived sample. From the 103 sequences, 44 alleles of the p67 gene were identified - the cattle and buffalo samples contained 26 and 27 different alleles, respectively, with 17 and 18 alleles being detected exclusively in either cattle or buffalo while nine alleles were detected in both hosts. The results demonstrate a similar level of diversity of alleles within each host population.

3.2. Similar numbers of p67 allele types from cattle and buffalo samples

Table 2 shows the different allele types detected in each animal. Type 3 alleles comprised 16 (62%) of the 26 different alleles found in cattle, and 20 (74%) of the 27 alleles found in buffalo, while type 2 alleles were represented nine (35%) and six (22%) times respectively. Two type 1 alleles differing at a single base pair were detected, one each from cattle and buffalo. There is no statistically significant difference in the representation of allele types between the cattle- and buffalo-derived parasite populations.

In terms of the absolute frequency of each allele type (*i.e.* counting an allele from every sample in which it was detected), type 3 alleles comprised 55 (72%) of the 76 alleles detected in all animals, compared to 19 (25%) and 2 (2.6%) for types 2 and 1, respectively.

3.3. Similar diversity within predicted protein sequences from cattle and buffalo samples

Predicted protein translations of the p67 sequences revealed 2, 11 and 27 distinct protein variants in the type 1, 2 and 3 allele types, respectively, (Supp. Data 1b, 2b and 3b), with protein:allele ratios of 1.0 (2:2), 0.85 (11:13) and 0.90 (27:30). As shown in Table 3, this is reflected in the ratios of non-synonymous SNPs to total SNP locations for each sequence, which were > 0.5, with the exception of KY912969.

A comparison of the protein sequence diversity in type 2 and 3 alleles between the cattle and buffalo populations showed that the type 2 sequences derived from cattle were slightly less diverse (mean diversity (d) = 0.033, +/- 0.007) than those from buffalo (d = 0.042, +/- 0.009), whereas there was equal diversity within the type 3 sequences from cattle and buffalo (d = 0.041, +/- 0.008). Interestingly, the type 3 protein sequences showed slightly more evolutionary diversity among each other (d = 0.040, +/- 0.008) than the type 2 sequences (d = 0.035, +/- 0.007).

3.4. Principal coordinates and phylogenetic analyses show no clustering into cattle- and buffalo-derived populations

PCoA revealed that the sequences clustered into the allele types described above, but there was no obvious clustering into sequences which were derived from buffalo, cattle or which were common to both (Fig. 1). It was noted that there was a clear separation of the type 2 sequences into two subclusters. The alleles in these subclusters are distinguished predominantly by a trinucleotide (AAA) insert (at residue

Table 3
SNP and predicted protein information.

Accession number	No. of Animals (cattle:buffalo)	Total No. SNP Locations[1]	Total No. Non-synonymous SNP Locations[2]	No. Non-synonymous SNP Locations/ Total No. SNP Locations	Protein ID
KY912962[3]	1 (1:0)	21	14	0.67	AVT43014[5]
KY912963[3]	1 (0:1)	20	13	0.65	AVT43015
KY912964[3]	1 (1:0)	0	0	–	**AVT43016**[5]
KY912965[3]	4 (3:1)	0	0	–	**AVT43017**[5]
KY912966[3]	1 (1:0)	1	1	1.00	AVT43018
KY912967[3]	3 (2:1)	1	1	1.00	**AVT43019**
KY912968[3]	1 (1:0)	2	2	1.00	AVT43020[6]
KY912969[3]	1 (1:0)	4	1	0.25	AVT43021[6]
KY912970[3]	2 (1:1)	27	16	0.59	**AVT43022**
KY912971[3]	1 (0:1)	31	17	0.55	AVT43023
KY912972[3]	1 (0:1)	31	19	0.61	AVT43024
KY912973[3]	1 (1:0)	28	17	0.61	AVT43025
KY912974[3]	1 (1:0)	32	18	0.56	AVT43026
KY912975[3]	1 (1:0)	35	21	0.60	AVT43027
KY912976[3]	1 (0:1)	34	20	0.59	AVT43028
KY912977[4]	1(0:1)	23	18	0.78	AVT43029[7]
KY912978[4]	1(1:0)	26	19	0.73	AVT43030[8]
KY912979[4]	10 (6:4)	37	26	0.70	**AVT43031**[9]
KY912980[4]	2 (1:1)	36	25	0.69	**AVT43032**
KY912981[4]	1 (0:1)	37	26	0.70	AVT43033
KY912982[4]	1 (0:1)	37	25	0.68	AVT43034
KY912983[4]	3 (1:2)	36	25	0.69	**AVT43035**
KY912984[4]	1 (1:0)	38	26	0.68	AVT43036
KY912985[4]	1 (1:0)	38	26	0.68	AVT43037[9]
KY912986[4]	1 (0:1)	36	25	0.69	AVT43038
KY912987[4]	2 (1:1)	29	21	0.72	**AVT43039**
KY912988[4]	1 (1:0)	34	24	0.71	AVT43040
KY912989[4]	1 (1:0)	26	19	0.73	AVT43041
KY912990[4]	1 (1:0)	26	20	0.77	AVT43042
KY912991[4]	1 (0:1)	31	23	0.74	AVT43043
KY912992[4]	1 (0:1)	35	25	0.71	AVT43044
KY912993[4]	1 (0:1)	38	26	0.68	AVT43045
KY912994[4]	1 (0:1)	35	24	0.69	AVT43046
KY912995[4]	7 (5:2)	29	21	0.72	AVT43047
KY912996[4]	1 (1:0)	30	22	0.73	AVT43048
KY912997[4]	6 (5:1)	28	20	0.71	**AVT43049**[7]
KY912998[4]	1 (1:0)	22	13	0.59	AVT43050
KY912999[4]	1 (0:1)	37	25	0.68	AVT43051
KY913000[4]	2 (0:2)	29	21	0.72	AVT43052
KY913001[4]	1 (0:1)	26	18	0.69	AVT43053
KY913002[4]	1 (0:1)	29	21	0.72	AVT43054
KY913003[4]	1 (1:0)	31	22	0.71	AVT43055
KY913004[4]	1 (1:0)	36	25	0.69	AVT43056
KY913005[4]	1 (0:1)	38	27	0.71	AVT43057
KY913006[4]	1 (0:1)	37	27	0.73	AVT43058

Accession numbers and protein IDs in bold indicate sequences found in both cattle and buffalo.
[1] Does not include the AAA insert as a SNP.
[2] Does not include the K from the AAA insert.
[3] Reference sequence: U40703.
[4] Reference sequence: JX442244.
[5] Identical to reference.
[6] Identical to each other except that AVT43021 has 2 more AA.
[7] Identical to each other.
[8] Sequence is shorter than, but identical to AVT43031, AVT43037, and AVT43058 in overlapping region.
[9] Identical to AVT43037.

425 of the reference sequence U40703), and a 33 bp region (at residue 592 of U40703) which consisted of sequences unique to each subcluster (Supp. Data 2a). The sequences were also displayed as a phylogenetic tree (Supp. Fig. S1), which identifies the individual alleles within each cluster.

3.5. Similar levels of mixed infections in cattle and buffalo

Mixed infections, as indicated by the presence of at least two different alleles of the p67 gene, were detected in samples from all animals except for calf 2104 (Table 2). A comparison of the level of mixed infections in the cattle or buffalo populations shows that the mean and median numbers of alleles detected in the individual cattle (3.6 and 3.5) were only slightly less than those from the individual resident buffalo (4.1 and 4.0).

3.6. Similar levels of polymorphism in epitope sequences expressed by cattle- and buffalo-derived parasites

The partial AR12.6 epitope sequences were all identical to the reference sequence (QTQSQVQ) from the Muguga stabilate, as shown in Supp. Data 1b, 2b and 3b. The TpM12 epitope in all type 1 and 2 alleles was identical to the reference sequence TKEEVPPADLSDQVP (Table 4). However, all but one (KY912998) of the type 3 sequences had five identical substitutions (TKEEVPPA**SS**SD**SEQ).** Allele KY912998, which was detected in one cattle sample (2310) had eight substitutions

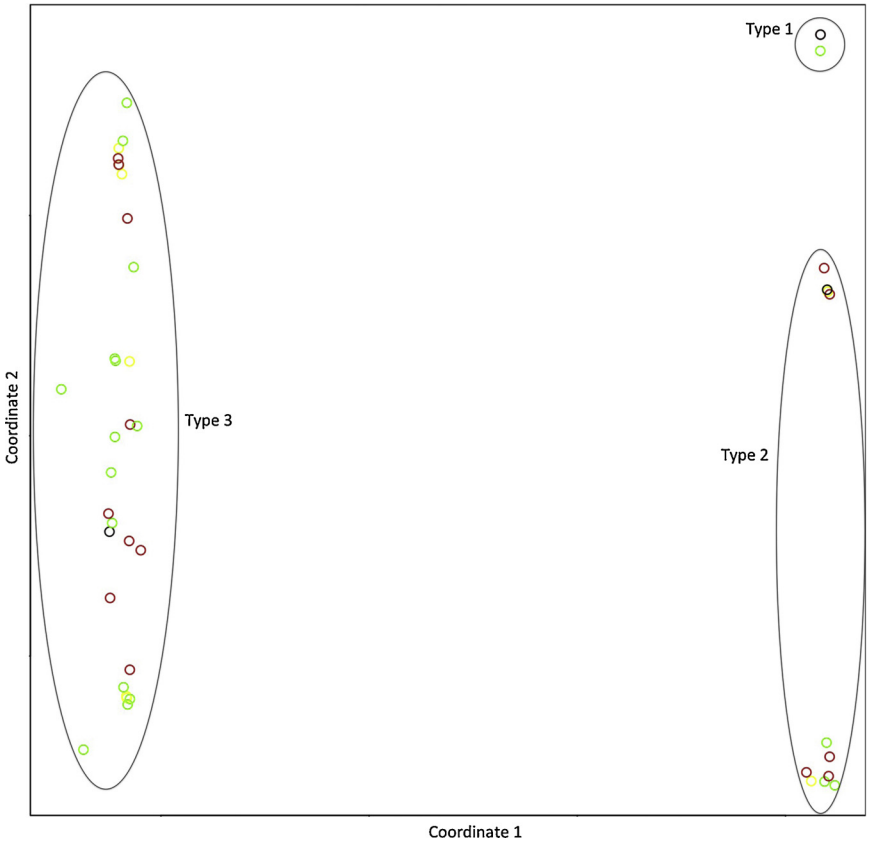

Fig. 1. The genetic relationships among the p67 sequences identified in the cattle and buffalo samples is illustrated by PCoA. The sequences clustered according to the presence/absence of indel sequences and labeled as type 1, 2 and 3. The circles represent sequences detected observed in blood samples from cattle (red circles), buffalo (green circles) or both cattle and buffalo (yellow circles). Reference sequences representative of the different allele types are shown as black circles. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 4
Predicted amino acid sequences for the TpM12 and AR22.7 epitopes found in the study population.

Accession number	TpM12 TKEEVPPADLSDQVP[1]	AR22.7 LQPGKTS[1]
KY912962	- - - - - - - - - - - - - - -	- - - - - - -
KY912965-KY912969	- - - - - - - - - - - - - - -	- - - - - - -
KY912970, KY912972-KY912976	- - - - - - - - - - - - - - -	- P- - - - -
KY912971	- - - - - - - - - - - - - - -	- - - - - - -
KY912979-KY912992	- - - - - - - - SS - -SEQ	not found[2]
KY912993-KY912997, KY912999-KY913006	- - - - - - - - SS - -SEQ	not found
KY912998	- - - - I - - KSD -ESEQ	not found

[1] Reference sequences from Nene et al. (1996).
[2] Orthologous sequences were not found in the Type 3 alleles.

(TKEE**I**PP**KSD**S**ESEQ**) and was identical to the sequence AFU34358 described by Sibeko et al. (2010). For the AR22.7 epitope, the type 1 alleles and six of the type 2 alleles were identical to the major recognition site of the epitope (LQPGKTS). The remaining six type 2 alleles displayed a single substition (L**P**PGKTS) and were detected in both cattle and buffalo. A sequence similar to this epitope was not identified in the type 3 sequences. In summary, the p67 sequences isolated from cattle and buffalo show a similar level of polymorphism within these epitopes.

4. Discussion

The aim of the current study was to use the diversity in the p67 gene to compare the level of heterogeneity in the *T. parva* population present in the blood of cattle soon after infection with buffalo-derived parasites, with that in a buffalo population from the same area. The cattle had been obtained from the ILRI ranch at Kapiti, a region of Kenya that is considered non-endemic for *T. parva*, and had undergone weekly acaricidal treatment at the ranch. No evidence of infection with *T. parva* was detected in the cattle prior to transport to the study site, and the cattle were introduced into an area grazed by buffalo but not cattle. Hence, the cattle infections were highly likely to have originated from the buffalo population.

The results show that, based on sequencing of the p67 gene, the amount of diversity in the parasite populations in the cattle and buffalo is similar. The levels of diversity were compared using several measures. First, the number of alleles detected in each host population was equivalent, with 17 and 18 alleles each being found only in the cattle and buffalo samples, respectively, and nine alleles common to both. Second, there were similar numbers of each of the previously defined allele types found in the cattle and buffalo populations. Third, there was an equal level of diversity of the type 3 protein sequences in the cattle and buffalo samples, and the protein diversity in the cattle type 2 sequences was only slightly less than those from buffalo. Fourth, PCoA and phylogenetic analysis of the p67 sequences obtained from the blood samples did not reveal any major clustering into cattle- or buffalo-derived parasites, suggesting that there was no emergence of parasites uniquely adapted to cattle in the blood of cattle during the acute phase of infection. Fifth, the level of mixed infections observed in the cattle and buffalo populations was similar. Finally, there was no major difference in the polymorphism of the three epitope sequences which were seen in samples from the cattle and buffalo populations.

T. parva is most likely to have originated in eastern Africa in association with the African buffalo (reviewed in Norval et al., 1992). The relationship in buffalo is one where the organism causes few if any clinical signs, with a persistent parasitaemia facilitating continued transmission of the parasite by the tick vector. Cattle are a relatively recent introduction into Africa (Freeman et al., 2006) and the relationship is not as benign. Cattle frequently die as a result of infection with *T. parva*, and infections of cattle with buffalo-derived parasites are characterised by low numbers of infected lymphocytes and limited progression of the parasite to the transmissible piroplasm stage (Neitz

et al., 1955; Jura and Losos, 1980). Based on the sequences of known *T. parva* antigens, it appears that the parasites maintained in cattle comprise a much more restricted population of *T.parva* than those in buffalo (Pelle et al., 2011). It is not known where in the parasite life cycle in cattle this restriction could occur, and whether the small numbers of infected lymphocytes and of piroplasms are causally related.

The results presented here have implications for these questions. As the p67 sequences derived from the cattle samples were no less diverse than those from the buffalo samples, it is difficult to explain the restricted heterogeneity and low parasitoses in the cattle by there being only a subset of parasites able to enter and transform bovine lymphocytes. The sequences obtained here were most likely derived from circulating, infected lymphocytes, as no piroplasms were observed in the cattle at the time of sampling, apart from cattle 2325 and 2227, both of which shared p67 alleles found in other cattle and buffalo. Thus, if there is selection occurring in the cattle host which results in emergence of a subset of cattle-adapted parasites, this selection appears to occur after the infected lymphocytes have migrated from the regional lymph nodes.

The data presented here provide insights into additional aspects of the biology of *T. parva*. More than one p67 allele was detected in the blood samples from all buffalo and 11 of the 12 cattle. As the p67 gene is present as a single copy in the haploid stages of the parasite found in the vertebrate host (Nene et al., 1992), this result indicates that all of the animals but one were infected with at least two *T. parva* strains. This has been observed before in buffalo (Sibeko et al., 2010; Oura et al., 2011; Hemmink et al., 2018) and cattle (Oura et al., 2005). The last study showed that the incidence of mixed infections increased with the age of the animal, suggesting that mixed infections result from a series of infection episodes. Interestingly, in the current study the cattle samples were all collected three to four weeks after the cattle were introduced to the field site. Thus, the mixed infections were the result of infection from the tick population soon after the field trial was initiated (as the animals were treated with acaricide every 3 to 4 days beginning on day 10 after introduction into the field site) and not the result of chronic re-infections as may be the case in the earlier studies.

In a previous publication (Sitt et al., 2015), data were presented showing that types 1, 2 and 3 were present in both the cattle and buffalo samples from Ol Pejeta, with type 3 alleles being the most abundant. No type 4 alleles were detected. The current study extends this by showing the numbers of allele types present in the individual animals. A comparison of the frequency of allele types found here and in the study from southern Africa (Sibeko et al., 2010) reveals differences in the predominance of the allele types between the two studies. The earlier study showed that type 2 were the most common sequences in the cattle and buffalo samples, with type 1 and 3 alleles being were relatively common in buffalo samples compared to cattle samples. In contrast there was only one buffalo sample with a type 1 allele in the current study, whereas 30 of the 44 alleles were type 3 and all of the buffalo in this study carried this allele type. Type 3 alleles were also the most predominant type detected in the other Kenyan study (Obara et al., 2015). To date, no type 4 sequences originating from eastern African samples have been published. These initial results point to a difference in the predominance of different parasites in eastern and southern Africa, which may have occurred after the separation of the two populations.

Sequence variation in epitopes recognized by anti-p67 monoclonal antibodies has been observed previously in Kenya in cattle co-grazing with buffalo (Obara et al., 2015). The PCR products in the current study contained three epitopes recognized by the sporozoite-neutralizing antibodies AR12.6 (partial), TpM12 and AR22.7 (Nene et al., 1999). Accordingly, the current study examined the sequences of these three epitopes to determine if there was any association of epitopes with host populations, and no major differences were observed. Of interest was the observation that the TpM12 epitope sequence in one allele (KY921998) differed markedly from all the others in this study, but was identical to one isolated from South Africa (Sibeko et al., 2010). This is in line with recently published results using sequences encoding antigens recognized by cytotoxic T cells, which also indicated that diversity in *T. parva* antigens arose before the parasites appeared in southern Africa (Hemmink et al., 2018).

In summary, the results presented here show that the level of diversity of the p67 gene is similar in the cattle and buffalo blood samples, suggesting that the ability to infect and transform bovine lymphocytes *in vivo* is not restricted to a subset of the parasites transmitted to cattle from buffalo and that any restriction in parasite development in cattle occurs at subsequent developmental stages. The study presents new insights into the differences in the development of cattle- and buffalo-adapted *T. parva* parasites in cattle, and indicates that stages of the life cycle after the early macroschizont should be targeted to identify the genes that regulate further development of the parasites in cattle and determine their transmissibility by ticks.

Conflict of interest statement

None.

Acknowledgements

This work was partially supported by a grant awarded jointly by the Department for International Development (UK Government) and the Biotechnology and Biological Sciences Research Council (BBSRC) UK grant number BB/H009515/1 of the Combating Infectious Diseases of Livestock for International Development (CIDLID) program. The study was conducted as part of the CGIAR Research Program on Livestock and Fish, led by ILRI, and is supported by contributors to the CGIAR Trust Fund.

The authors would like to thank Giles Prettejohn and the Ol Pejeta Conservancy for approval and support for conducting our field studies on site, and the Kenya Wildlife Service for conducting the buffalo sampling. The authors would also like to thank Lucy Muthui and Ben Kiawa at the sequencing facility at the BECA- ILRI hub.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.04.006.

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