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Polymorphisms of Selected Antigen-Coding Genes of *Theileria parva* Isolates from Cattle in Southwestern Uganda

Davies Paul Kasaija[1], Nsubuga Julius[2], Lubega George William[2] and Ann Nanteza[3*]

[1]National Livestock Resources Research Institute (NaLIRRI)/National Agricultural Research Organisation (NARO), Tororo, Uganda

[2]Department of Biomolecular Resources and Biolab Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Kampala, Uganda

[3]Department of Biotechnical and Diagnostics, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, Kampala, Uganda

[*]**Corresponding author:** Ann Nanteza, Department of Biotechnical and Diagnostics, College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB), Makerere University, P.O. Box 7062, Kampala, Uganda

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Abstract

Background: Theileria parva is a complex tick-borne haemoprotozoan parasite causing an acute and often fatal disease in cattle called East Coast fever (ECF). Buffaloes are the major wild reservoirs of the parasite and may harbour multiple *T. parva* parasite strains. The success of vaccination control of the disease by the Infection and Treatment Method (ITM) using the Muguga cocktail live vaccine requires cross-protection against parasite field strains. However, the parasite strains in Uganda have not been fully characterized to ensure cross-protection. We investigated polymorphisms at three important *Theileria parva* antigenic loci among Ankole cattle from southwestern Uganda, where cattle-keeping is the major source of human livelihood and where a cattle-buffalo interface also occurs.

Results: Fifty *Theileria parva*-positive cattle blood samples from Kiruhura district were selected by nested p104 PCR and assessed for allelic diversity using PCR-RFLP for the p104 and PIM genes. The Bionumerics software was used to analyze the restriction digestion profiles and generate dendrograms. Diversity at the p67 locus was investigated by sequencing of PCR products and DNA multi-sequence alignment analysis. Theileria parva isolates exhibited moderate and high allelic diversity for the p104 and PIM loci respectively and there was a dominant allele for each of the genes. The p67 locus was however homogeneous in all the isolates and homologous to that of *T. parva Muguga* reference strain. The study also revealed multiplicity of *T. parva* genotypes with respect to the above antigen-coding genes for individual samples.

Conclusions: Theileria parva parasites circulating in Kiruhura district are generally markedly diverse at antigenic-gene loci with the variations bearing undefined effects on vaccine cross-protectivity. Multiplicity of *T. parva* genotypes as demonstrated here may further complicate disease epidemiology and confound vaccination strategies.

Keywords: Alleles; Antigenic loci; Fingerprints; Muguga cocktail vaccine; Multiple infections; Polymorphism; Theileria parva

Background

Bovine theileriosis is a tick-borne protozoan disease caused by six species of the genus Theileria, two of which- *T. parva* and *T. annulata* are pathogenic and cause significant economic and production losses in affected ungulates (Siege, et al. 2006) [1]. Theileria parva, transmitted by the brown ear hard tick (Ixodidae) Rhipicephalus appendiculatus is by far the most pathogenic and economically significant Theileria species in Eastern, Central and Southern Africa (Mukhebi, et al. 1992) [2]. An international research group (including Washington State University) revealed that ECF killed more than 1 million cattle in 11 countries in one year and costed ranchers more than $300 million. The disease also currently threatens up to 28 million cattle in the region (Mallot,

2014) [3].

East Coast fever vaccination-control using the Immunisation and Treatment Method (ITM) involves inoculation of the animal with a potentially lethal dose of cryo-preserved sporozoites (live parasites) and simultaneous treatment with long-acting oxytetracycline (Radley, 1981). This leads to mild reaction and full immunity against subsequent hoel,kmologous infections. It is the $CD8^+$ T-lymphocyte responses that mediate protection and several parasite antigens recognized by $CD8^+$ T cells have been identified (Pelle, et al. 2011) [4]. However, the antigenic diversity expressed at the schizont stage of the parasite together with a wild reservoir of the organism in buffalo have complicated the development of effective disease control by immunization (Nene, et al. 1996) [5]. In Uganda, *T. parva* parasite strains have not been fully characterized at antigenic loci, yet immunity acquired from ITM is partially strain-specific (Odongo, et al., 2006) [6]. Cross protection by the Muguga cocktail vaccine against locally circulating strains of *T. parva* may therefore not be possible in case of genomic polymorphisms that give rise to multiple strains. There is thus urgent need to establish allelic diversity of putative antigen genes of the circulating parasites for recommendation of use or improvement of the currently used live vaccine in Uganda. The Tp1 and Tp2 antigens are major targets of the $CD8^+$ T-cell response in cattle expressing the A18 and A10 class I Major Histo-Compatibility (MHC) haplotypes, respectively (Pelle, et al. 2011) [4]. Investigations of the nature and extent of polymorphisms in the two antigens using partial and full-length sequencing revealed high allelic diversity for both antigens. The diversity was observed both between geographically distinct isolates and those in a single population of *T. parva*.

Theileria parva genes coding for antigen proteins PIM, p67, p104 and p150 have been previously analyzed in search for discriminatory differences between *T. parva* isolates (Baylis, et al., 1993; Toye, et al., 1996) [7,8]. These surface proteins are usually encoded by single copy genes and contain a polymorphic central region of amino acid sequences flanked by conserved amino and carboxyl termini (Skilton, et al., 1998; Toye, et al., 1995) [9,10]. Size polymorphisms displayed by these genes have been used to develop several molecular tools for characterization of *T. parva* stocks, exploiting the variable regions of the genes (Bishop, et al., 2001; Geysen et al., 1999b) [11,12]. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of these genes demonstrated polymorphism in field stocks of *T. parva* in Kenya, but majority of field stocks isolated from two regions of Zambia were relatively homogeneous (Geysen, et al., 1999a) [13]. The study herein sought to establish allelic diversity at these three antigenic loci of *T. parva*. The data predicts a diverse population of *T. parva* strains with respect to the p104 and PIM genes and presence of dominant alleles that could be further evaluated for potential usefulness in vaccine design. However, the p67 locus was homogeneous in all the parasite isolates and identical to the *T. parva* Muguga reference strain (cattle-derived).

Methods

Study site

The study was carried out in two neighboring sub-counties in Kiruhura district in southwestern Uganda. Cattle keeping is a major activity for the livelihood of the people in this area in a livestock-wildlife interface area.

Sample collection and storage

Blood samples were obtained from 250 Ankole long-horn cattle between February and June, 2012. Poorly managed farms (relaxed tick control practices) with history of recent outbreaks of ECF and buffalo interface were selected for sampling. Three to four milliliters of blood were collected from the jugular vein of each animal into EDTA vacutainer tubes for genomic DNA extraction. Samples were kept on ice prior to delivery at the Molecular Biology Laboratory (MOBILA), Makerere University where they were stored at -20°C until required for DNA extraction and subsequent analysis in the same laboratory.

Cattle blood DNA extraction

BBlood was processed for DNA extraction as described by d'Oliviera, et al. (1995) with minor modifications. Briefly, 200µl of thawed blood was washed three times in 0.5ml PBS (pH 7.4) by centrifugation at 16000g for 5 minutes. The resulting cell pellet was re-suspended in 100µl of lysis mixture (10mM Tris-HCl (pH 8.0), 50mM KCl, 0.5% Tween 20, 100µg/ml of proteinase K) followed by overnight incubation at 56°C. The mixture was then boiled for 10 minutes to inactivate proteinase K and centrifuged for 5 minutes to recover the supernatant containing DNA which was aliquoted into a new tube and stored at -20°C until use.

The p104 gene-based screening for *T. parva* -positive cattle blood samples

The extracted genomic DNA was used for *T. parva* parasite detection and characterization. Nested PCR for an invariable region of the p104 gene (np104) was used to screen blood samples for presence of *T. parva* DNA as described by Odongo, et al. (2006) [6]. The method generates a 496bp fragment using forward and reverse primers IL3231 (5′-ATTTAAGGAACCTGACGTGACTGC-3′) and IL755 (5′-TAAGATGCCGACTATTAATGACACC-3′) respectively (Skilton et al., 2002) in the first reaction. The secondary PCR uses forward and reverse primers 5′-GGCCAAGGTCTCCTTCAGATTACG-3′ and 5′-gTGGGTGTGTTTCCTCGTCATCTGC-3′ respectively to amplify a 277bp internal fragment located between bases 2784 and 3061 of the p104 gene (Figure 1). Five microliters (5µl) of genomic DNA template was used in the primary PCR while 1µl of template (product

of the primary PCR) was used for the secondary PCR. The amplification was carried out in a 25µl reaction containing 1x DreamTaq Green PCR master mix (Thermo Scientific), 50ng of each of specific primers. The cycling conditions were; initial denaturation at 95˚C for 5 minutes, followed by 35 cycles of denaturation at 95˚C for 1 minute, annealing at 58˚C for 1 minute and extension at 72˚C for 1 minute then a final extension at 72˚C for 7 minutes before cooling at 4˚C. For the secondary PCR, the reaction components and cycling conditions were the same as those used in the primary PCR except for the annealing temperature which was 55˚C for 1 minute and 30 cycles were performed. Five microliters of the PCR products were separated on 2% agarose gels in Tris-Acetate-EDTA (TAE) buffer containing 0.5µg/ml ethidium bromide. The DNA bands were visualized in a UV light box and photographed (Figure 1). The PCR thermocycler used throughout the study was GenAmp® while the BIORAD power pac™ was used for agarose gel electrophoresis. All primers used in this study were supplied by Eurofins, Germany. A 277bp PCR product was observed in 127 (50%) *T. parva* infected (positive) cattle blood samples.

The PCR-RFLP analysis of *T. parva* isolates at the p104 gene locus

Fifty (50) *T. parva* -positive samples were randomly selected for further analysis at the p104, PIM and p67 loci. For the p104 gene, semi-nested PCR on the variable region of the gene was performed as previously described (De Dekens, et al., 2007; Sibeko, et al., 2011) [14,15]. Briefly, primers p104F2, p104R and p104nF were used for the PCR reactions with the following modifications: for the secondary PCR, 2µl of DNA template were used instead of 0.5 µl, and annealing temperature of 58˚C was adopted for both rounds of PCR instead of 60˚C to increase on PCR sensitivity. DreamTaq Green PCR Master Mix (Thermo scientific) was used for the PCR reactions. Cycling conditions were as for np104 *T. parva* -detection PCR above. Successful amplification of the p104 gene was determined by visualization of an approximately 800bp band on a 0.5 µg/ml ethidium bromide pre-stained 1% agarose gel. Products of the semi-nested PCR for the p104 gene were digested overnight with the restriction enzyme AluI (Thermo Scientific). Restriction digestion was carried out in a final volume of 10µl consisting of 7µl amplified DNA, and 1 µl of restriction enzyme (1U), 1µl of double distilled water and 1µl of 10x Tango enzyme buffer (supplied in the kit). Incubation was done in a water bath at 37°C overnight. Eight microliters of the digestion product of each sample was mixed with 3 µl of 6x DNA loading buffer and transferred into each well of a 10% non-denaturing polyacrylamide gel. Five microliters of a 50bp DNA ladder (Sigma-Aldrich) was also loaded onto the gel in an adjacent well for fragment size determination. A 1x Tris Borate EDTA (TBE) (pH 8.0) electrophoretic buffer was used at 80 volts for 2 hours and 30 minutes and the gel stained with ethidium bromide (0.005%) for 30 minutes before UV photography. Bionumerics software was used to calculate Dice coefficients of similarity, cluster the RFLP profiles and generate dendrograms (Figure 2).

The PCR-RFLP analysis of *T. parva* isolates at the PIM locus

Semi-nested PCR-RFLP analysis was performed on the PIM locus of the same fifty *T. parva* -positive samples as for the p104 gene locus. Primers Pim1 and Pim R4 were used for the forward and reverse reactions respectively for the primary PCR while primers Pim Fm and Pim R4 were used for the secondary PCR. DreamTaq Green Master Mix was used at reagent concentrations and volumes as for the p104 gene characterization above. For the primary PCR reaction, 5µl of the DNA template was used while 1µl of the primary PCR product was used for the secondary PCR. Cycling conditions used were as for the p104 gene above with annealing temperature of 60˚C maintained for both cycles. For the secondary PCR, 30 cycles were performed instead of 35 cycles. Five microliters of the PCR products and DNA ladder were each loaded onto 1% agarose gel and separated in 1X TAE buffer containing 0.5 µg/ml ethidium bromide. The DNA ladder used was λ DNA-HindIII/Φx- HaeIII DNA (Finnzymes) and the expected band sizes of the PIM gene amplicons (700-1200 bp). The PIM gene PCR products were digested overnight with the restriction enzyme BclI (10U/µl) (Thermo Scientific) at 50°C (De Deken, et al., 2007) [14], the restriction reaction set up was as for the p104 gene above. Similarly, the restriction digestion products were separated on a 10% non-denaturing polyacrylamide gel with apparatus and conditions as for the p104 gene above. The PCR-RFLP profiles were inspected visually and then analyzed using the Bionumerics software (Figure 3).

Multi-sequence alignment analysis of *T. parva* isolates at p67 gene locus

Synthetic oligonucleotides p67 IL613 and p67 IL792 which flank the 5′ and 3′ ends of the p67 gene, respectively, were used in a standard PCR (Nene, et al. 1996) [5] to amplify 800bp DNA fragment (variable region of the p67 gene) from the same parasite genomic DNA samples analyzed above. DreamTaq Green Master Mix was used at reagent concentrations and volumes as for the p104 gene above. Five microliters of extracted DNA were used in a 25 µl PCR reaction volume. Cycling conditions used were also as for the p104 gene above, while annealing at 50˚C. The PCR products were analyzed on a 1% agarose gel by electrophoresis. The PCR amplicons were sent for sequencing at Macrogen Inc., Seoul, Korea. The p67 gene sequence data obtained were aligned with published *T. parva* Muguga isolate sequence and four buffalo sequences in the Gene bank. Phylogenetic analysis was performed using the MEGA 5.10 software.

Analysis of *T. parva* p104 or PIM PCR-RFLP profiles and p67 DNA sequences for allelic diversity

The gel RFLP profiles were inspected visually and using the gel documentation system to enumerate the alleles produced per gene from the field isolates. Gel pictures were scanned and imported into the Bionumerics software which calculates the Dice coefficients of similarity, clusters the RFLP profiles and generates dendrograms by the Unweighted-Pair Group Method Using Average Linkages (UPGMA). The most appropriate settings for optimisation and tolerance as determined by the software were calculated. Samples with similar RFLP profiles obtained from different isolates were defined as clusters.

Results

Screening for *T. parva* infected blood samples by p104 nested PCR analysis

Two hundred fifty (250) cattle blood samples were screened for Theileria parva DNA using p104 nested PCR. A 277bp PCR product was observed in 127 (50%) *T. parva* infected (positive) samples (Figure 1).

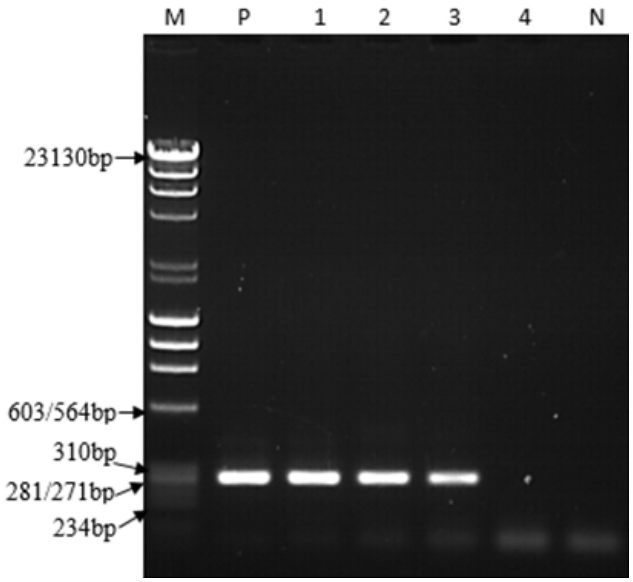

Figure 1: Detection of *Theileria parva* DNA by agarose gel analysis of p104 gene nested PCR products.

Lane **M**: λ DNA-HindIII/Φx- HaeIII DNA marker from Finnzymes Lane **P**: positive control (*T. parva Katete* isolate), Lanes **1-3**: positive field samples. Lane **4**: negative field sample, Lane **N**: negative control (distilled water).

The PCR-RFLP analysis of *T. parva* field isolates at the p104 locus

The semi-nested PCR was carried out on 50 *T. parva* -infected samples to amplify the p104 gene and characterize the products by RFLP. Only 17 out of the 50 samples yielded distinct RFLP profiles and these domnstrated two alleles (polymorphism). One allele was observed in 14(82%) samples (dominant) while the other allele was exhibited in 3(18%) isolates. However, both alleles were present in one sample suggesting mixed infections (data not shown). The *T. parva Katete* (reference isolate) profile deviated slightly from the dominant allele but significantly from the other allele. Cluster analysis of the p104 PCR-RFLP profiles using the Bionumerics software also demonstrated two main clusters (Figure 2). Cluster (A) consisted of thirteen (13) isolates while cluster (B) contained three isolates (3). There was limited profile difference within each cluster (homogeneous fingerprints) but a major contrast was observed between profiles in the two clusters. Restriction profiles of the mixed infection can be seen at the

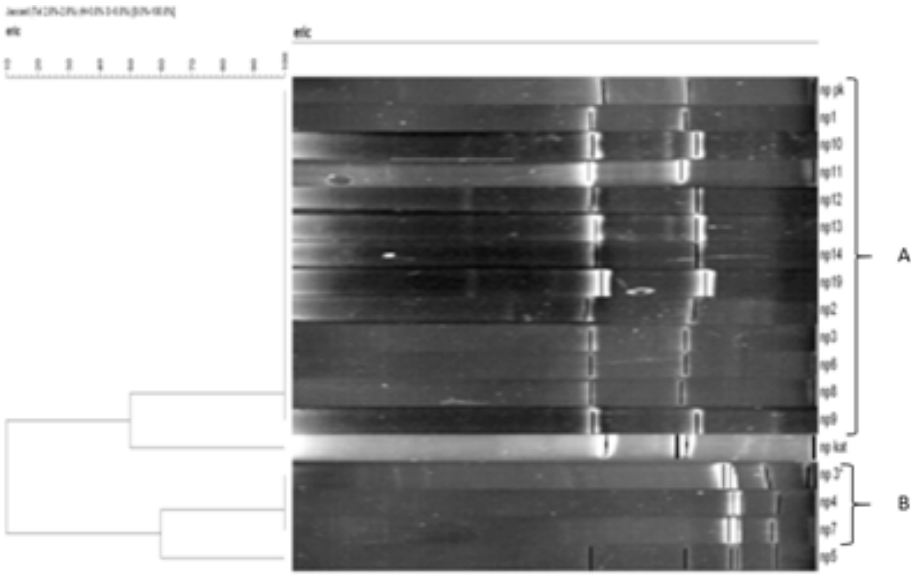

Figure 2: Restricting profiles of amplified p104 loci from 17 representative *T. parva* isolates generated using Alu1 restriction enzyme. Polyacrylamide gel pictures of PCR-RFLP products were imported into the Bionumerics software to generate the dendrograms by UPGMA. The profiles were clustered into two clades (A & B). The bottom profiles show the mixed infection.

The PCR-RFLP analysis of *T. parva* field isolates at the PIM locus

The semi-nested PCR was carried out on 50 *T. parva* -infected samples to amplify the PIM gene and analyse the products by RFLP (Figure 3). Polymerase Chain Reaction (PCR) products of the PIM gene varied in size ranging from 0.7kb to 1.1kb (data not shown). Twenty two samples were successfully amplified at the PIM gene locus of which 20 samples each displayed at least two bands on 1% agarose gel suggestive of mixed infections (data not shown). Cluster analysis of PIM PCR-RFLP profiles by the Bionumeric software identified two clusters (Figure 3). Restriction profiles in a given cluster were markedly different from those in the other cluster but profiles within the same cluster were relatively homogeneous (Figure 3).

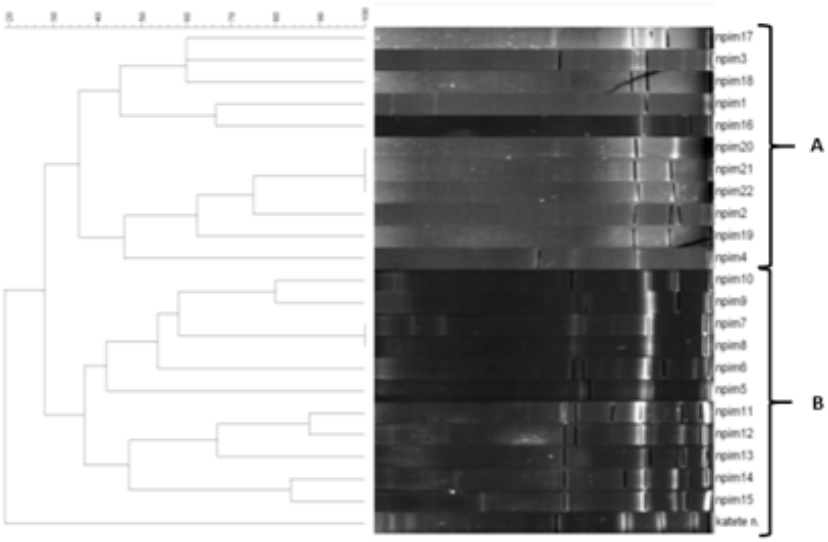

Figure 3: Restricting profiles of amplified PIM loci from 22 representative *T. parva* isolates generated using Bcl1 restriction enzyme. Polyacrylamide gel pictures of the PIM gene restriction-digest products were imported into the Bionumerics software and dendrograms were generated by UPGMA. The profiles were clustered into 2 clades.

Multi-sequence alignment analysis of *T. parva* field isolates at the p67 locus

The DNA sequences obtained from 8 PCR products (900bp) out of fifty (50) Theileria parva-infected samples analyzed for the p67 gene were aligned with reference *T. parva Muguga* sequence (accession number M67476) (Nene, et al. 1996) [12], *T. parva Katete* sequence (isolate amplified in this study) and four reference buffalo-derived *T. parva* sequences (Figure 4). All field p67 gene sequences were similar to the reference *T. parva Muguga* and *T. parva Katete* sequences (cattle-derived) and possessed a characteristic 129bp DNA deletion, which was not present in the buffalo-derived *T. parva* sequences.

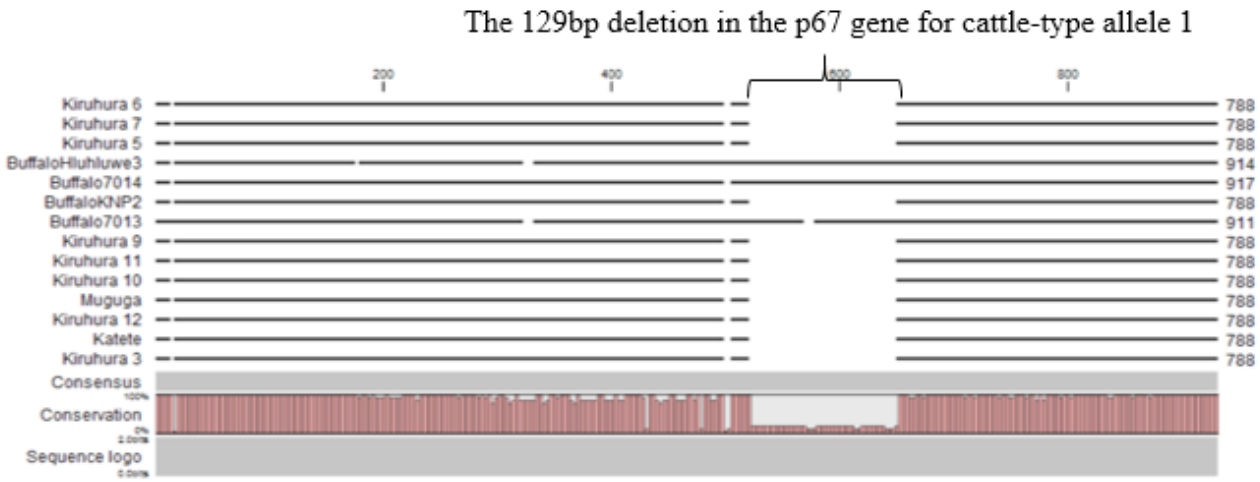

Figure 4: Multiple alignment of *T. parva* DNA sequences of the p67 gene-central variable region. The DNA was amplified from 8 representative cattle *T. parva* field isolates and five (5) sequences derived from gene bank reference data. Sequence alignments in MEGA 5.10 were imported into CLC Main Workbench version 7.0 to generate the alignment.

When a phylogenetic tree was calculated, all the field *T. parva* p67 gene sequences clustered closely together with *T. parva Muguga* and *T. parva Katete* sequences (cattle-derived) while buffalo-derived sequences grouped separately without forming a distinct cluster. Buffalo-derived *T. parva* p67 gene sequences also formed a basal clade for the cattle-derived *T. parva* p67 gene sequences (Figure 5).

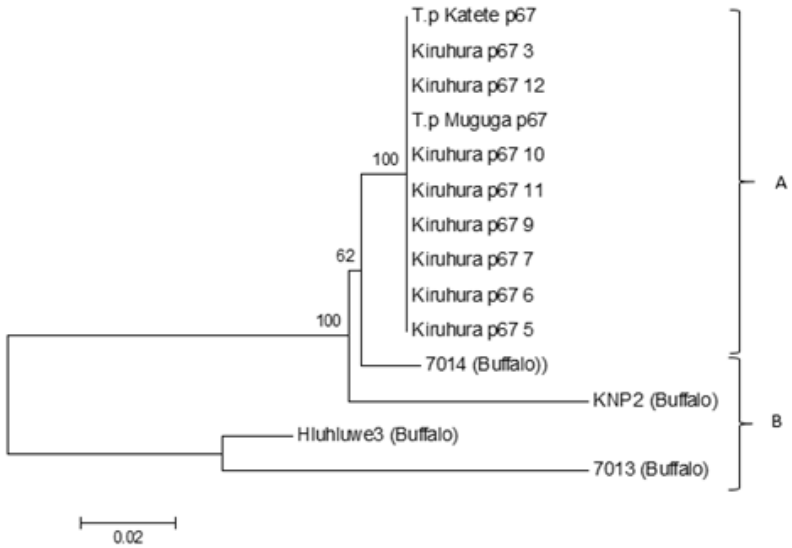

Figure 5: Phylogenetic relationship of p67 gene sequences of 8 representative *T. parva* field isolates. The *T. parva* partial p67 locus PCR products of field isolates were sequenced (Marogen Inc.) and aligned using MEGA v 5.10 program. The phylogenetic tree was calculated by Neighbor-joining analysis in MEGA 5.10 (*fasta) with 1000 bootstrap replicates.

Discussion

Ankole long-horn cattle blood samples were collected between February and June, 2012 from two sub counties of Kiruhura district in southwestern Uganda where ECF is endemic. The cattle selected for the study belonged to farms with history of recent outbreaks of the disease and/or were communally grazed. The cattle also shared grazing grounds with buffaloes and there was laxity in tick control on the farms due to high unaffordable costs of the acaricides. According to this study, prevalence of *T. parva* infection by np104 PCR (Odongo, et al., 2006) [6] was high, being 50% (127/250), thus confirming disease endemicity in the Ankole long horn cattle in the study area. Prevalence of the parasite in Western Uganda by reverse line blot hybridization assay, according to Muhanguzi, et al., (2010) was 19.8%, the difference probably being due to the higher sensitivity of np104 PCR and sample selection whereby exposed cattle were targeted. The high prevalence here may be due to the prevailing high transmission intensities in the area whereby the climatic conditions are favorable for the survival of the vector tick, Rh. appendiculatus and other tick species of livestock and wildlife.

Some of the *T. parva* positive samples that were selected for assessment of polymorphisms at the p104, PIM and p67 antigen gene loci did not successfully amplify in the subsequent PCRs. The low yield of the p104, PIM or p67 gene PCR products could be due to the usually low parasitaemia among local cattle in endemic areas. This confirms the high sensitivity of the np104 PCR screening test (Odongo, et al., 2006) [6]. Visual inspection and cluster analysis of the p104 gene PCR-RFLP profiles from 17 PCR amplicons revealed two sets of fingerprints (Figure 2) representing two alleles of the p104 gene. Theileria parva field isolates14(82%) possessed a common allele while 4(18%) isolates exhibited a different allele. This is comparable to PCR-RFLP characterization of *T. parva* antigen genes p104 and p150 in Zambia in which each of the genes demonstrated two alleles (moderate polymorphism) (Geysen et al., 1999b) [12]. Sibeko et al (2011) [15]. The p104 cluster analysis also revealed two major groups mainly correlating with geographic origins of the samples. In this study, the allele identified in 14 isolates could thus represent the most abundant (dominant) strain of *T. parva* circulating in Kiruhura district with respect to the p104 gene. One isolate possessed both alleles. This could indicate presence of multiple *T. parva* infections in cattle. None of the two identified alleles of the p104 gene was identical to that of *T. parva* Muguga strain demonstrated in similar analysis by Sibeko, et al (2011) [15]. This observation could suggest occurrence of *T. parva* strains circulating in southwestern Uganda that are distinct from the *T. parva* Muguga strain with respect to the p104 antigen gene. The effect of the situation above on ITM vaccine cross-protection among the local cattle *T. parva* populations still needs to be investigated. Considering that all study samples were obtained from the same geographic region, the study results thus predict a population of *T. parva* parasites exhibiting moderate polymorphism (2 alleles) for the p104 gene in Kiruhura district.

The *T. parva* PIM gene is known to be a single copy gene and is therefore expected to yield single-sized PCR amplicons (Toye, et al., 1995) [10]. This study demonstrated a multiplicity of *T. parva* infections in cattle as 20 out of 22 (91%) PIM gene PCR products from single (discrete) field samples produced multiple bands (data not shown). This is supported by findings by Sibeko, et al. (2011) [15] where four out of seven cattle and all 27 buffalo samples were observed to have multiple infections. Multiple infections observed could be due to shared infections of *T. parva* strains as a result of the high transmission rates and recombination within the ticks. However, buffaloes are known wild reservoirs of multiple *T. parva* parasite strains hence a potential source of diversity of the organism to cattle. There is also evidence that recombination occurs in ticks resulting in parasites that have characteristics of both buffalo- and cattle-derived parasites (Geysen, et al., 2000) [16]. However, Oura, et al. (2003) [17] observed that only a limited subset of the total *T. parva* gene pool present in buffalo had become established in cattle. Visual analysis of the PIM gene PCR-RFLP profiles revealed up to eleven (11) different fingerprints for the 22 samples analyzed. This is comparable to results from similar work done in Zambia where the PIM locus was the most polymorphic of the three antigen gene loci, p104, p67 and PIM with ten (10) alleles and marked size polymorphisms in both DNA and protein lengths (Geysen, et al., 1999a) [13]. In this study, PIM gene PCR-RFLP profiles were clustered into two clades in the dendrogram. There was marked contrast between PCR-RFLP profiles of samples distributed in the two clusters but variations within each cluster were moderate. Observations above suggest high diversity of alleles of the PIM gene among *T. parva* isolates circulating in Kiruhura district. Such diversity could yield variants of the PIM antigen in the field that are distinct from those in the immunising live vaccine cocktail, bearing undefined implications on vaccine cross protection against parasite field strains. The PIM gene PCR-RFLP profiles could not be directly compared with those of reference *T. parva Muguga* strain generated under similar analysis because of the multiple infections as revealed by PIM gene PCR amplicons.

No parasite field isolate grouped closely with *T. parva Katete* (an isolate from Zambia) in the PIM gene dendrogram. This could be attributed to the difference in the geographical origins of the parasite isolates. This finding is supported by the study in which the PIM gene polymorphism was effectively used to differentiate isolates originating from different geographical regions in Zambia. It was thus suggested that the PIM gene alone could characterize subdivisions in the *T. parva* populations in the field (Geysen, et al., 1999b) [12]. However, related studies in South Africa by Sibeko, et al. (2011) [15] did not establish any correlation between the PIM gene variations among isolates and geographical distribution. The

PIM gene which is mainly expressed during the schizont stage of the parasite is believed to code for a putative antigen that induces the cytotoxic T-cell response (Katende, et al., 1998) [18]. The high polymorphism observed in the PIM gene exons is associated with selective pressure from the host protective immune response (Toye, et al. 1995) [10].

The p67 gene of cattle-derived *T. parva* in East Africa has previously been suggested to be conserved. The p67 gene sequence conservation could be attributed to the presumed role of the p67 protein in sporozoite-host interaction during parasite invasion of host lymphocytes. In this study, all the eight p67 gene sequences from field isolates were shown to be identical to the published *T. parva* Muguga strain sequence (allele 1) (accession number M67476) (Nene, et al., 1996) [5] following multi-sequence alignment (Figure 4). The isolates therefore represent a single strain of *T. parva* circulating in Kiruhura district with respect to the p67 gene. This is consistent with a report by Nene, et al.(1996) [5] where sequences of the p67 gene from four cattle-derived parasite stocks (Boleni, Marikebuni, Mariakani and Uganda) were stated to be identical to Muguga p67 gene sequence. The p67 gene sequence common to cattle-derived *T. parva* parasites in East Africa is characterized by a 129bp deletion in the central region of the gene and is designated allele 1, while buffalo-derived *T. parva* does not have a deletion and is designated allele 2 (Nene, et al., 1996) [5]. It is likely that p67 gene allele type is associated with the parasite's potential to infect a specific host, hence the apparent selection of allele 1 among cattle-infective *T. parva* parasites in East Africa (Sibeko, et al., 2010) [19]. The p67 locus confirmed that all the isolates were cattle-derived. However, it remains to be shown whether transmission of *T. parva* between cattle and buffalo is present in the study area. A cross-sectional or longitudinal survey in the same region among wildlife, including buffalo would be required to provide data to make informed conclusions. The phylogenetic analysis also revealed that all the cattle-derived *T. parva* p67 sequences including *T. parva* Muguga and *T. parva Katete* strains cluster together, indicating maintenance of a single *T. parva* strain with respect to the p67 gene. The basal clade formed by the buffalo-derived *T. parva* p67 sequence suggests that the cattle-derived *T. parva* p67 sequence (allele 1) evolved from the former (allele 2).

According to this study, the allelic characteristics observed for a given isolate at a specific antigen locus were not necessarily maintained at other antigen loci. This observation could indicate that these antigen genes evolve at different rates. Polymorphisms among *T. parva* antigen genes reported here range from absent (p67), through moderate (p104 gene) to high (PIM gene) depending on the gene considered. This diversity is partly attributed to genetic recombination and random assortment of alleles of different *T. parva* stocks that occurs during the parasite's sexual reproduction in the tick vector (Gauer et al., 1995) [20,21]. This genetic mixing is confounded by the diverse buffalo-derived *T. parva* parasites in areas where cattle and buffalo co-graze, of which is the study's scenario. In areas of high endemicity such as East Africa, individual hosts can be infected with multiple clones of *T. parva* of different genotypes. This results in extensive recombination taking place in the tick and subsequently a high level of diversity in the *T. parva* populations (Oura, et al., 2003) [17]. Such parasite diversity could result in incomplete cross-protection by live vaccines.

Conclusions

Overall, polymorphisms are moderate in the p104, high in the PIM and absent in the p67 antigen-coding genes of *T. parva* circulating in Kiruhura district in southwestern Uganda. The p67 gene sequence among local parasite isolates is identical to that of the *T. parva Muguga* isolate and this could emphasize potential usefulness of the gene in vaccine design. For each of the polymorphic genes p104 and PIM, there is a dominant allele (strain) in circulation. The PIM gene significantly contributes to the high antigenic diversity among *T. parva* populations. Multiple *T. parva* infections occur in cattle and may compound antigenic diversity. Certain field variants of *Theileria parva* possess alleles of the p104 and PIM genes that are distinct from those of reference *T. parva* Muguga isolate and this could probably undermine ITM cross-protective potential. In order to have a more comprehensive view of the *T. parva* interactions along the buffalo-cattle interface, a larger set of isolates from cattle and buffaloes need to be characterized and sequenced. Parasite strains possessing dominant alleles of polymorphic antigens should be identified extensively and incorporated in the immunizing live cocktail for the local cattle population.

References

1. Shanna Siegel, Elizabeth Howerth, Bruce E LeRoy (2006) East Coast fever (Theileria parva) Veterinary Clinical Pathology Clerkship Program.
2. Mukhebi AW, Perry BD, Kruska R (1992) Estimated economics of theileriosis control in Africa. Preventive Veterinary Medicine 12: 73-85.
3. Mallot S (2014) WSU Researchers work to Create Cattle Vaccine. The Spokesman review 2014.
4. Pelle R, Graham SP, Njahira MN, Osaso J, Saya RM, et al. (2011) Two Theileria parva CD8 T Cell Antigen genes are more variable in buffalo than cattle parasites, but differ in pattern of sequence diversity. PLoS One 6: e19015.
5. 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. Infection and Immunity 64: 2056.
6. Odongo DO, Oura CAL, Spooner PR, Kiara H, Mburu D, et al. (2006) Linkage disequilibrium between alleles at highly polymorphic mini- and micro-satellite loci of Theileria parva isolated from cattle in three regions of Kenya. International Journal for Parasitology 36: 937-946.

7. Baylis HA, Allsopp BA, Hall R, Carrington M (1993) Characterisation of a glutamine-and proline-rich protein (QP protein) from Theileria parva. Molecular and Biochemical parasitology 61: 171-178.

8. Toye P, Nyanjui J, Goddeeris B, Musoke AJ (1996) Identification of neutralization and diagnostic epitopes on PIM, the polymorphic immunodominant molecule of Theileria parva. Infection and Immunity 64: 1832-1838.

9. Skilton RA, Bishop RP, Wells CW, Spooner PR, Gobright E, et al. (1998) Cloning and characterization of a 150 kDa microsphere antigen of Theileria parva that is immunologically cross-reactive with the Polymorphic Immunodominant Molecule (PIM). Parasitology 117: 321-330.

10. Toye PG, Metzelaar MJ, Wijngaard PL, Nene V, Iams K, et al. (1995) Characterization of the gene encoding the polymorphic immunodominant molecule, a neutralizing antigen of Theileria parva. The Journal of Immunology 155: 1370-1381.

11. Bishop R, Geysen D, Spooner P, Skilton R, Nene V, et al. (2001) Molecular and immunological characterisation of Theileria parva stocks which are components of the Muguga cocktail'used for vaccination against East Coast fever in cattle. Veterinary Parasitology 94: 227-237.

12. Geysen D, Bishop R, Skilton R, Dolan TT, Morzaria S (1999b) Molecular epidemiology of Theileria parva in the field. Tropical Medicine & International Health 4: A21-A27.

13. Geysen D, Bishop R, Skilton R, Dolan TT, Morzaria S (1999a) Molecular epidemiology of Theileria parva in the field. Tropical Medicine & International Health 4: A21-A27.

14. De Deken R, Martin V, Saido A, Madder M, Brandt J, et al. (2007) An outbreak of East Coast fever on the Comoros: A consequence of the import of immunised cattle from Tanzania? Veterinary Parasitology 143: 245-253.

15. Sibeko KP, Collins NE, Oosthuizen MC, Troskie M, Potgieter FT, et al. (2011) Analyses of genes encoding Theileria parva p104 and polymorphic immunodominant molecule (PIM) reveal evidence of the presence of cattle-type alleles in the South African T. parva population. Veterinary Parasitology 167: 244-254.

16. Geysen D (2000) The application of molecular biology techniques to analyse diversity in Theileria parva populations in Zambia. School of Health Sciences and Social Care.

17. Oura CAL, Odongo DO, Lubega GW, Spooner PR, Tait A, et al. (2003) A panel of microsatellite and minisatellite markers for the characterisation of field isolates of Theileria parva. International Journal for Parasitology 33: 1641-1653.

18. Katende J, Morzaria S, Toye P, Skilton R, Nene V, et al. (1998) An enzyme-linked immunosorbent assay for detection of Theileria parva antibodies in cattle using a recombinant polymorphic immunodominant molecule. Parasitology Research 84: 408-416.

19. Sibeko KP, Geysen D, Oosthuizen MC, Matthee CA, Troskie M, et al. (2010) Four p67 alleles identified in South African Theileria parva field samples. Veterinary Parasitology 167: 244-254.

20. Gauer Martina, Mackenstedt Ute, Mehlhorn Heinz, Schein Eberhard, Zapf Frank, et al. (1995) DNA measurements and ploidy determination of developmental stages in the life cycles of Theileria annulata and T. parva . Parasitology Research 81: 565-574.

21. Geysen D, Bazarusanga T, Brandt J, Dolan TT (2004) An unusual mosaic structure of the PIM gene of Theileria parva and its relationship to allelic diversity. Molecular and biochemical parasitology 133: 163-173.