Molecular epidemiology of *Theileria parva* in Eastern Zone of Tanzania

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SUMMARY

Theileria parva is a haemoprotozoan parasite that causes fatal lymphoproliferative disease of cattle, East Coast fever (ECF), transmitted by *Rhipicephalus appendiculatus* tick. There are many stocks of *T. parva* differing in various features. Immunity elicited by immunization is stock specific and therefore, it is appropriate to characterize *T. parva* to find most relevant stocks for inclusion in the vaccine. This study determined molecular epidemiology of *T. parva* parasite in Eastern Zone of Tanzania by conventional polymerase chain reaction (PCR) and semi-nested PCR to amplify antigenic genes coding for sporozoite surface protein (p67) and piroplasm proteins (p104) respectively using DNA extracted from cattle blood samples naturally infected with *T. parva.* Restriction fragments length polymorphism (RFLP) profiles of p104 were generated by *Arthrobacter luteus* I (*Alu* I) restriction enzyme. Results demonstrated two types of p67 bands, 750 bp and 950 bp. Profiles for p104 PCR-RFLP revealed 5 types of stocks; the first and the second stocks produced PCR-RFLP profiles identical to *T. parva* Katete and *T. parva* Muguga. The p104 PCR-RFLP profiles of the rest three stocks were distinct novel variant stocks never reported in previous studies. The significance of the different p67 alleles and the novel variants stocks detected by p104 PCR-RFLP in the epidemiology of theileriosis in Eastern Zone of Tanzania and the key aspects for vaccine developement is discussed.

Keywords: p104, p67, molecular epidemiology, *Theileria parva.*

INTRODUCTION

Theileria parva is a haemoprotozoan apicoplexan parasite which causes East Coast fever (ECF) which is also known as bovine theileriosis; a fatal lymphoproliferative bovine disease transmitted by *Rhipicephalus appendiculatus* tick. In Tanzania the losses due to tick and tick borne diseases (TTBD) mount to US$ 364 million annually as a result of deaths of more than 1.3 million cattle of which 68% of these deaths are caused by bovine theileriosis (Kivaria, 2006). The clinical prevalence of theileriosis in calves in traditional cattle herds in Tanzania during cool months of the year (May-July) is high (Mbassa *et al*., 2008) and without treatment almost equal mortalities may be observed. In addition, theileriosis causes indirect losses in form of costs of control of the disease and reduced production capability (Mbassa *et al*., 2009). Depending on severity of infection, lactating cows may take 30 days and above delay in attaining normal milk production after recovery from ECF (Mbwambo *et al*., 2002).

Evolutionally, genetically, phylogenetically and immunologically there are many stocks of *Theileria* within the same species, differing in genome structure, pathogenicity, immunogenicity and molecular markers (Geysen *et al*., 1999; Bishop *et al*. 2001; Oura *et al*., 2003; Matete *et al*., 2004; De Deken *et al*., 2007; Katzer *et al*., 2010; Sibeko *et al*., 2010; Sibeko *et al*., 2011; Patel *et al*., 2011). Cross-immunity trials (Geysen *et al*., 1999) and monoclonal antibodies (Matete *et al*., 2004) have been used for determination of *T. parva* population diversity. Nevertheless, there have been some difficulties in determining the change in the characteristics of the parasite after tick-cattle passage and cross reactivity are among the drawbacks in the use of these methods. Restricted fragment length polymorphism (RFLP) of the gene coding for piroplasma antigenic protein (p104) had been used to elucidate the diversity of *T. parva* population in different localitions with success (Bishop *et* al., 2001; De Deken et al., 2007; Sibeko et al., 2011).

The aim of this study was to determine the population diversity of *T. parva* in Eastern Zone of Tanzania by examining the banding pattern of the gene coding for sporozites surface antigenic protein (p67) and RFLP profiles of the gene coding for piroplasm antigenic surface protein (p104). The gene for the p104 antigen was selected for PCR-RFLP analysis due to its limited polymorphism (Geysen *et al*., 1999).

MATERIALS AND METHODS

Study locations

Eastern Zone of Tanzania is composed of four administrative regions namely Dar es Salaam, Pwani, Tanga and Morogoro. The study locations were selected from the four regions which include:- Twatwatwa, Itete-njiwa, Mtimbila, Mzumbe, Wami-Dakawa, Muhoro, Muyuyu, Siasa, Kiwanga, Kimara and Segerea (Figure 1). All the locations were in rural areas except Kimara and Segerea which are found 25 km and 22 km away from Dar es Salaam city centre, respectively. The livestock production system in these areas was transhumance with the exception of Kimara and Segerea which ranged from semi-intensive to zero grazing. Animal breeds used were the local cattle breed; Tanzanian short-horned Zebu and crosses of dairy cattle, Friesian, Ayrshire & Jersey.

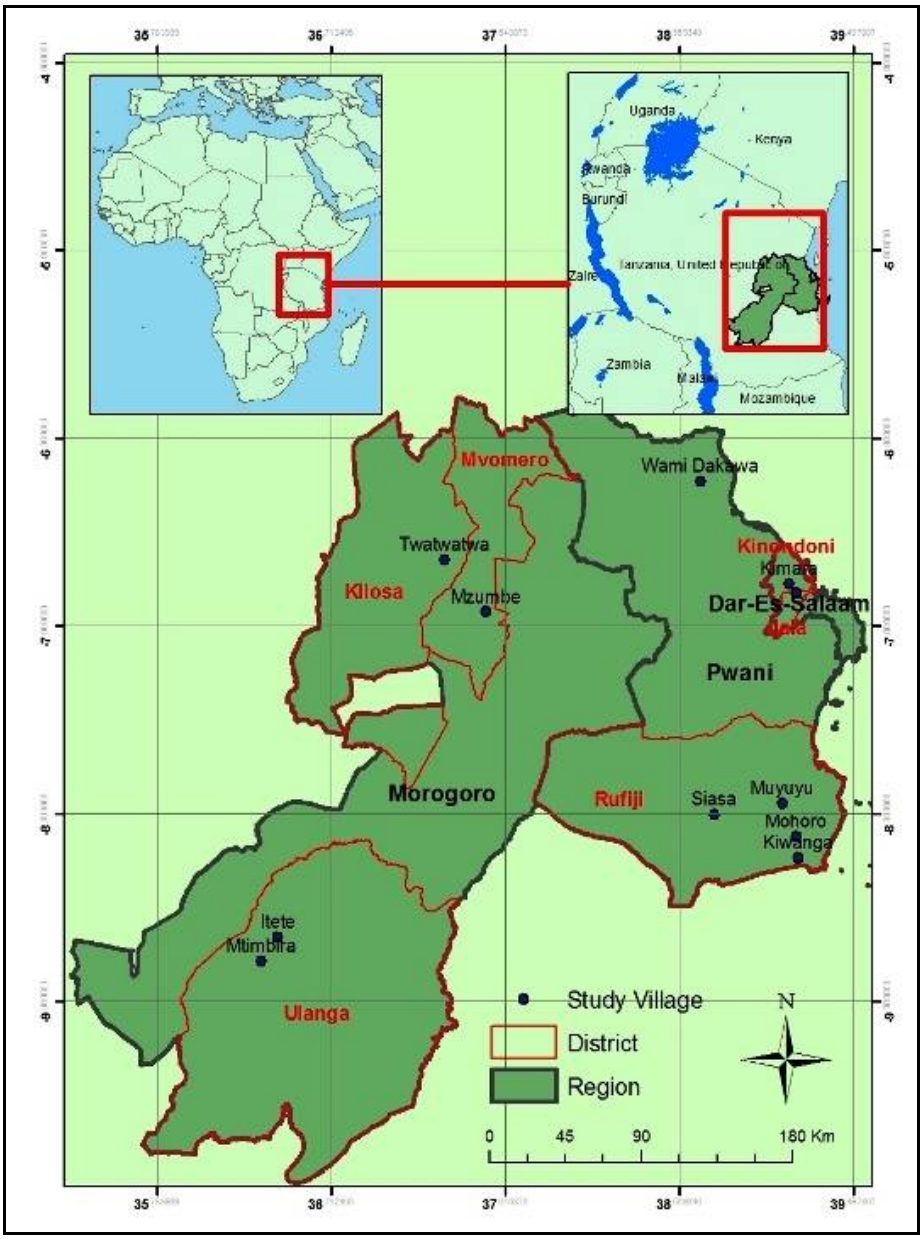

Figure 1. A study area map showing sampling locations

Sample collection

Blood was sampled from cattle with enlarged either one or all of parotid and pre-scapular lymph nodes. The blood samples (3-10 ml) were collected aseptically from the jugular vein into 10 ml vacuum tubes (Vacutainer® Dickinson B-D United Kingdom) coated with potassium ethylene amino-tetracetate (K_3EDTA) using 21 gauge needles. A total of 102 blood samples were collected and placed in a cool box with ice pack, then refrigerated before transportation to the Faculty of Veterinary Medicine, Sokoine University of Agriculture, Tanzania for DNA extraction. The DNA sample extracted from *T. parva* Muguga sporozoites at the Institute of Tropical Medicine, Antwerp, Belgium and kept at the Genome Science Centre of Sokoine

University of Agriculture was used as positive control during running PCR.

DNA extraction and amplification of the p67 and p104 genes

DNA was extracted from blood samples by using Quick-gDNA™ MiniPrep DNA extraction kit according to manufacturer instructions. Semi-nested polymerase chain reaction was performed using themocycler (Takara, Tokyo, Japan) to amplify p104 locus using p104 forward (5′CCACCATCTAAACCACCGT-3′) and p104 reverse (5′TAAGATGCCGACTATTGACACCA CAA3′) primers in the first round under the following amplification conditions; pre-denaturation at 94°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, initial extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. Primers p104nF (5′ACCACCGTTTGATCC ATCATTCA3′) and p104 R (5′TAAGATGCCGACTATTGACACCA CAA3′) were used during the second round using first round amplification conditions except in annealing step where the condition was adjusted to 60°C for 45 seconds. The numbers of reaction cycles for first and second round were 35 and 25 respectively. All samples were screened for *T. parva* using p104 semi-nested PCR; later on all positive samples for p104 amplification were subjected to conventional PCR for p67 amplification.

Amplification of p67 gene was done by conventional PCR using p67F (5′CAGGTGATAC- ATCGG3′) and p67R (5′TACTCAAAAAAACAAACC3′) primers under the same condition used during amplification of p104 with excepation of annealing step whereby 52°C and 45 seconds amplification temperature and time respectively was used. In p104 first round and p67 amplifications, each PCR reaction was performed in a final volume of 25 µl, contained 5 µl of genomic DNA, 12.5 µl of HotStat Master Mix (Bioystem, Foster City, Calfornia, USA), 0.4 µl of each primer at 10 pmole/µl concentration and 6.7 µl nuclease free water. During second round amplification of p104, 0.5 µl of the first round PCR amplicons was used as a template, other reaction components, their volumes and total reaction volume were maintained except nuclease free water which was increased to compensate decreased template volume.

After amplification for both genes, the PCR amplicons were run in the agarose gel stained with ethidium bromide (Sigma-Aldrich, USA), and normalization of the bands was done using 1 kbp DNA marker (O'GeneRuler™ 100bp DNA Ladder Plus, Fermentas Life Science, EU), which was run in lane one for each gel. The DNA bands were observed on the gel under UV light illumination then photographed. The p104 positive samples were recorded accordingly then subjected to p67 gene amplification as previously described. Second round positive p104 amplicons were kept for restriction enzyme analysis.

Generation and analysis of p104 PCR-RFLP profiles

The amplicons of the samples that produced bands, were analyzed using restriction enzyme specific for p104. The final volume of each restriction enzyme analysis reaction was 15 µl, containing 1.5 µl of reaction buffer, 0.5 µl of *Arthrobacter luteus* I (*Alu* I) (Fast Digest®, Fermentas Life Science, EU), 6 µl of amplicons and 7 µl nuclease free water. The reaction mixtures were incubated in water bath at 50°C for 12 hours. Ten µl of the restriction enzyme

digests were loaded in wells of 10% polyacrylamide gel, followed by electrophoresis at 80 volts for 1 hour and 30 minutes. After electrophoresis the gel was stained with SYBR® green (Sigma-Aldrich, USA) for 40 minutes, followed by observation under UV light illumination and photographing.

RFLP patterns were analyzed by visual inspection. Normalization of the RFLP profiles was done using 1 kbp DNA marker (O'GeneRuler™ 100bp DNA Ladder Plus, Fermentas Life Science, EU), run parallel with the samples. The DNA fragments with less than 100 bp were ignored because they could not be estimated accurately using 100 bp DNA ladder. Samples with similar final RFLPs profiles obtained from blood samples of different animals were regarded as similar to *T. parva* stocks.

RESULTS

Bands generated after amplification of p104 and p67 genes

A total of 102 samples were screened, out of which 32 (31.4%) samples were positive for p104. All positive samples for p104 showed ~800 bp band (Figure 2), which was the expected band size. All 32 positive samples for p104 were amplified for p67. Ninenteen (59.4%) samples were positive and 13 were negative (Table 1). Single allele per sample of band sizes ~750 bp (9/19) and ~950 bp (10/19) were obtained in samples which were positive for p67 amplification (Figure 3). There were no samples showing more than one allele. Samples with ~750 bp allele originated from Twatwatwa (2/9), Mzumbe (3/9), Wami-Dakawa (2/9), Kimara (1/9) and Kiwanga (1/9) (Table 1). Samples with 950 bp originated from Mzumbe (1/10), Mtimbila (2/10), Kiwanga (2/10), Muhoro (3/10), and Siasa (2/10) (Table 1).

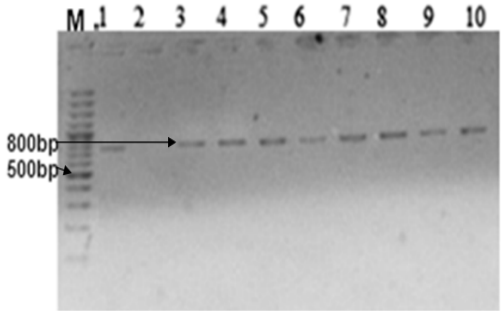

Figure 2. Representatives of positive samples for p104 amplification obtained from different geographical areas of the Eastern zone of Tanzania. The allele band size is approximately to 800 bp which was the expected band size. Lanes M is 1kbp DNA marker (O'GeneRuler™ 100bp DNA Ladder Plus, Fermentas Life Science, EU), lane 1 is positive control, lane 2 is negative control (instead of adding template, nuclease free water was added), lane 3 is sample Mak/C 10, lane 4 is sample Seg/M 79, lane 5 is sample Twa/W, lane 6 is sample Mti/28, lane 7 is sample Vik/B 56, lane 8 is sample Ute/U 22, lane 9 is sample Kis/A 08, lane 10 is sample Isu/T 31 which were collected from Muhoro, Segerea, Twatwatwa, Mtimbila, Vikindu, Siasa, Kiwanga and Muyuyu respectively.

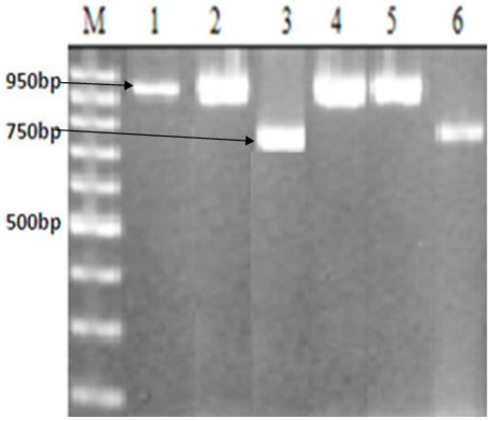

Figure 3. Representatives of positive samples for p67 amplification obtained from different geographical areas of Eastern zone of Tanzania. The allele band

size were approximately to 750 bp and 950 bp. Lane M is 1 kbp DNA marker (O'GeneRuler™ 100bp DNA Ladder Plus, Fermentas Life Science, EU), lane 1 is sample Mbi/G 09, lane 2 is sample Mli/P 22, lane 3 is sample Mzu/76, lane 4 is sample Mbu/A 65, lane 5 is sample Mti/14, lane 6 is sample Wam/11 collected from Kiwanga, Muhoro, Mzumbe, Kiwanga, Mtimbila and Wami-Dakawa respectively.

Table 1. Number of positive samples for p104 and p67 with their respective areas of origin in the Eastern zone of Tanzania

Location	No. of p104 positive samples	No. of p67 positive samples	
		7.5 kbp Allele	9.5 kbp Allele
Twatwatwa	5	2	**
Mzumbe	5	3	1
Wami-Dakawa	2	2	**
Mtimbila	3	*	2
Segerea	3	*	**
Kimara	2	1	**
Siasa	3	*	2
Kiwanga	4	1	2
Muhoro	4	*	4
Muyuyu	2	*	**
Itete njiwa	***	****	****

*No sample from this area showed 7.5 kbp allele on amplification of p67
**No sample from this area showed 9.5 kbp allele on amplification of p67
***All samples where negative on p104 amplification
****Amplification of p67 was not done

PCR-RFLP profiles of p104 gene generated and analysis

RFLP profiles generated after digesting p104 positive amplicons with *Alu* I restriction enzyme revealed 5 different types of stocks; one stock showed 320 bp and 500 bp band sizes in lanes 2, 3 and 6 (Figure 4), the band numbers and sizes identical to *T. parva* Katete, a stock first isolated in Eastern province of Zambia. This type of stock was isolated in Twatwatwa, Muhoro, Mtimbila and Wami-Dakawa. The second type of stock was shown in lane 1 and 4 having 220 bp and 280 bp band sizes, a band number and size identical to *T. parva* Muguga, a stock first isolated in Central highlands of Kenya in 1950s. The stocks with these PCR-RFLP profiles were isolated from Twatwatwa, Mtimbila, Mzumbe, Wami-Dakawa, Muhoro, Muyuyu, Siasa, Kiwanga, Kimara and Segerea.

Lane 4 and 6 containing the sample from Siasa and Mtimbila respectively, apart from having the bands identical to *T. parva* Muguga and *T. parva* Katete respectively they contain other types of bands which were 120 bp and 500 bp in lane 4 and 120 bp and 280 bp bands in lane 6 . The band size in these two lanes was more than ~800 which was the size of the original band before being digested with restriction enzyme, an indication of the presence of two different types of populations in each isolate. Lane 5 contain the isolate from Kiwanga with band size close to ~800 bp, an indication of the presence of single population but with banding pattern distinct from other isolates in other lanes.

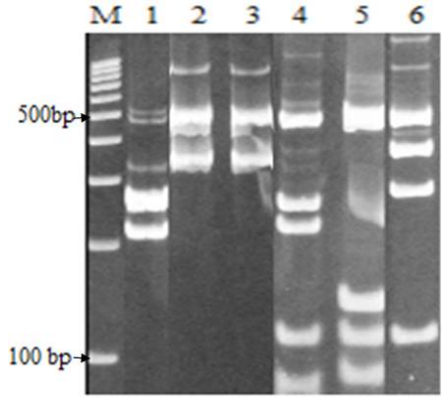

Figure 4. *Alu* I PCR-RFLP profiles of p104 gene. Lanes: M is 1kbp DNA marker (O'GeneRuler™ 100bp DNA Ladder Plus, Fermentas Life Science, EU), lane 1 is sample Mak C/10 sampled at Muhoro, with PCR-RFLP profiles which have two bands of 220 bp and 280 bp sizes, the profile similar to *T. parva* Muguga. Lane 2 is sample Twa/ M 54 and lane 3 is sample

Shi/J 51 sampled at Twatwatwa and Muhoro respectively, with two bands of, 320 bp and 500 bp sizes, the band sizes resembling *T. parva* Katete. Lane 4 is sample Ute/Y 44 sampled at Siasa with *T. parva* Muguga like bands and two additional unidentified bands of 120 bp and 500 bp sizes, lane 5 is sample Das/X 14 sampled at Kiwanga with 4 bands sizes ranging from below 100 bp to 500 bp and banding pattern distinct from other lanes, lane 6 is sample Mti/23 collected from Mtimbila with *T. parva* Katete like bands and two additional unidentified bands of 120 bp and 280 bp.

DISCUSSION

Analysis of p67 PCR product profiles indicated that two p67 alleles of ~750 bp and ~950 bp are present in *T. parva* parasites in cattle in Estern zone of Tanzania. These results indicate the presence of a mixture of two different stocks of *T. parva* in the zone. The allele ~750 bp was more common in the Northern part of the Eastern Zone in areas including Twatwatwa, Mzumbe and Wami-Dakawa. The allele ~950 bp was more common in the Southern part of the Eastern zone in areas including Mtimbila, Siasa, Kiwanga and Muhoro. The difference in occurrence with regards to p67 allelic size may represent different stocks of *T. parva* which are specific to a particular geographical area. Sequencing of this gene in these two stock is required in order to elucidate if other differences exist than allele sizes between the two types of stocks.

Nested PCR of p104 gene revealed only one allele of ~800 bp in all samples. The allele is similar to *T. parva* Muguga; a stock first isolated in Central highland of Kenya. The other 4 samples were similar to *T. parva* Katete; a strain which was first isolated in the Eastern province of Zambia. The *T. parva* Muguga like p104 PCR-RFLP profiles generated in this study were closely similar to *T. parva* Katete like p104 PCR-RFLP profiles reported in previously studies (Bishop *et al.*, 2001; De Deken *et al.*,2007 and Sibeko *et al.*,2011).

The band sizes shown in lane 4 and 6 were obtained from samples originating from Siasa and Mtimbila. The band sizes were more than ~800 bp but less than ~1600 bp. Band sizes of ~800 bp detected in lane 5 were from samples originating from Kiwanga. These results show that the former two isolates contain two and the later contain one type of *T. parva* populations. *Theileria parva* Muguga like stock profiles were identified in lane 4 and *T. parva* Katete like stock profiles were identified in lane 6. The remaining band size profiles in lanes 4 and 6 and all in lane 5 were not identical to any profiles previously reported and hence, remained unidentified.

The possibility of having *T. parva* Serengeti-transformed; a stock isolated in Tanzania in these samples with RFLP profile similar to *T. parva* Muguga cannot be ignored, since RFLP profile of these two stocks are closely related (Bishop *et al*., 2001; Oura *et al*., 2004; Oura *et al*., 2007). *Theileria parva* Muguga stock isolated may be derived from exotic trivalent vaccine ("Muguga cocktail") used for vaccination campaign in some of the study locations. Isolation of *T. parva* Muguga stock in areas where there was no immunization exercise before such as Siasa cannot exclude the possibility of this stock to be vaccine derived as there is an extensive normadisms practiced among these areas. Thus sharing of *T. parva* stocks among these cattle population is likely to occur. Cases of introduction of a vaccine-derived stock have also been reported in the Southern Province of Zambia (Geysen *et al*., 1999). However, there

was no evidence of importation of cattle from Zambia, a place where *T. parva* Katete stock was first reported, signifying that this stock is indigenous to Siasa area where it was isolated.

Unidentified PCR-RFLP profiles in sample Ute/Y 44, Das/X 14 and Mti/23 originating from Siasa, Kiwanga and Mtimbila respectively may be representatives of the subpopulation of the *T. parva* Katete and *T. parva* Muguga which have undergone mutation leading to dfferent band size after digestion with *Alu* I. The unidentified *T. parva* stocks in the field may complicate the epidemiology and immunization exercise of the disease since there is no guarantee of cross-protection of those stocks by the *T. parva* stocks currently present in a trivalent vaccine ("Muguga cocktail"), which is used for immunization in Tanzania.

In conclusion, conventional PCR of p67 gene and PCR-RFLP profiles of p104 gene revealed differences in genetic structure of *T. parva* in Tanzania isolated in different localitions. The study recommends that a detailed study be done using other molecular techniques including sequencing of the two genes . Also use of mini and microsatellites markers for *T. parva* characterization in order to prepare *T. parva* stabilates for manufacturing industry and efficient control of theireliosis. In addition, other molecular methods be used to confirm specifically the existence of *T. parva* Katete stock in the Eastern Zone in order to include the stock in the multivalent vaccine used for ECF immunization in the Esastern Zone of Tanzania.

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