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Original Article

Molecular characterization of *Theileria parva* parasites from South Sudan using the PCR-RFLP approach on antigen genes

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ABSTRACT

In an attempt to characterize *Theileria parva* parasites circulating in South Sudan cattle , polymerase chain reaction (PCR)-based assays were carried out using four single copy encoding antigen genes p104, PIM, p150 and p67 in addition to one microsatellite MS321. A total of 20 bovine DNA samples from two locations in South Sudan were included in this study, in addition to two references strains, Muguga and Katete. A total of nine alleles were identified for the polymorphic immunodominant molecule (PIM) locus using restriction fragment length polymorphism (RFLP), against 2 alleles each for the p150 and p104 loci, respectively. This confirms that differences in the polymorphic PIM genes alone could be used to characterize subdivisions in the *T. parva* populations in the field. On the other hand, 4 alleles were identified for the MS321 locus and 2 alleles for the p67 locus. The data indicate that the studied parasites are genetically closely related, mainly cattle derived and genetically quite distinct from the Muguga strain.

Keywords: Characterization, PCR-RFLP, South Sudan, *Theileria parva*

INTRODUCTION

Theileria parva, a protozoan parasite causing East Coast fever (ECF), poses one of the major challenges to the development of cattle industry in many South, Central and Eastern African countries including South Sudan (Dolan 1989). The disease is transmitted by the brown ear tick *Rhipicephalus appendiculatus*. The first outbreak of ECF in Sudan was reported in 1950 (Hoogstraal 1956) in Kajo Kaji and Yei River districts. After this outbreak, the disease was not reported again until 1981 (Morzaria *et al* 1981), when ECF was believed to have been reintroduced from Uganda in 1979 through uncontrolled movement of cattle by Ugandan refugees (Julla 1994). In 1999, there was a severe outbreak of ECF in and around Juba, and findings indicated that the morbidity due to this disease ranged between 20-60% with mortality reaching up to 30% (Marcellino 2008). East Coast fever is ranked high in terms of its impact on the livelihood of resource poor farming communities in South Sudan (Marcellino *et al* 2011). Currently, management of ECF is primarily through control of the tick vector using acaricides, although this is unsustainable due to increasing

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acaricide resistance and public and environment health safety concerns. A highly effective "infection and treatment" live vaccine based on the injection of sporozoites together with a dose of long acting tetracycline has long been available for control of ECF in many Central and Western African countries (Radley *et al* 1975). However, there are fears that the introduction of foreign parasites by vaccinated cattle into a region might results in novel genotypes (De Deken *et al* 2007, Geysen 2008). Therefore, before deciding to use such vaccine in South Sudan, there is certainly a need to characterize the strains of *Theileria parva* parasite circulating in the region. The introduction of molecular techniques has enabled development of markers that has provided insight into many issues associated with ECF epidemiology in the field. Restriction fragment length polymorphism (RFLP) is a technique that exploits variations in homologous DNA sequences. This technique has been often used to characterize field isolates of *Theileria parva* as well as the Muguga cocktail used in the infection and treatment method (Geysen *et al* 1999, Bishop *et al* 2001, De Deken *et al* 2007, Oura *et al* 2003, 2005 and 2007).

The present study was aimed at molecular characterization of *Theileria parva* parasites circulating in South Sudan cattle using the PCR-RFLP approach.

MATERIALS AND METHODS

***T. parva* samples.** Seven samples (JUBA-1 to JUBA-7) were collected from cattle in Gumba (east of Juba town), while the remaining 13 (JUBA-8 to JUBA-20) were collected from cattle in Khor Rumla (south-west of Juba town) (Table 1). All samples were proven to be *Theileria parva* single infection in reverse line blot hybridization technique (RLB) (Salih *et al* 2007). Two additional reference strains were included in this study. *Theileria parva* Katete is a Zambian isolate from Eastern Province isolated in 1983 by D. Geysen (Geysen *et al* 1999). *T. parva* Muguga (Kenya, stabilate 73), is one of the components of the trivalent vaccine and its origin has been described by Radley et al. (1975). Genomic DNA of these 2 isolates was derived from *T. parva* schizont-infected lymphocyte cultures.

PCR conditions. Polymerase Chain Reaction (PCR) was performed using different primer pairs as described below. PCR master mix for one reaction was prepared as follows: 5xPCR buffer, 1.65mM $MgCl_2$, 0.2mM dNTPs, 10pmol forward primer, 10pmol reverse primer, 1UTaq, molecular grade water and 20 ng genomic DNA. The final volume was 25 µl for a single reaction. A semi-nested PCR was performed in an automated DNA thermal cycler (Biometra, Germany) for 40 cycles, and 25 cycles for the second round. Each cycle consisted of a denaturing step of 30 seconds at 94 °C, an annealing step (the temperature was set up according to the primers used) and extension step of 1 min at 72 °C. A final extension step of 10 min at 72 °C completed the program.

PCR primers. *T. parva* strain characterization was carried out by multilocus genotyping. A PCR-RFLP approach was used on four polymorphic antigen loci, p104, PIM, p150 (Geysen *et al* 1999) and p67 in addition to one microsatellite MS321 (Oura *et al* 2003). The RFLP profiles of the amplicons by the appropriate restriction enzymes were compared with the theoretical values as well as with Katete and Muguga strain profiles (De Deken *et al* 2007). Primer sequences are shown in table 2. First round p104 PCR was carried out using primers p104/F2 and p104/5 at an annealing temperature of 58 °C, followed by second round PCR using p104/5 and p104/2nF primers at an annealing temperature of 60 °C followed by a digestion using *Alu*I as the restriction enzyme at a temperature of 37 °C. First round PIM PCR was carried out using primers PIM1 and PIMR4 with an annealing temperature of 60 °C, while those used in the second round were PIM Fm and PIMR4 with an annealing temperature of 62 °C. The enzyme used was *Bcl*I at a temperature of 50 °C. The p150 locus was amplified using first round primers NP150F and NP150R at 64 °C and the seminested primers were NP150nF and NP150R at 60 °C. The RFLP profile was obtained by using *Eco57*1 as the

restriction enzyme at a temperature of 37 °C. First round MS321 PCR was carried out using primers MS321nF6 and MS321R at an annealing temperature of 62 °C, and second round primers was followed using MS321nF4and MS321R at an annealing temperature of 57 °C. The RFLP profile was obtained by using *Alu*I as the restriction enzyme at a temperature of 37 °C. The digested fragments were visualized on 2% high resolution agarose, after running for 40 min at 100 V. The p67 locus was amplified using first round primers p67F30 and p67Ri at 55 °C and the seminested primers were p67Fi and p67Ri at 55 °C. The PCR product was visualized on 2% agarose after running for 40 min at 100 V.

Cloning and sequencing experiments. To verify the amplicons, amplified fragments from four samples positive in the PIM PCR were purified using a PCR purification kit (Qiagen, Germany). Thereafter, they were cloned using the TOPO® TA cloning kit (Invitrogen, Germany) and sequenced by MWG (Germany) after confirmation of the fragments by colony PCR. In total seven PIM clones were sequenced.

RESULTS

All the 20 samples showed single infections of *T. parva* in all investigated four loci. Depending on the PCR sensitivity and specificity of the respective locus, some samples remained negative. The p104 and p150 antigen loci (Figures 1 and 2) exhibited low polymorphism with two alleles identified among the parasite samples studied (Table 3). The p104 locus was the most sensitive assay where all samples could be characterized, on one hand. On the other hand, MS321 and to a higher extend, the PIM locus demonstrated more diversity, but was less sensitivity than p104 locus. With respect to the PIM locus, this could be attributed to the fact that this locus is the most polymorphic among the loci investigated in this study. All p104 profiles were identical to the Katete profile, with exception of one sample (JUBA-7) that exhibited a Muguga profile (Figure 1). Regarding the p150 locus, 6 samples exhibited a Muguga profile and 10 samples showed a Katete profile (Figure 2). The PIM locus was the most polymorphic with 9 alleles among the parasite studied samples (Figure 3) and one sample showing the Muguga PIM profile. Using the combination of the loci results per parasite sample, a moderate differentiation of the parasites originating from the two South Sudanese geographical areas was possible. On the other hand, the MS321 profile is illustrated in figure 4, with four identified alleles, although 9 samples revealed no PCR products after the seminested PCR (Table 3). The amplified p67 products generated from 19 samples from South Sudan, including the product from the *T. parva* Muguga and Katete strains were identical in size being approximately 320 bp (Figure 5), whereas the JUBA-2 sample showed a higher band of around 450 bp. The nearly total length sequence of the PIM gene for *T. parva* obtained in this study from four samples showed the highest identity of 100% with four database entries of *T. parva* PIM surface protein gene (GenBank™ accession numbers EF469603.1, L06323.1, L41148.1 and XM_758593.1) using ClustalW alignment. According to these results, sequence analysis confirmed the specificity of PCR-RFLP analysis. When aligning using the Gene Jockey II software and analyzing the PIM gene sequences, it was possible to distinguish 3 groups: 1 similar to Zambia/Burundi (Juba-6 “K-1” and Juba-18”K-2”),1 similar to another cluster of Zambia/Burundi (Juba-18 “K-1”, Juba-19 “K-1” and Juba-19 “K-2”) and 1 similar to Rwanda (Juba-3 “K-2” and “K-1”) (Tables 4, Figure 6).

Table 1. *Theileria parva* strains used in this study

Strains	Origin	References
Theileria parva Muguga	Kenya, stabilate 73	Radley et al. 1975
Theileria parva Katete	Zambia, Eastern Provine	Geysen et al. 1999
Theileria parva JUBA -1	South Sudan, Gumba	
From 1 to 7 Theileria parva JUBA -8 From 8 to 20	South Sudan, Khor Ramla	Salih et al. 2007

Table 2. Primer sequences used in semi-nested PCR assays for the p104, PIM, p150, p67 and MS321 loci of Theileria parva

Primer name	Sequence ′5-3′	References
p104 F2	CCACCATCTCCTAAACCACCGTT	
p104-5	TAAGATGCCGACTATTAATGACACCACAA	
p104 2nF	AACCACCGTTTGATCCATCATTCA	
PIM 1	GTGAATGTTGTGATCTTAATCC	De Deken et al. 2007
PIM R4	CCCACAACCGTGGAATGGCGTA	
PIM Fm	ATTCCACTGGTTCTTCCGATSTA	
NP150 R	TTACCATCTTCACCGCGAAC	
NP150 F	GATATTCCTTTACTTGCTCGAC	
NP150 nF	CGACTTGAAGAAGAAGATTACAGT	
p67F30	ccgtcaaacggctcagactcc	Nene et al., 1996
p67Ri	Tactcaaaaaaacaaacc	Bishop et al. 1998
p67Fi	Caggtgaaactacatcgg	
MS321 nF6	taagagcgtgattggaga	
MS321 R	CAGACTCCAACATTGCAGG	
MS321 nF4	GAGATGACTGTTGGTCGTAATTGCT	

NS*: Non-specific bands in the PCR
-ve: Negative results in the PCR

Table 3. Multilocus genotypes results from various T. parva isolates. Genotypes are defined by letters. Identical letters stands for identical genotypes.

Isolate		Allelic markers					
		p104	p150	PIM	MS32.1	p67	
Exotics	Muguga	A	C	G	P	V	ACGPV
	Katete	B	D	H	Q	V	BDHQV
Local	JUBA-1	B	D	H	Q	V	BDHQV
	JUBA-2	B	-ve	NS*	P	W	BPW
	JUBA-3	B	C	H	Q	V	BCHQV
	JUBA-4	B	D	J	Q	V	BDJQV
	JUBA-5	B	D	I	-ve	V	BDIV
	JUBA-6	B	D	N	R	V	BDNRV
	JUBA-7	A	NS	-ve	-ve	V	AV
	JUBA-8	B	C	H	P	V	BCHPV
	JUBA-9	B	-ve	-ve	-ve	V	BV
	JUBA-10	B	D	-ve	-ve	V	BDV
	JUBA-11	B	-ve	H	P	V	BHPV
	JUBA-12	B	C	K	-ve	V	BCKV
	JUBA-13	B	D	L	S	V	BDLSV
	JUBA-14	B	D	M	-ve	V	BDMV
	JUBA-15	B	C	N	-ve	V	BCNV
	JUBA-16	B	D	NS	-ve	V	BDV
	JUBA-17	B	C	H	P	V	BCGP
	JUBA-18	B	C	O	P	V	BCOPV
	JUBA-19	B	D	H	Q	V	BDHQV
	JUBA-20	B	NS	-ve	-ve	V	BEV
Numbers of alleles		2	2	9	4	2	

NS*: Non-specific bands in the PCR -ve : Negative results in the PCR

Table 4. PIM sequences identification and their corresponding samples and clones

Sequence name	Sample	Clone
Sudan1	Juba-3	K1
Sudan2	Juba-3	K2
Sudan3	Juba-6	K1
Sudan5	Juba-18	K1
Sudan6	Juba-18	K2
Sudan7	Juba-19	K1
Sudan8	Juba-19	K2

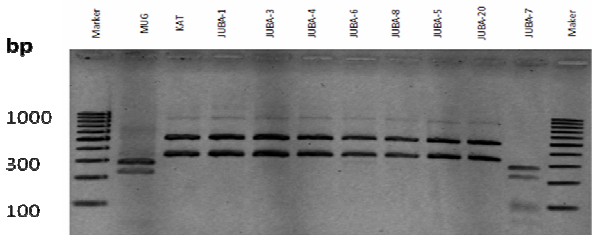

Figure 1. PCR-RFLP characterisation of South Sudan sample using the p104 locus and digested with the restriction enzyme AluI. Genomic DNA derived from T. parva schizont-infected lymphocyte cultures (Muguga and Katete) was used as control. Samples were resolved by electrophoresis through 2% high resolution agarose.

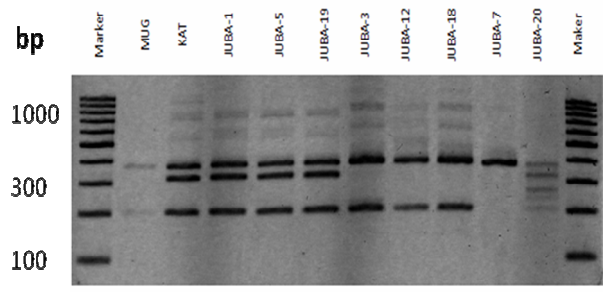

Figure 2. PCR-RFLP characterisation of South Sudan samples at p150 locus and digested with the restriction enzymeEco57I. Genomic DNA derived from T. parva schizont-infected lymphocyte cultures (Muguga and Katete) was used as control. Samples were resolved by electrophoresis through 2% high resolution agarose.

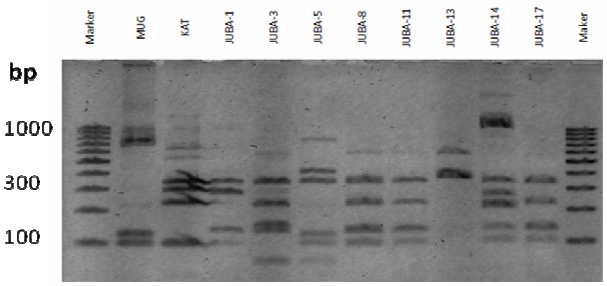

Figure 3. PCR-RFLP characterisation of South Sudan samples at PIM locus and digested with the restriction enzyme BclI. Genomic DNA derived from *T. parva* schizont-infected lymphocyte cultures (Muguga and Katete) was used as control. Samples were resolved by electrophoresis through 2% high resolution agarose.

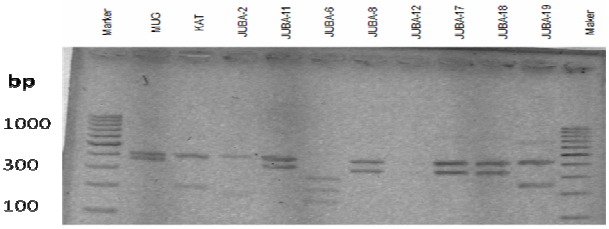

Figure 4. PCR-RFLP characterisation of South Sudan samples at MS321 locus and digested with the restriction enzymes AluI. Genomic DNA derived from *T. parva* schizont-infected lymphocyte cultures (Muguga and Katete) was used as control. Samples were resolved by electrophoresis through 2% high resolution agarose.

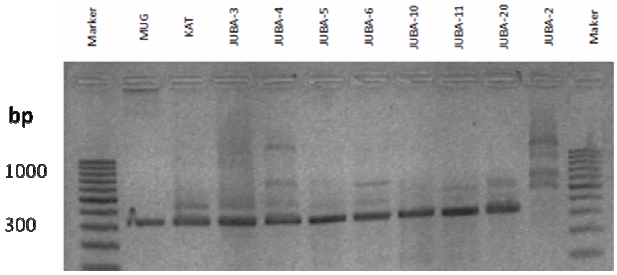

Figure 5. PCR characterisation of South Sudan samples at p67 locus. Genomic DNA derived from *T. parva* schizont-infected lymphocyte cultures (Muguga and Katete) was used as control. Samples were resolved by electrophoresis through 2% agarose.

DISCUSSION

Characterization of field strains of *Theileria parva* in a particular area is a pre-requisite before any control method could be adopted in that area. Different epidemiological regions are known to exhibit different parasite population dynamics and degrees of diversity. In identical situation, as shown for parts of Zambia and Zimbabwe (Geysen *et al* 1999), the introduction of an exotic, multivalent vaccine could create major problems as ticks feeding on these immunized animals harboring different parasite genotypes could be the basis for a new recombinant genotype in the region. At a more practical level, the outcome will depend on the cross immunity characteristics of the new variant in relation to the local strains. This can only be checked by performing cross immunity trials between the vaccine strain(s) and the dominant representative genotypes in the field. Molecular characterization is a first step for evaluating parasite relations. Using antigen

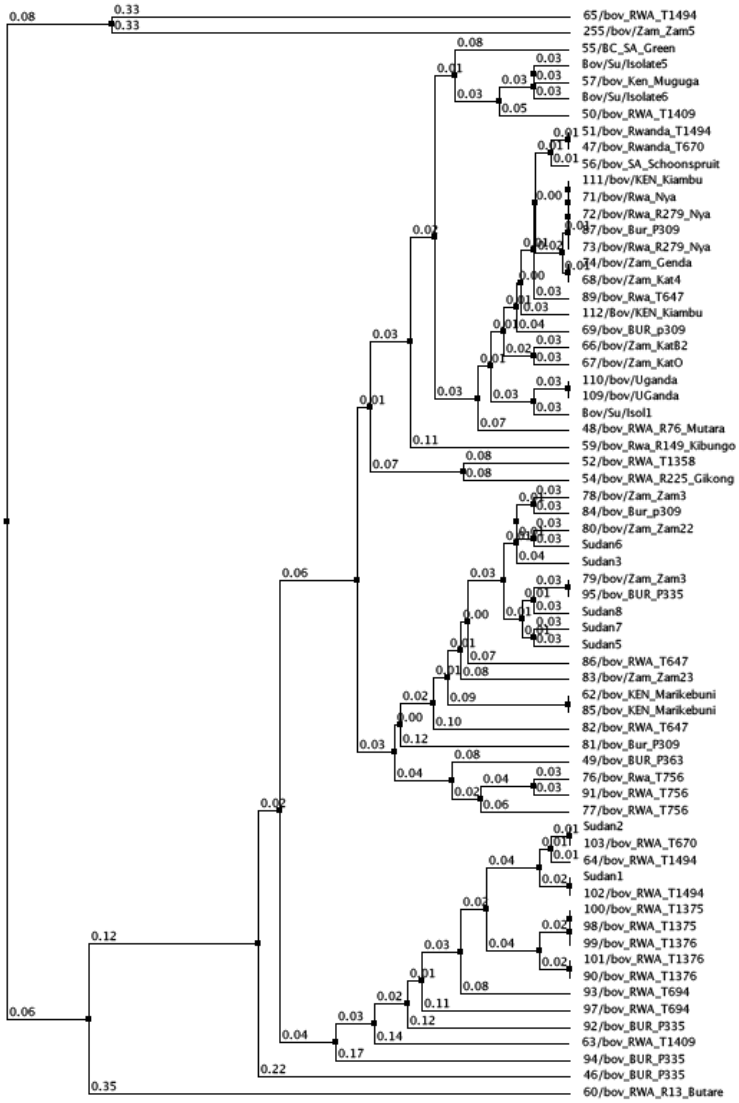

Figure 6 . Phylogenetic relationship of *T. parva* parasites as revealed by PIM gene sequences. Results indicated 3 groups, group similar to Zambia/Burundi (Sudan3 = Juba-6 "K-1" and Sudan6 = Juba-18"K-2"), 1 similar to another cluster of Zambia/Burundi (Sudan7 = Juba-18 "K-

related genes have shown a rough idea of parasite genotype diversity in the field of South Sudan. Cattle derived *T. parva* parasites have only 2 alleles for the p150 or p104 genes as compared to buffalo-derived parasites with specific and polymorphic p150 and p104 sequences. The cattle sequences for both genes can be divided in 2 subgroups based on their alleles and the majority was different from the Kenyan derived Muguga isolate. Most (99%) p104 profiles were identical to the Katete cluster but a 40/60 percent ratio of the two respectively Muguga and Katete p150 profiles was found. The present study demonstrated that the local strains of *Theileria parva* in South Sudan are more diverse than was expected. It is not known if this is due to import of various genotypes from Uganda or to prevailing high transmission intensities in the area

so that there is broad scope for recombination. The first hypothesis seems the more plausible as the area is less suitable for *R. appendiculatus* ticks being only seasonally present in low numbers. The MS321 locus illustrated a moderate diversity, with 4 alleles identified, but this assay was only moderately sensitive. This could be due to the low level of parasitemia in the examined animals, as the samples were collected from apparently healthy cattle (Salih *et al* 2007). With respect to PIM, it was observed that only one sample (JUBA-17) shared the same profile with Muguga, while 5 samples sharing the Katete profile (Table 3). On the other hand, 4 samples showed a negative result and two sample showed a non-specific band. When comparing the partial to full data on multi locus genotypes (MLG), one can see that most are similar to the Katete haplotype, including 2 identical MLGs. The p67 locus confirmed that all the samples were cattle derived except the JUBA-2 sample being a buffalo-derived strain. The p67 PCR product of 320 bp is typically observed in cattle-derived *T. parva* stocks, whereas a 130 bp insert is present in most buffalo-derived *T. parva* stocks (Nene *et al* 1996). It remains to be shown whether transmission of *Theileria parva* between cattle and buffalo is still present in the study region or this is an import of a buffalo-derived but cattle circulating genotype from Uganda. However, a cross-sectional and longitudinal survey in the same region among wildlife, including buffalo would be required to answer the fore-going questions. The only conclusion now is that it appears there is no geographical correlation between the PIM families and the location in Sudan, beside the fact that one sample has a PIM similar to Muguga, a finding of particular interest. It is important to state that results of this study do not represent the actual field situation of *T. parva* genotypes in South Sudan because of the small numbers of tested samples. Molecular tools could also be used to determine the various parasite population structures present in the field in South Sudan. Thus, further genotyping studies of *Theileria parva* isolates using a larger numbers of samples and testing with either the same approach or the high-throughput capillary electrophoresis system (Patel *et al* 2011) using satellite markers would give a more global understanding of *T. parva* strains diversity in this region. This would help to establish detailed and up to date knowledge of *Theileria parva* in the region and help in formulating appropriate control strategies.

Ethics

I hereby declare all ethical standards have been respected in preparation of the article.

Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

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