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Identification of *Trypanosoma vivax* subtypes isolated from cattle and goats using microsatellite markers

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ABSTRACT

Microsatellite DNA polymorphisms can be utilised to assess intra-specific genetic diversity and hence are useful for characterisation of species and strains of trypanosomes. Here, we present four new microsatellite markers specific for *T. vivax* isolated from Ugandan cattle and goats. The GeneDB partial shotgun 5x coverage sequence of *T. vivax* available as of 1st August 2005 was used and targeted the genomic sequence of *T. vivax* that has no cross amplification with other livestock-infective trypanosomes. Only di-; tri-; tetra;- and pentanucleotide microsatellites not less than five units were selected. Although pentanucleotide repeats on screening appeared to have the desired variability, they gave poorer PCR products compared to di-, tri- and tetranucleotide repeats. Mononucleotide repeats presented difficulty in detecting visible bands on agarose gels from their amplification and were omitted from this study. Clear length polymorphism was obtained with guanine, thymine and adenine repeated 16 times $(GTA)_{16}$ while cytosine, adenine, cytosine and thymine $(CACT)_{15}$ gave size and length variability. Bands of similar size were obtained from thymine and adenine $(TTA)_{24}$ microsatellite, approximately 150 base pairs long and 180-200 base pairs from the cytosine and adenine $(CA)_{26}$ microsatellite. These findings suggest that different subtypes of *T. vivax* exist in Uganda; the polymorphic forms derived from microsatellite band size differences may suggest this parasite exhibits virulence differences as has been shown in *T. congolense* subtypes.

Key words: microsatellites, *Trypanosoma vivax*, subtypes

Introduction

Livestock trypanosomes exhibit diversity in their genetic makeup. This considerable intra-specific genetic diversity at the level of individual genes has been useful for

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characterisation of the parasite species and strains and has been used extensively for epidemiological analyses. The most useful target sequences / genes are those that are presented in multicopy form, compared to those existing as a single copy, because the latter are more difficult to amplify (MACLEOD et al., 1999).

Primers have been developed for specific detection of *T. congolense* subtypes basing on minichromosomes of the nuclear DNA, which apparently contain satellite DNA (MASIGA et al., 1992). Mini-exon genes have been used for detection of *T. vivax* (VENTURA et al., 2002); kinetoplastic mini-circle DNA sequences have also been useful for *T. evansi* detection (ARTAMA et al., 1992). DELESPAUX et al. (2003) using a single PCR-RFLP assay detected mixed infections with trypanosomes' species in cattle. Chromosome polymorphisms (karyotye) have been demonstrated in *T. brucei* (MELVILLE et al., 1998) and in *T. cruzi* (VARGAS et al., 2004). Additional PCR-based techniques have also been developed (MASAKE et al., 1997; Mc NAMARA et al., 1995) but one pair of primers is required to detect each subspecies or type of trypanosomes. For *T. congolense* subtypes, the process requires processing three to five different PCRs per sample (DESQUESNES et al., 2001).

Microsatellites are specific DNA sequences of tandemly repeated short basic motifs generally less than five nucleotides in length. These microsatellites are ubiquitous among eukaryotes, from mammals to parasites (BITEAU et al., 2000) and most of them occur interspersed in the genome, depending on the repeated motif and the species considered. Microsatellites can occur anywhere in the genome: in both protein-coding and non-coding regions (TOTH and GASPARI, 2000). Generally, microsatellites arise from slippage mechanisms which occur during replication or DNA repair according to the Stepwise Mutation Model (LEVINSON and GUTMAN, 1987). Microsatellites were chosen in this study because they present a number of advantages since they enable direct study of a parasite at the level of nuclear DNA where the most important evolutionary phenomena take place. They are also the most powerful Mendelian markers for eukaryotes, combining specificity and high variability; low quantities of template DNA were required to get good PCR products hence allowing comparisons across different gels derived from different samples from Ugandan livestock.

Materials and methods

Selection of livestock for bleeding. Cattle and goats of all ages and sex were bled from two districts in Uganda: Kasese and Jinja, located in the western and eastern regions of the country respectively (Fig. 1). At least 10% of the animals on each farm were randomly selected and bled between October and December, 2004. Overall, the number of cattle bled was 584 and 807 from Kasese and Jinja districts respectively, while 213 and 185 goats were bled from the former and latter districts respectively. From each animal, 2-3

millilitres of blood were collected into Ethyl diamine tetra acetic acid (EDTA)-coated tubes from the jugular vein in cattle and goats. Twenty microlitres of blood were expressed onto Whatman FTA cards for subsequent extraction of template DNA that was required for testing out selected primer sets in detection of microsatellite sequences specific for *T. vivax*.

Extraction of DNA from the Whatman FTA cards. The DNA was extracted using the QIAamp DNA mini kit (2003) protocol with minor modifications. Three or four 2 mm in diameter circles of whole dried blood spots were punched out of the FTA cards and placed in a 1.5 mL microfuge tube, covered with cell lysis buffer, incubated at 85 ºC for 10 minutes. Genomic DNA was extracted by adding proteinase K and incubating the extract at 56 ºC for 1 hour. The DNA was precipitated with ethanol and subsequently eluted in 110 µL of buffer AE, aliquoted and stored at -20 ºC until required for use as template DNA for testing out microsatellite sequences specific for *T. vivax*.

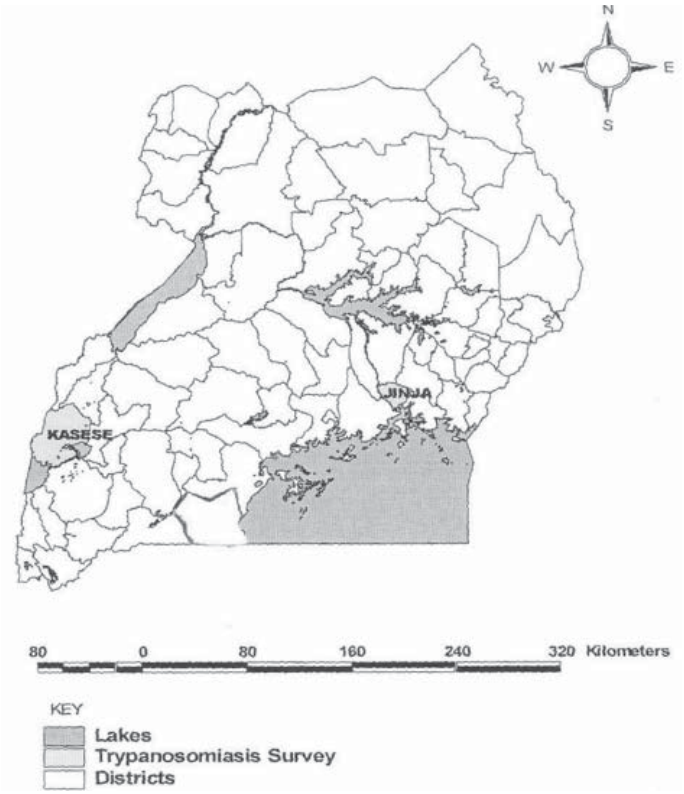

Fig. 1. Map of Uganda showing the districts where the livestock were bled

Bioinformatic selection and categorisation of microsatellite DNA markers. Microsatellites were selected from the latest GeneDB 5x *T. vivax* contig releases as of 1st August 2005. The *T. vivax* sequence file transfer protocol (ftp) data set was used and was obtained from The Sanger Institute website at http://www.sanger.ac.uk/Projects/*T_vivax*/ and was visualised in Artemis version 7 (RUTHERFORD et al., 2000). The data set, in Fasta format was searched for sequences not exceeding five tandem repeats with Tandem Repeats Finder software (BENSON, 1999).

The computer output was manually scrutinised and the repeats classified as di, tri, tetra or pentanucleotides. Mononucleotides were not scrutinised. Each microsatellite was also examined for consensus sequences and classified as perfect if it was entire with no nucleotide substitution or deletion. If a substitution or deletion of one or more nucleotides was detected, the microsatellite was classified as imperfect. Repeats were tested for evidence of existence in other genomes namely of *T. congolense* and *T. brucei* by performing an OmniBLAST search of the repeat in sequenced genomes of other pathogenic trypanosomes.

Positive control T. vivax: Positive control DNA was derived from *T. vivax* strain IL 2569 and was kindly provided by Dr. F. Majiwa of International Livestock Research Institute, Nairobi, Kenya.

The PCR protocol for application of target microsatellites from T. vivax genomicDNA. The reactions were made in 25 µL total volume PCR mix and each reaction contained 2.5 µL of 10x PCR buffer containing 15 mM $MgCl_2$; 0.25 µL of 10 mM of each dNTP; 0.5 µL of 10x forward and reverse primer, 0.125 µL of HotStar Taq DNA polymerase and 5 µL of DNA extracted from field-derived blood samples. All the reactions were performed in a PTC-100 Peltier Thermal Cycler which was programmed as follows: Lid preheating at 96 ºC for 15 minutes followed by the denaturation step at 94 ºC for 30 seconds; annealing at 55 ºC for 1 minute. However, the annealing conditions were varied to suit different primers and in particular, the best temperature was determined by the absence of non-specific PCR fragments in the optimisation step. This was followed by the extension step at 72 ºC for 1.30 minutes. The reaction was cycled 39 times before the mixture was incubated at 72 ºC for 10 minutes in a final extension step.

The products were kept at 4 ºC in the thermocycler pending running them on a 1.5% or 2.0% w/v agarose gel stained with ethidium bromide (0.5 µg / mL) and flooded with 1x TBE buffer. In order to track the amplicons on the gel, 8 µL of the PCR products stained with 2 µL of 1:10 10x loading dye (0.25% w/v bromophenol blue; 0.25 w/v xylene cyanol, 20% Ficoll 400; 0.1M Na_2EDTA, pH 8.0; 1% SDS) and were run side by side with 100 bp and 1 kb DNA ladders. Electrophoresis was done at the following conditions: 55 V, 250 mA and run for 30 minutes.

Results

The results presented here describe the selection of *T. vivax* microsatellite markers basing on size polymorphism (in approximate number of base pairs) and the number of DNA bands generated per field-derived trypanosome DNA. A summary of the numbers and frequencies of different types of repeats from analysis of the *T. vivax* sequence with Tandem Repeats Finder and Artemis version 7 is shown in Table 1.

Table 1. Summary of the numbers and frequency of simple sequence repeats as searched in the August 2005 GeneDB *T. vivax* contigs

Nucleotide type	Frequency of repeats in the genome	Number of perfect repeats	Number of imperfect repeats	Repeats per megabase of nucleotides
Di-	494	354	140	36.5
Tri-	247	134	113	18.4
Tetra-	73	51	22	5.4
Penta-	205	70	135	15.1
Total	1.019	609	410	-

Overall, the di-, tri-, tetra and penta nucleotides from GeneDB database returned 494, 249, 85 and 191 repeats for each category respectively. The total number of repeats was 1,019 of which 609 were perfect and 410 imperfect. Dinucleotide repeats were the most frequent (36.5 repeats per megabase of genome) whereas tetranucleotides were the least frequent (5.4 repeats per megabase of genome).

Several microsatellites were scrutinized from which twenty were selected for further evaluation during PCR optimisation reactions. Further analysis of the selected microsatellites with GeneDB omniBLAST search revealing the upstream and downstream sequence homology against *T. congolense* and *T. brucei* is shown in Table 2. Representative gels showing typical banding patterns of individual microsatellite primer sets are shown in Fig. 3.

Table 2. Sequence homology comparing sequences (primers) flanking selected *T. vivax* microsatellites with those of *T. brucei* and *T. congolense* at equivalent loci

Microsatellite	Trypanosome species *	Downstream homology (%)	Upstream homology (%)
$(CA)_{26}$	*T. vivax*	100	100
	T. brucei	0	0
	T. congolense	0	0
$(GTA)_{16}$	*T. vivax*	100	100
	T. brucei	0	0
	T. congolense	0	0
$(TTA)_{24}$	*T. vivax*	100	100
	T. brucei	0	67
	T. congolense	0	59
$(CACT)_{15}$	*T. vivax*	100	100
	T. brucei	70	0
	T. congolense	90	0

The upstream and downstream flanking sequences are those adjacent to the 5' and 3' ends of each microsatellite as viewed in Artemis, respectively. The $(CA)_{26}$ and $(GTA)_{16}$ microsatellites returned 100% homology from *T. vivax* contig reads, showing that no similarity in both upstream and downstream sequences were noted in either *T. brucei* or *T. congolense* sequences. Searches with $(CACT)_{15}$ and $(TTA)_{24}$ returned some similarity in the downstream and upstream sequences of *T. brucei* and *T. congolense* respectively.

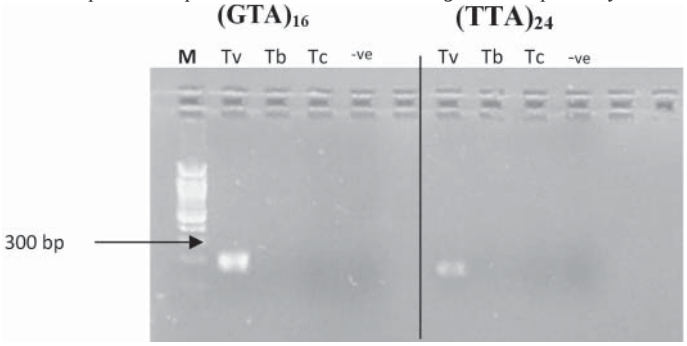

Fig. 2. Specific amplification of $(CAT)_{12}$ and $(TTA)_{16}$ microsatellites from *T. vivax* genomic DNA. The PCR was performed with positive control genomic DNA derived from *T. vivax* IL 2569, *T. congolense*, IL 3000 and *T. brucei* TREU 427 and specific primers in Table 3. For both primer sets amplicons were observed in the PCR with *T. vivax* DNA template but not *T. congolense* or *T. brucei*, implying the specificity of the microsatellites in *T. vivax* genome. Similar reactions were performed with each of the selected primer sequences (gels not shown). The lane Tv = *T. vivax*; Tb = *T. brucei*; Tc = *T. congolense*; -ve = negative control. M = Lambda DNA size marker.

Table 3. Oligonucleotide sequences designed for amplification of five selected microsatellites for *T. vivax*

Primer name	Primer sequence (5' end to 3' end)	Tm
BS $(CA)_{26}$	F TTG TCA TCT TTA TTC CTG TAA GTC A	60.4 ºC
	R TTA TTA CTC CGC GCT TGC TC	64.1 ºC
BS $(CACT)_{15}$	F CGA AGG AGA CTT GCT TGT TT	61.0 ºC
	R TGC ATG ATT GAA ATT AGA TGA T	62.9 ºC
BS $(TTA)_{24}$	F CAG GGT GTC GTC TCA TGA CAT	62.6 ºC
	R TCT TGT TGC ACC ACA AAT	64.1 ºC
BS $(CAT)_{16}$	F CGC TCA CAA CAC ACC TTT TC	63.3 ºC
	R GGG GTA ACC TTA TTT CGC AGT	62.8 ºC

T_m: The melting temperature values for each forward and reverse strand; •F = forward strand; R = reverse strand; BS is primer name derived from the abbreviation of the principal author Savino Biryomumaisho

Sequence homology searches between T. vivax, T. brucei and T. congolense genomes. Sequences flanking the selected microsatellites were blasted (Table 2) against contig reads of *T. brucei* and *T. congolense* in GeneDB webpage to verify whether these regions (potential primers) were unique and likely to be specific to *T. vivax* without cross-amplification with other livestock-infective trypanosomes. $(CA)_{26}$, $(GTA)_{16}$ and $(ACAT)_{10}$ microsatellites returned 100% sequence homology to *T. vivax* while $(TTA)_{24}$ and $(CACT)_{15}$ microsatellites had some upstream and downstream homology with *T. brucei* and *T. congolense* respectively.

The selected primer sets for characterization of T. vivax isolates. Table 3 shows the sequences of selected primers generated from Primer 3 software presenting the forward (F) and reverse strands (R) of each set. In order to have a good choice of primers to amplify the targeted regions of the genome efficiently, sequences in excess of 25 nucleotides and the forward and reverse strand G+C ratios out of 45- 55% range were not selected from the six possible primer set output of each set. Likewise, primer sequences shorter than 18 nucleotides were rejected**.**

Optimisation of PCRs for T. vivax-specific microsatellite sequences. The gel electrophoretic patterns for each microsatellite were made to authenticate the specificity of the selected primer sets in the *T. vivax* genome and their absence in either *T. congolense* or *T. brucei*. The reactions were made to fortify the sequence homology omniBLAST search results in Table 2. An annealing temperature that gave clear bands (Fig. 2) with no stutter bands was used for subsequent PCRs with template DNA from *T. vivax* isolates from cattle bled from the Kasese and Jinja districts and the single goat number 89 from the Kasese district.

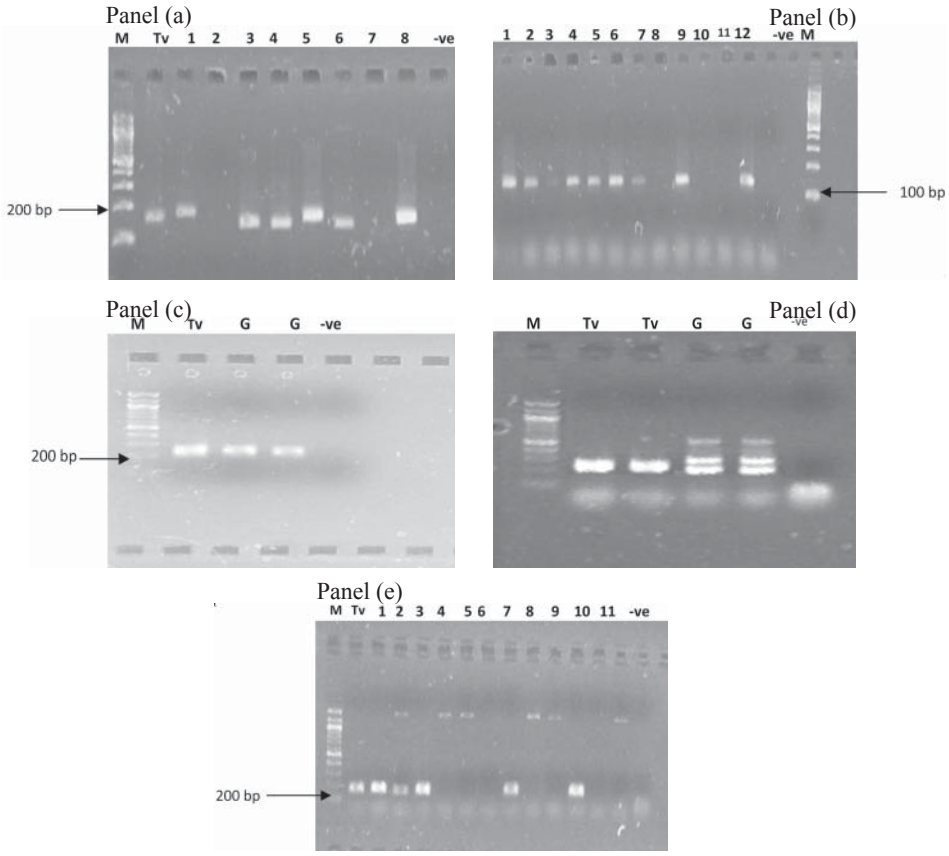

Fig. 3. Ethidium bromide staining analysis of PCR products of five microsatellites for *T. vivax* monolithic infection in representative isolates of cattle from Jinja and Kasese districts and goat number 89 from Kasese district. The panels were run on 2% Metaphor agarose gel. Both positive control and test DNA of goat number 89 in duplicate was run in Panel (c) with $(TTA)_{24}$ microsatellite while the positive control *T. vivax* DNA and DNA extract of the same goat were run on the gel in duplicate and presented in Panel (d) with $(CACT)_{15}$ microsatellite. The gel shown in Panel (e) is for the $(CA)_{26}$ microsatellite on DNA extracted from cattle from Jinja district.

Discussion

In this study, the availability of bioinformatics tools was exploited to develop four new microsatellite markers specific for *T. vivax*. The agarose gel banding pattern of $(GTA)_{16}$ showed clear length variation; bands for $(CACT)_{15}$ microsatellite gave clear double bands compared to *T. vivax* positive control single bands. Although the exact band sizes could not be resolved with 2% Metaphor agarose gel, the banding pattern observed with the $(CACT)_{15}$ microsatellite suggests that the flanking sequence of this microsatellite exhibits allelic dimorphism. The presence of bands of constant size, approximately 160 base pair in $(TTA)_{24}$ microsatellite suggests that this microsatellite is monomorphic and homozygous; thus, the forward and reverse strand flanking sequences of this locus has not undergone genetic mutation. The $(TTA)_{24}$ banding pattern is similar in *T. vivax* circulating in cattle in the Jinja district and the goat from the Kasese district. The microsatellite amplified well, gave no stutter bands and is hence a reliable marker for diagnostic and epidemiological studies of *T. vivax*. However, in these samples, the microsatellite is monomorphic, and therefore does not distinguish between the Ugandan field isolates studied here. A similar result is shown in Panel (c); the PCR was done on duplicate DNA extracts from goat number 89. Likewise, bands generated from $(CA)_{26}$ gave constant size bands approximately 200 bp hence demonstrating the homozygosity of this microsatellite. Basing on band size, this microsatellite can be useful when band size variations *T. vivax* from different geographical regions are obtained and genotyped.

The allelic size variation observed in $(GTA)_{16}$ and $(CACT)_{15}$ and the diploid alleles observed in $(CACT)_{15}$ microsatellites suggests that different genotypes of *T. vivax* exist in Uganda. Further work identifying polymorphisms in microsatellites may allow us to correlate genotype variation with phenotype variation in *T. vivax*, such as varying pathogenicity. Similar observations were reported with three genotypes of *T. congolense* (BENGALY et al., 2002). The demonstration of genetic diversity at the level of individual DNA segments in *T. congolense* and the subsequent characterisation into three genetically distinct types suggested that the defined subtypes might also differ in pathogenicity. Genotypic variation could be the basis of varying pathogenicity and correlation with specific microsatellite markers would be a useful marker for such phenotypic variation. It would be interesting to study specific microsatellites of *T. vivax* drawn from wider geographical locations and also explore the possible genetic basis of the varying pathogenicity observed between West African and East African livestock trypanosomosis with *T. congolense* being the most pathogenic species in East Africa and *T. vivax* in West Africa (KAAYA et al., 1978). Morphological variations in *T. vivax* were earlier linked to variations in pathogenicity of this parasite (FAIRBAIRN, 1953).

The success and usefulness of microsatellite markers depended on whether individual microsatellites had highly conserved flanking sequences, a phenomenon that

has been demonstrated in other organisms including work on cataecians SCHLOTTERR et al. (1991) and in turtles (FITZSIMMONS et al., 1995). This phenomenon has allowed cross amplification from species that diverged as long ago as 470 million years. In this study, cross-amplification would be a disadvantage and this was scrutinised in two ways: first by BLAST searches of the chosen microsatellite considering both the 5 ' and 3 ' flanking sequences against sequences of other trypanosomes species in GeneDB database (Table 2). Sequences which returned 0% homology were preferred; only two of the four microsatellites $(CACT)_{15}$ and $(TTA)_{24}$ returned relatively low homology. The second way was by PCR optimisation of individual reactions (Fig. 2). A microsatellite was chosen when bands were obtained from *T. vivax* DNA and not from *T. congolense* or *T. brucei* DNAs.

While the search for *T. vivax* microsatellites is not complete, the results presented here show that specific microsatellite DNA sequences have been identified and can be used as markers to unequivocally genotype *T. vivax* with no cross amplification with either *T. congolense* or *T. brucei* for use in molecular epidemiology studies. The application of new microsatellite markers will be useful in resolving the complex issue of the diversity because new *T. vixax* genotypes have been discovered in East Africa (ADAMS et al., 2010).

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BIRYOMUMAISHO, S., E. KATUNGUKA-RWAKISHAYA, G. W. LUBEGA, S. E. MELVILLE: Identifikacija podtipova protozoona *Trypanosoma vivax* izdvojenih iz goveda i koza pomoću mikrosatelitskih markera. Vet. arhiv 81, 13-24, 2011.

SAŽETAK

Polimorfizam mikrosatelitske DNA može se rabiti za procjenu unutarvrsne genetske raznolikosti pa tako i za karakterizaciju vrsta i sojeva tripanosoma. Prikazana su četiri nova mikrosatelitska markera specifična za vrstu *T. vivax* izdvojenu iz goveda i koza u Ugandi. GenDB kratka i specifična sekvencija *T. vivax* dostupna nakon 1. kolovoza 2005. bila je ciljano rabljena za određivanje genomskoga slijeda za protozoon *T. vivax* koji nije pokazivao križne reakcije s drugim tripanosomama zaraznima za stoku. Izabrani su bili samo di-, tri-, tetra- i pentanukleotidni mikrosateliti. Premda se činilo da pentanukleotidne ponavljajuće sekvencije u probirnom testu imaju potrebnu varijabilnost, one su dale lošije PCR proizvode u odnosu na di-, tri- i tetranukleotidne ponavljajuće sekvencije. Mononukleotidne ponavljajuće sekvencije nisu dale jasno vidljive trake na agaroznom gelu pa nisu bile dalje istražene. Jasan polimorfizam postignut je upotrebom gvanina, timina i adenina sa šesnaesterostrukim ponavljanjem $(GTA)_{16}$ dok je sekvencija citozin, adenin, citozin i timin $(CACT)_{15}$ bila varijabilna u odnosu na veličinu i dužinu. Sekvencije slične veličine bile su dobivene od mikrosatelita koji su sadržavali timin i adenin $(TTA)_{24}$, a one od 150 parova baza te 180 - 200 parova baza od mikrosatelita citozina i adenina $(CA)_{26}$. Ovi nalazi govore u prilog postojanju različitih podtipova protozoona *T. vivax* u Ugandi, koji bi se mogli odlikovati i različitom virulencijom kao što je dokazano za podtipove *T. congolense*.

Ključne riječi: mikrosateliti, *Trypanosoma vivax*, podtipovi