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## THE USE OF MULTIPLE DISPLACEMENT AMPLIFICATION TO INCREASE THE DETECTION AND GENOTYPING OF *TRYPANOSOMA* SPECIES SAMPLES IMMOBILISED ON FTA FILTERS

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### Abstract

Whole genome amplification methods are a recently developed tool for amplifying DNA from limited template. We report its application in trypanosome infections, characterised by low parasitaemias. Multiple Displacement Amplification (MDA) amplifies DNA with a simple *in vitro* step, and was evaluated on mouse blood samples on FTA filter cards with known numbers of *Trypanosoma brucei* parasites. The data showed a twenty-fold increase in the number of PCRs possible per sample, using primers diagnostic for the multi-copy ribosomal ITS region or 177 bp repeats, and a twenty-fold increase in sensitivity over nested PCR against a single copy microsatellite. Using MDA for microsatellite genotyping caused allele dropout at low DNA concentrations, which was overcome by pooling multiple MDA reactions. The validity of using MDA was established with samples from Human African Trypanosomiasis patients. The use of MDA allows maximal use of finite DNA samples and may prove a valuable tool in studies where multiple reactions are necessary, such as population genetic analyses.

### INTRODUCTION

African trypanosomes cause a disease syndrome across a wide geographic area of sub-Saharan Africa. In domestic livestock disease is caused by *Trypanosoma congolense*, *Trypanosoma vivax*, and, to a lesser extent, *Trypanosoma brucei*, while in humans, disease is caused by *T. brucei gambiense* and *T. brucei rhodesiense*. Diagnosis is routinely by microscopic techniques, which are relatively insensitive, or by serology in the case of humans, which may not necessarily indicate current infection, and is reliant upon the broad specificity of the antigen used as the basis for the assay.<sup>1</sup> The causative agent of 97% of human cases is *T. b. gambiense*,<sup>2</sup> infections of which are characterised by chronic infections with very low parasitaemias that are therefore difficult to detect by microscopy.<sup>3-5</sup>

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Prevalence as measured by traditional microscopic diagnosis has been shown to be a significant underestimation both in animal and human infections, as shown by comparative Polymerase Chain Reaction (PCR) surveys,<sup>6</sup> and by an epidemiology and modelling study<sup>7</sup>. Current methods for the analysis of *T. b. gambiense* are unsatisfactory, as they depend on either inoculation and amplification in rodents, or growth *in vitro*.<sup>8, 9</sup> In both of these cases, the methodology is difficult to use in field settings and is associated with the inevitable selection of strains and potential distortion of our understanding of the genetic structure of parasite populations.<sup>9</sup> Thus a method that enables analysis of field samples directly, even those occurring at a low level in the host, would allow a range of studies that include parasite and host population genetics.

DNA samples, such as those collected in the field or hospital situations, are often one-off finite resources, and the amount of DNA available is usually the limiting factor in the extent and sensitivity of analyses that can be performed. With available resources such as sequenced genomes, there is an increase in numbers of markers such as micro- and minisatellites and single nucleotide polymorphisms (SNPs) that can be analysed. This in turn leads to much improved power in studies such as those involving genotyping and genetic association. These analyses will only prove possible if the bottleneck of restricted starting template is overcome. In addition, replica tests are often required and retrospective studies are commonly undertaken so that maximising the number of tests that can be performed on a single sample is a critical issue to address.

PCR has allowed detection of low concentrations of DNA and the threshold of detection is further increased when nested PCR is utilised. However, even with these techniques, if the target DNA concentration of a sample is low, for example in the case of a haemoparasite blood sample with a low circulating parasite burden, the number of reactions possible is limited. Recently, Whole Genome Amplification (WGA) methods have been developed for increasing the amount of template available.<sup>10</sup> Multiple Displacement Amplification (MDA)<sup>11</sup> employs the bacteriophage  $\phi$ 29 DNA polymerase, which efficiently replicates large stretches (>10 kb) of double stranded DNA utilising random hexamer primers and strand displacement.<sup>10</sup> The fact that the reaction takes place at 30 °C means that the genome(s) present in a sample can effectively be replicated using a simple *in vitro* reaction. This method has been demonstrated to be efficient at amplifying some 93% of loci 250-fold from a single human cell.<sup>12</sup> Although the  $\phi$ 29 DNA polymerase has a very low error rate, estimated as 1 in 10<sup>6</sup> bp,<sup>13</sup> and replicates large stretches of DNA, there is a potential drawback. Failure to amplify both alleles at heterozygous loci ('allele dropout') from single human cells has been detected at up to 31% of heterozygous loci.<sup>14</sup> If higher quantities of template DNA are used however, the successful amplification of both alleles at heterozygous loci increases to 97%, using 10 or 100 ng of human DNA.<sup>15</sup> As quantitative or population genetic studies require the identification of heterozygous loci, this is an important issue to address when considering the application of this technique.

Infected blood samples on FTA® filters (Whatman BioSciences Ltd.) are commonly used as a resource in many disciplines for transporting DNA samples from the field, and so we have used such samples as a source for this study to optimise the analysis of *Trypanosoma brucei* infections. These filters are easy to use as they automatically lyse cells, inactivate viruses, bind the target DNA, and provide a safe and stable matrix for transport. The filters represent a finite sample resource when used with standard PCR approaches. The first approach taken was to use mouse blood with known dilutions of trypanosomes in order to define the sensitivity of PCR, and to estimate the benefits of utilising MDA in terms of multiplying the number of reactions that can be carried out on any one sample. The analysis was undertaken with oligonucleotide primers that were trypanosome-specific,<sup>16, 17</sup> *T. brucei* species-specific,<sup>18</sup> and finally with primers that targeted a single copy heterozygous microsatellite

locus.19 This allowed an analysis of the sensitivity with both multiple- or single-copy target sequences, and the fidelity of amplification of both alleles for a single-copy heterozygous locus. We then analysed a set of field samples from Human African Trypanosomiasis (HAT) patients in the Democratic Republic of Congo (DRC) to test both the findings from the laboratory-based experiments and the potential benefits of using MDA on field samples.

## MATERIALS AND METHODS

### FTA filter preparation

To examine thresholds of detection, TREU 927 (genome reference strain) trypanosomes were grown in an ICR mouse (Harlan) from a cryopreserved stabilate, and the blood was collected at the first peak of parasitaemia (approximately  $1 \times 10^8$  parasites per mL) by cardiac puncture. The parasites were counted in triplicate using an improved Neubauer haemocytometer, and dilutions made in fresh, unparasitised mouse blood of  $1 \times 10^1$ ,  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ , and  $1 \times 10^5$  parasites per mL. Two hundred  $\mu\text{L}$  of each dilution was spotted onto an FTA filter (Whatman), and allowed to air-dry in the dark overnight. Two hundred  $\mu\text{L}$  of uninfected mouse blood was also spotted onto a filter for use as a parasite-negative control. Filters were routinely stored at  $4^\circ\text{C}$  in the dark, with silica desiccant. The maintenance and care of experimental animals complied with the appropriate legislation; the UK Animals (Scientific Procedures) Act, 1986, and with the national and University of Glasgow maintenance and care guidelines.

The field samples were blood samples collected by venepuncture from consenting patients, with approximately 200  $\mu\text{L}$  spotted onto an FTA filter, and allowed to air-dry. The samples were collected in Maluku, 80 km north of Kinshasa, Democratic Republic of Congo, in 2003. The parasitaemia of the samples was estimated at the point of collection by the capillary tube centrifugation (CTC) technique,<sup>20</sup> and all samples used in this study were positive by microscopy. Ethical permission for this study has been granted by OMS/IRD and the University of Glasgow.

For use in PCR, discs were punched out of the FTA blood spot using a clean 2 mm diameter Harris Micro-punch<sup>TM</sup> (Whatman), the discs were washed 3 times with 200  $\mu\text{L}$  FTA purification reagent (Whatman), and twice with 200  $\mu\text{L}$  1 mM TE (10 mM Tris, 1 mM EDTA) buffer pH 8.0, with incubation for 5 minutes at each wash as per the manufacturer's instructions. The washes were necessary to reduce the many potential PCR inhibitors present in blood.<sup>21</sup> The washed discs were air-dried for an hour, and then used as substrate in MDA reactions or PCRs.

### MDA reactions

MDA reactions were carried out directly on washed FTA punched discs. The MDA protocol followed was as described by the manufacturer, in a final volume of 20  $\mu\text{L}$  (GenomiPhi<sup>TM</sup> DNA Amplification kit; Amersham Biosciences). When MDA reactions were pooled, 3 independent reactions were carried out at each trypanosome concentration, the reactions at each concentration were pooled and mixed, and 1  $\mu\text{L}$  of the pooled reactions used as template for PCR. MDA products were routinely stored at  $-20^\circ\text{C}$ .

### PCR primers and conditions

PCR amplification was carried out directly on washed FTA discs using the following conditions, PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM  $(\text{NH}_4)_2\text{SO}_4$ , 4.5 mM  $\text{MgCl}_2$ , 6.7 mM 2-mercaptoethanol, 4.4  $\mu\text{M}$  EDTA, 113  $\mu\text{g}$  per mL BSA, 1 mM of each four deoxyribonucleotide triphosphates), 1  $\mu\text{M}$  of each oligonucleotide primer, and 1 unit of Taq polymerase (ABgene) per 20  $\mu\text{L}$  reaction, or 2.5 U per 50  $\mu\text{L}$  reaction. Two single copy

heterozygous microsatellite markers (PLC and JS2) were amplified in this study, using the PCR primers and conditions described previously,<sup>19</sup> and new primer pairs were designed in order to amplify the outer fragment for the nested PCRs; (sequences 5' to 3') JS2-C AGTAATGGGAATGAGCGTCACCAG and JS2-D GATCTTCGCTTACACAAGCGGTAC, PLC-G2 TTAAGTGGACGACGACGAAATAACAACA and PLC-H4 TTCAAACACCGTCCCCCTCAATAAT. PCR amplification of two different multi-copy genes was also carried out, one set directed against the ribosomal DNA internal transcribed spacer (ITS),<sup>16, 17</sup> and the other targeting the 177 base pair (bp) repeats found in the trypanosome genome,<sup>18</sup> using the conditions described above. PCR amplification was carried out using one disc (PCR volume 20 µL), and 5 discs per blood dilution (PCR volume 50 µL; for nested PCR - 50 µL first round amplification, 20 µL second round). For PCR amplification of MDA products, 1 µL of amplification product was used as template, in a volume of 20 µL. In the case of the nested PCRs, 1 µL of a 1/100 dilution of first round product was used as template in the second round PCR, except for the nested ITS primers, where 1 µL of first round PCR was carried over.<sup>17</sup> Negative controls were included for both PCR on discs and MDA reactions, using as template an FTA disc spotted with uninfected mouse blood, and also a reaction with no template. PCR products were resolved by electrophoresis on a 3% Nusieve® GTG® agarose gel (Cambrex), and were stained with 0.2 µg/mL ethidium bromide in order to allow visualisation under UV light. All reactions were carried out at least 5 times, in order to ensure reproducibility of results.

### Genescan© analysis

When analysing the field samples, one primer of the second round nested PCRs targeting the single copy heterozygous microsatellite (PLC) included a 5' FAM modification. This enabled the accurate sizing and the sensitive detection of alleles by the separation of products using a capillary-based sequencer (ABI 3100 Genetic Analyser; Applied Biosystems). DNA fragment size was determined relative to a set of ROX-labelled size standards (GS500 markers; Applied Biosystems) using Genescan© software, which allowed resolution to the level of 1 bp.

## RESULTS

The first approach was to directly compare standard and nested PCR amplification, using either reactions carried out directly on FTA filters or on the MDA product from FTA filters (Table 1). Sensitivity was measured in two ways. One was 'absolute sensitivity', referring to the trypanosome concentration of the infected blood spotted onto the FTA filter. The second was 'effective sensitivity', which takes into account the dilution factor involved inherent in the MDA method. Effective sensitivity is a measurement of the number of trypanosomes per PCR reaction, more accurately reflecting the multiplication of template by MDA. The threshold of reproducible detection in terms of effective sensitivity for both alleles of the single copy microsatellite marker PLC by single round PCR was 10 trypanosomes per reaction. Using nested PCR, the sensitivity was increased ten-fold to 1 trypanosome per reaction. The sensitivity when using the product of an MDA reaction from a single FTA disc as template was at the level of 0.05 trypanosomes per reaction, a twenty-fold increase in sensitivity compared to nested PCR. However, when 5 discs were used as a template for MDA, allele dropout was observed at 1 trypanosome per reaction (Table 1). In the cases of allele dropout, the allele that failed to amplify was not consistently the same allele, suggesting no homologue bias in the MDA reaction. This problem was overcome by the pooling of 3 independent MDA reactions, with sensitivity observed down to 0.05 trypanosomes per reaction, and amplification of both alleles for reactions from both 1 and 5 discs. Although the absolute sensitivity does not increase between nested PCR on FTA disc

and nested PCR using MDA as template, both having a threshold of  $1 \times 10^3$  trypanosomes per mL, the increase in effective sensitivity for MDA represents the increase in number of reactions able to be carried out at the same threshold of absolute sensitivity. All negative controls, using either uninfected mouse blood or no DNA as template, did not result in amplification of product for any reaction. Overall, for single copy sequences, MDA increases effective sensitivity twenty-fold over nested PCR, and one hundred fold over single round PCR.

The second approach examined whether the sensitivity could be increased by amplification of multi-copy sequences, using primers specific to the ITS and 177 bp repeats, and the results are shown in Table 2. Using the 177 bp repeat as a target, amplifications by PCR18 were able to detect the presence of trypanosomes down to the level of 0.025--0.1 trypanosomes per reaction (corresponding to 100 trypanosomes per mL absolute sensitivity) both with 1 washed disc or with the MDA products (1 or 5 discs) as template. This is possibly the limit of detection, as determined by the probability of a single trypanosome being present in the original blood volume captured in 1 or 5 discs of an FTA filter. The single round ITS PCR amplifications16 were less sensitive, detecting only to the level of 2.5--10 trypanosomes per reaction, for 1 washed disc or MDA products from 1 or 5 discs. Nested primers to the ITS sequence17 were also used in an attempt to overcome this lack of sensitivity. The nested ITS primers improved the level of sensitivity 10-fold over the non-nested ITS primers, to 0.25--1 trypanosomes per reaction, still representing a sensitivity 10-fold less than that observed for the 177 bp repeats. The sensitivity when PCR amplification was carried out on 5 washed discs was 5-fold less sensitive with all sets of primers than PCR amplification using 1 washed disc, and also less sensitive than using MDA product from 1 or 5 discs. The decreased sensitivity when 5 discs are used is very likely due to the presence of increased amounts of PCR inhibitors in the reaction. These results indicate that the use of the products of whole genome amplification in PCRs does not greatly increase sensitivity when multi-copy genes are used as the target for PCR, in contrast to the improved sensitivity using single copy markers. PCRs using the product from MDA reactions from 5 discs are equally as sensitive as nested PCR directly on 1 disc.

The use of Whole Genome Amplification to effectively increase the available material for genotyping was examined using blood samples from HAT patients from the Democratic Republic of the Congo spotted onto FTA filters. An estimate of the parasitaemia was undertaken at the point of collection by Capillary Tube Centrifugation technique (CTC), which gave an approximate measure of trypanosome concentrations, ranging from 251--1000 trypanosomes per mL (Table 3). Discs from 11 samples spotted on FTA cards were used as template for MDA reactions. Nested PCRs using primers for the microsatellites JS2 and PLC were then carried out using 5 discs, and on 3 pooled independent MDA reactions using 5 discs each. Genescan analysis of the PCR products allowed sizing of individual alleles, and also detection of low levels of product. Products were amplified from all samples with both sets of primers revealing two alleles at the PLC locus and three alleles at the JS2 locus; results are shown in Table 3. The fidelity of the MDA reaction in terms of consistency of detection of both alleles of heterozygous samples was examined (Table 3). When products from single MDA reactions were used, 1 out of 11 samples were homozygous for JS2, and 4 were homozygous in the case of PLC (Table 3 bold). However, when the reactions were repeated using 3 pooled MDA products, only one sample gave a homozygous genotype (MAL122 for PLC). Thus, 4 of the 5 homozygous results detected using single MDA reactions were pseudo-homozygous and it was only by pooling MDA reactions that they could be scored correctly. These data highlight the problem of allele dropout when using MDA, and the necessity for repeated analysis or the use of pooled MDA reactions in order to ensure accuracy of results with this technique, particularly in instances when the detection of heterozygosity is an important feature.

## DISCUSSION

This study examined the potential use of Multiple Displacement Amplification, the most robust of the currently available Whole Genome Amplification methods in terms of unbiased genome amplification,<sup>22</sup> in optimising the analysis of finite *Trypanosoma brucei* DNA samples in the form of infected blood spotted onto FTA filters. The analysis by PCR amplification of single- or multi-copy genes confirmed that the multi-copy 177 bp repeat target was the most sensitive method for detecting the presence of trypanosomes, some 10-fold more sensitive than the ribosomal internal transcribed spacer target DNA. The 177 bp satellite repeats are found in large arrays, mainly in the mini- and intermediate chromosomes of *T. brucei*,<sup>23</sup> and so represent a battery of at least tens of thousands of target sequences in the genome of each individual parasite. The ITS sequences on the other hand, are fewer in number, approximately 20--40 copies per cell,<sup>24</sup> and this difference in copy number is likely to be the basis behind the difference in sensitivity. In addition, the relatively low sensitivity of the ITS PCRs, which has been noted previously,<sup>16</sup> may also be due to the fact that the oligonucleotide primers are degenerate, as they are designed to amplify across trypanosome species and so may not be completely homologous to the target sequence. The lack of sensitivity, even after MDA, may limit the use of the ITS primers for field diagnosis of samples with low parasitaemia. The ITS primers were designed in order to generate bands of diagnostically different size for each trypanosome species, allowing the detection of multiple species in a single PCR. However, the ITS PCRs are demonstrably less sensitive than the 177 bp PCRs, although the 177 bp PCRs require an individual reaction per trypanosome species. The use of MDA removes any restriction upon number of reactions per sample, and allows the use of multiple reactions using the very sensitive species-specific 177 bp PCR.

The use of MDA can increase the possible number of reactions 20-fold, and improves the effective sensitivity of detection of single copy genes. Nested PCR amplification of the single copy heterozygous microsatellite was 5-fold more sensitive than single round PCR amplification of the same target. When MDA products were used in nested PCR, there was a 20-fold increase in effective sensitivity over direct nested PCR on washed discs for the amplification of the single copy microsatellite. The issue of detection of single copy genes is important for two reasons. Firstly, the detection of human infective trypanosomes relies upon the amplification of single copy genes, *SRA* for *T. b. rhodesiense*,<sup>25</sup> and *TgsGP* for *T. b. gambiense*<sup>26</sup> and both targets have been used to identify human infective trypanosomes from field samples on FTA filters, and to analyse the epidemiology of the disease.<sup>27</sup> Secondly, single copy target sequences, such as microsatellites, minisatellites and SNPs are the most widely used loci for population genetic analyses.<sup>28</sup> The use of field samples that can be stably stored on FTA filters is a potentially invaluable tool for population genetics, in terms of accurately defining the parasite population. This is particularly useful as it requires no amplification of the trypanosomes in culture or rodents. Current methods for the isolation of the trypanosomes in a sample all potentially lead to selection of particular genotypes. This problem seems to be particularly noteworthy in the case of *T. b. gambiense*, where it has been demonstrated that different genotypes can be isolated from the same sample depending on whether the *in vitro* KIVI isolation kit or rodent inoculation methods were used.<sup>9</sup> Indeed it has been suggested that the narrow range of genotypes that will grow using the KIVI isolation method may be a significant reason<sup>9</sup> for the conclusion that *T. b. gambiense* populations show low genetic variability, and are clonal.<sup>29, 30</sup> Therefore the use of FTA filters will be important in terms of determining the extent of genetic exchange and rigorously examining the population structure of the African trypanosomes.

The availability of polymorphic markers (microsatellite, minisatellite and SNPs) for both host and parasite is increasing as a result of genome sequence resources, so the need to be

able to undertake multiple genotype analyses on a single small sample will increase in order to be able to undertake association studies between pathogen and host genotypes, and disease. The use of MDA as a tool to amplify the genomes present in a sample is potentially extremely useful in this area, as the available template can be amplified twenty-fold allowing multiple analyses to be undertaken on a single sample. An important feature of this study, which is relevant to other pathogens, is that even in the presence of much greater quantities of host DNA, there is effective amplification of low levels of the parasite genome. When the increased sensitivity provided by MDA is combined with the increased number of reactions, the benefits in terms of increased information per sample are substantial. In terms of laboratory research as well as detailed retrospective studies, MDA may well prove invaluable. While this study has focussed on trypanosomes, obviously the application of this technique to any pathogen samples will have considerable value.

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**Table 1**

Sensitivity analysis for a single copy gene. The number of trypanosomes per reaction at which the microsatellite marker PLC was reproducibly detected by PCR amplification. 1 disc or 5 discs indicates the number of washed FTA discs used as the initial template. + indicates reproducible successful amplification and detection of both alleles, +\* indicates the concentrations at which allele dropout was observed, - indicates no product observed, ND indicates not determined. Effective sensitivity is the calculated number of trypanosomes present in the individual PCR. Absolute sensitivity is the initial concentration of trypanosomes per mL of blood spotted onto the FTA card. There are two different values of effective sensitivity at the same absolute sensitivity value, due to the use of 1 or 5 FTA discs, or 1  $\mu$ L (1 disc) or 0.4  $\mu$ L (5 discs) or 0.4  $\mu$ L (5 discs) or 2  $\mu$ L (pooled) of MDA product, as template in the PCR.

Effective sensitivity	Absolute sensitivity	Single round PCR	Nested PCR	Absolute sensitivity	MDA and nested PCR		
					1 disc	5 discs	pooled
500	$1 \times 10^5$	+	+	ND	ND	ND	ND
100	$1 \times 10^5$	+	+	ND	ND	ND	ND
50	$1 \times 10^4$	+	+	ND	ND	ND	ND
10	$1 \times 10^4$	+	+	ND	ND	ND	ND
5	$1 \times 10^3$	-	+	$1 \times 10^5$	+	+	+
1	$1 \times 10^3$	-	+	$1 \times 10^5$	+	+*	+
0.5	$1 \times 10^2$	-	-	$1 \times 10^4$	+	+*	+
0.1	$1 \times 10^2$	-	-	$1 \times 10^4$	+	+*	+
0.05	$1 \times 10^1$	-	-	$1 \times 10^3$	+*	+*	+
0.01	$1 \times 10^1$	-	-	$1 \times 10^3$	-	-	-
0	0	-	-	0	-	-	-

**Table 2**

Sensitivity analysis for multicopy target sequences. Lowest concentrations of trypanosome at which multicopy targets, 177 bp repeats (177) and internal transcribed spacer region (ITS) of ribosomal DNA, were reproducibly detected by PCR amplification. Absolute sensitivity (AS) equates to the number of trypanosomes per mL of blood spotted onto the FTA filter, and effective sensitivity (ES) is the number of trypanosomes calculated per individual PCR. PCR= PCRs carried out on the specified number of washed discs. MDA= Multiple Displacement Amplification reactions carried out on the specified number of washed discs, and PCR undertaken using MDA product as template.

Primers	PCR (1 disc)		PCR (5 discs)		MDA (1 disc)		MDA (5 discs)	
	AS	ES	AS	ES	AS	ES	AS	ES
177	$1 \times 10^2$	0.1	$1 \times 10^2$	0.5	$1 \times 10^3$	0.05	$1 \times 10^2$	0.025
ITS	$1 \times 10^4$	10	$1 \times 10^4$	50	$1 \times 10^5$	5	$1 \times 10^4$	2.5
ITS nested	$1 \times 10^3$	1	$1 \times 10^3$	5	$1 \times 10^4$	0.5	$1 \times 10^3$	0.25

**Table 3**

Detection of heterozygosity of microsatellite markers JS2 and PLC by PCR, from either single or 3 pooled MDA reactions on 5 FTA discs of HAT cases from the DRC. Heterozygosity was analysed both by visualisation of PCR product on ethidium bromide stained agarose gel, and by separation of FAM-labelled products on an ABI300 sequencer. Individual alleles were identified by size in bp by GENESCAN© software, and allocated an identifying number. The numbers in the table indicate the alleles identified in each case. Homozygous samples are highlighted in bold. CTC count indicates the estimation of trypanosome numbers (trypanosomes per mL) by the CTC technique.

Sample	Markers						CTC count
	JS2		PLC		Pooled	CTC count	
	Single	Pooled	Single	Pooled			
MAL100	1 2	1 2	<b>2 2</b>	1 2	1 2	251	
MAL200	1 2	1 2	1 2	1 2	1 2	251	
MAL300	1 2	1 2	1 2	1 2	1 2	251	
MAL400	1 2	1 2	1 2	1 2	1 2	1000	
MAL500	1 2	1 2	1 2	1 2	1 2	251	
MAL600	1 2	1 2	1 2	1 2	1 2	1000	
MAL800	1 2	1 2	1 2	1 2	1 2	251	
MAL900	1 2	1 2	<b>2 2</b>	1 2	1 2	251	
MAL10a	<b>2 2</b>	1 2	<b>2 2</b>	1 2	1 2	251	
MAL111	1 4	1 4	1 2	1 2	1 2	251	
MAL122	1 2	1 2	<b>1 1</b>	<b>1 1</b>	<b>1 1</b>	501	