

USE OF MULTIPLE DISPLACEMENT AMPLIFICATION TO INCREASE THE DETECTION AND GENOTYPING OF *TRYPANOSOMA* SPECIES SAMPLES IMMOBILIZED 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, characterized by low parasitemias. 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 20-fold increase in the number of PCRs possible per sample, using primers diagnostic for the multicopy ribosomal ITS region or 177-bp repeats, and a 20-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*, whereas 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.¹ The causative agent of 97% of human cases is *T. b. gambiense*,² infections of which are characterized by chronic infections with very low parasitemias that are therefore difficult to detect by microscopy.^{3–5} Prevalence as measured by traditional microscopic diagnosis has been shown to be significantly underestimated in both animal and human infections, as shown by comparative polymerase chain reaction (PCR) surveys,⁶ and by an epidemiology and modeling study.⁷ Current methods for analysis of *T. b. gambiense* are unsatisfactory as they depend either on inoculation and amplification in rodents or on growth *in vitro*.^{8,9} 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.⁹ Thus a method that enables direct analysis of field samples, 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 analyzed. This in turn leads to much improved power in studies that, for example, involve 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 maximizing 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 utilized. However, even with these techniques, if the target DNA concentration of a sample is low, for example in the case of a hemoparasite 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.¹⁰ Multiple displacement amplification (MDA)¹¹ employs the bacteriophage ϕ 29 DNA polymerase, which efficiently replicates large stretches (> 10 kb) of double-stranded DNA utilizing random hexamer primers and strand displacement.¹⁰ 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.¹² Although the ϕ 29 DNA polymerase has a very low error rate, estimated as 1 in 10⁶ bp,¹³ 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.¹⁴ If higher quantities of template DNA are used, however, the successful amplification of both alleles at heterozygous loci increases to 97% when 10 or 100 ng of human DNA is used.¹⁵ Because quantitative or population genetic studies require identification of heterozygous loci, this is an important issue to address when considering the application of this technique.

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Infected blood samples on FTA filters (Whatman Bio-Sciences Ltd., Brentford, UK) 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 optimize the analysis of *T. brucei* infections. These filters are easy to use because 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 approaches taken were to use mouse blood with known dilutions of trypanosomes to define the sensitivity of PCR and to estimate the benefits of utilizing 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,^{16,17} *T. brucei* species-specific,¹⁸ and finally with primers that targeted a single-copy heterozygous microsatellite locus.¹⁹ This allowed analysis of the sensitivity with both multiple- or single-copy target sequences and of the fidelity of amplification of both alleles for a single-copy heterozygous locus. We then analyzed 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, Bicester, UK) from a cryopreserved stabilate, and the blood was collected at the first peak of parasitemia ($\approx 1 \times 10^8$ parasites per mL) by cardiac puncture. The parasites were counted in triplicate using an improved Neubauer hemocytometer, and dilutions were made in fresh, unparasitized mouse blood with 1×10^1 , 1×10^2 , 1×10^3 , 1×10^4 , and 1×10^5 parasites per mL. Two hundred microliters of each dilution was spotted onto an FTA filter (Whatman) and allowed to air-dry in the dark overnight. Two hundred microliters of uninfected mouse blood was also spotted onto a filter for use as a parasite-negative control. Filters were routinely stored at 4°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 $\approx 200 \mu\text{L}$ spotted onto an FTA filter and allowed to air-dry. The samples were collected in Maluku, 80 km north of Kinshasa, DRC, in 2003. The parasitemia of the samples was estimated at the point of collection by the capillary tube centrifugation (CTC) technique,²⁰ 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 micropunch (Whatman), and the discs were then washed 3 times with 200 μL of FTA purification reagent (Whatman) and twice with 200 μL of 1 mM TE (10 mM Tris, 1 mM EDTA) buffer, pH 8.0, with incubations for 5 minutes at each wash as per the manufac-

turer's instructions. The washes were necessary to reduce the many potential PCR inhibitors present in blood.²¹ The washed discs were air-dried for an hour and were 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 μL (GenomiPhi DNA Amplification kit; Amersham Biosciences, Piscataway, NJ). 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 μL of the pooled reactions used as template for PCR. MDA products were routinely stored at -20°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 MgCl_2 , 6.7 mM 2-mercaptoethanol, 4.4 μM EDTA, 113 μg per mL BSA, 1 mM of each 4 deoxyribonucleotide triphosphates), 1 μM of each oligonucleotide primer, and 1 unit of *Taq* polymerase (ABgene, Epsom, UK) per 20- μL reaction or 2.5 U per 50- μL reaction. Two single-copy heterozygous microsatellite markers (PLC and JS2) were amplified in this study, using the PCR primers and conditions described previously,¹⁹ and new primer pairs were designed to amplify the outer fragment for the nested PCRs (sequences from 5' to 3'): JS2-C, AGTAATGGGAATGAGCGTCACCAG, and JS2-D, GATCTTCGCTTACA-CAAGCGGTACI; PLC-G2, TTAAGTGGACGACGACGAAATAACAACA, and PLC-H4, TTCAAACACCGTCCCCTCAATAAT. PCR amplification of 2 different multicopy genes was also carried out: one set directed against the ribosomal DNA internal transcribed spacer (ITS),^{16,17} and the other targeting the 177-base pair (bp) repeats found in the trypanosome genome,¹⁸ using the conditions described above. PCR amplification was carried out using 1 disc (PCR volume, 20 μL), and 5 discs per blood dilution (PCR volume, 50 μL ; for nested PCR, 50 μL in the first-round amplification and 20 μL in the second-round amplification). 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 from the first-round PCR was carried over.¹⁷ 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, East Rutherford, NJ), and were stained with 0.2 $\mu\text{g}/\text{mL}$ ethidium bromide to allow visualization under UV light. All reactions were carried out at least 5 times, to ensure reproducibility of results.

GeneScan analysis. When analyzing the field samples, 1 primer of the second-round nested PCRs targeting the single-copy heterozygous microsatellite (PLC) included a 5' FAM modification. This enabled accurate sizing and sensitive detection of alleles by the separation of products using a capillary-based sequencer (ABI 3100 Genetic Analyser; Applied Biosystems, Foster City, CA). DNA fragment size was determined relative to a set of ROX-labeled size standards (GS500 markers; Applied Biosystems) using GeneScan software, which allowed resolution to 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 2 ways: "absolute sensitivity," referring to the trypanosome concentration of the infected blood spotted onto the FTA filter, and "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 10-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 trypanosome per reaction, a 20-fold increase in sensitivity compared with 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 trypanosome 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 1×10^3 trypanosomes per mL, the increase in effective sensitivity for MDA represents the increase in number of reactions that can 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 20-fold over nested PCR and 100-fold over single-round PCR.

The second approach examined whether sensitivity could be increased by amplification of multicopy sequences, using primers specific to the ITS and 177-bp repeats; 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 trypanosome 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 may be 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 trypanosome per reaction, still representing a sensitivity 10-fold less than that observed for the 177-bp repeats. PCR amplification was 5-fold less sensitive with all sets of primers when carried out on 5 washed discs than was PCR amplification using 1 washed disc; it was also less sensitive than using MDA product from 1 or 5 discs. The decreased sensitivity with 5 discs is very likely due to the presence of increased amounts of PCR inhibitors in the reaction. These results indicate that use of WGA products in PCRs does not greatly increase sensitivity when multicopy 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 WGA to effectively increase the available material for genotyping was examined using blood samples from HAT patients from the DRC spotted onto FTA filters. An estimate of the parasitemia was undertaken at the point of collection by the capillary tube centrifugation (CTC) technique, which gave an approximate measure of trypanosome concentrations, ranging from 251 to 1,000 trypanosomes per mL (Table 3). Discs from 11 samples spotted on FTA cards were used as the templates 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 detec-

TABLE 1

Sensitivity analysis for a single-copy gene; numbers of trypanosomes per reaction, and trypanosome concentration in initial blood at which the microsatellite marker PLC was reproducibly detected by PCR amplification are shown

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

"1 disc" or "5 discs" indicates the number of washed FTA discs used as the initial template; "+," reproducible successful amplification and detection of both alleles; "+*" concentrations at which allele dropout was observed; "–," no product observed; ND, 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 μ L (1 disc) or 0.4 μ L (5 discs) or 2 μ L (pooled) of MDA product, as PCR template.

TABLE 2

Sensitivity analysis for multicopy target sequences; the 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 are shown*

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

* Absolute sensitivity (AS) equates to the number 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.

tion of low levels of product. Products were amplified from all samples with both sets of primers revealing 2 alleles at the PLC locus and 3 alleles at the JS2 locus (see 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 sample out of 11 was 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 1 sample gave a homozygous genotype (MAL122 for PLC). Thus, 4 of the 5 homozygous results detected using single MDA reactions were pseudo-homozygous, and only by pooling MDA reactions could they 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 to ensure accuracy of results with this technique, particularly in instances when detection of heterozygosity is an important feature.

DISCUSSION

This study examined the potential use of MDA, the most robust of the currently available WGA methods in terms of unbiased genome amplification,²² in optimizing the analysis of finite *T. brucei* DNA samples in the form of infected blood

spotted onto FTA filters. Analysis by PCR amplification of single- or multicopy genes confirmed that the multicopy 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*,²³ 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, at ≈ 20 –40 copies per cell,²⁴ 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,¹⁶ could 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 parasitemia. The ITS primers were designed 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. 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 amplification of single-copy genes (*SRA* for *T. b. rhodesiense*²⁵ and *TgsGP* for *T. b. gambiense*²⁶), and both targets have been used to identify human-infective trypanosomes from field samples on FTA filters and to analyze the epidemiology of the disease.²⁷ Secondly, single-copy target sequences—such as microsatellites, minisatellites, and SNPs—are the most widely used loci for population genetic analyses.²⁸ 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

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*

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

* Heterozygosity was analyzed both by visualisation of PCR product on ethidium bromide-stained agarose gel, and by separation of FAM-labeled products on an ABI3000 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 text. CTC count indicates the estimation of trypanosome numbers (trypanosomes per mL) by the CTC technique.

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 a kit for *in vitro* isolation (KIVI) or rodent inoculation methods were used.⁹ Indeed, it has been suggested that the narrow range of genotypes that will grow using the KIVI isolation method may be a significant reason⁹ for the conclusion that *T. b. gambiense* populations show low genetic variability and are clonal.^{29,30} Therefore, 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 for association studies between pathogen and host genotypes and disease. 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 20-fold, allowing multiple analyses to be undertaken from 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. Although this study has focused on trypanosomes, obviously the application of this technique to any pathogen samples will have considerable value.

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