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NOVEL PRIMER SEQUENCES FOR POLYMERASE CHAIN REACTION–BASED DETECTION OF *TRYPANOSOMA BRUCEI GAMBIENSE*

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Abstract. Progress in diagnosis, treatment, and epidemiology of human African trypanosomiasis (sleeping sickness) depends on the existence of specific and sensitive diagnostic tools. Inherent shortcomings of serologic and parasitologic diagnostic methods can be overcome by molecular techniques. Therefore, we have developed a new polymerase chain reaction (PCR) test using primers derived from the recently identified sequence of the *Trypanosoma brucei gambiense*–specific glycoprotein (TgsGP). The specificity of the TgsGP-PCR was evaluated on DNA extracted from 73 different trypanosome populations belonging to diverse taxonomic groups that were isolated from various host species, and from different geographic origins. The TgsG-PCR was shown to be specific for *T. b. gambiense* and was suitable for detection of trypanosome DNA in blood samples of patients with confirmed sleeping sickness.

INTRODUCTION

Trypanosoma brucei gambiense and *T. b. rhodesiense* are extracellular protozoan parasites causing human African trypanosomiasis (HAT or sleeping sickness). *Trypanosoma b. brucei* is not infectious to human but causes nagana in domestic ruminants. All three subspecies are morphologically indistinguishable and can be harbored by domestic animals as well as wildlife.[1–4] *Trypanosoma b. brucei* is sensitive to lysis by normal human serum (NHS) while *T. b. gambiense* and *T. b. rhodesiense* are resistant, although the latter subspecies can revert to a human serum-sensitive phenotype.[5,6] The parasites are transmitted by tsetse flies (*Glossina spp*.) in sub-Saharan Africa. Therefore, *T. b. gambiense* is present in western and central Africa, while *T. b. rhodesiense* is restricted to eastern Africa.[7]

Epidemics of *T. b. gambiense* are currently threatening approximately 50 million people, mainly in the Democratic Republic of the Congo, Angola, southern Sudan, and northern Uganda, with an estimated number of 300,000–500,000 infected patients.[8] Control of *T. b. gambiense* sleeping sickness relies heavily on active case detection and correct treatment. Screening of the population at risk is done by antibody detection with the card agglutination test for trypanosomiasis (CATT) and subsequent parasitologic detection performed on the seropositive individuals.[9,10] Although proven to be very useful, the CATT has its limitations. A variable percentage of the screened population that is seropositive in the CATT shows no clinical sign of infection and/or cannot be confirmed by parasite detection. As shown by Simarro and others[11] and Garcia and others,[12] the parasitologic detection techniques have limited sensitivity; thus, it is possible that at least some of these unconfirmed CATT-seropositive individuals are indeed infected. Furthermore, cured patients can remain CATT seropositive for up to three years due to persisting circulating antibodies, thus prohibiting the use of antibody tests for assessment of treatment success.[13]

For reasons of erroneous stage determination or treatment refractoriness, patients sometimes relapse after treatment. Studies on improved stage determination, therapy, drug resistance, and new drugs are being undertaken but are hampered by the low sensitivity of the parasitologic diagnostic methods.[14–17] In principle, DNA detection techniques such as the polymerase chain reaction (PCR) might partially overcome at least some of these diagnostic problems due to their alleged increased sensitivity and specificity. Indeed, PCR tests for detection of *T. brucei* DNA have been developed and used in several studies on sleeping sickness.[18–23] The primers used in these studies were derived from the repetitive nuclear DNA or the expression site–associated genes (ESAGs) ESAG 6 and 7.[24–27] Although, their specificity is restricted to the subgenus *Trypanozoon,* they are unable to distinguish between *T. brucei* subspecies.

However, for epidemiologic studies, discrimination of the three *T. brucei* subspecies within the host or the vector is essential.[4] Within this context, various molecular techniques have been applied, such as isoenzyme analysis, restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA analysis (RAPD), karyotype analysis, polymorphism analysis within the sequences of the variant surface glycoprotein (VSG), mini-satellites and microsatellites, kinetoplast DNA, and an internal transcribed spacer 1 of rDNA.[28–42] These techniques often require prior expansion of trypanosome populations in laboratory animals or in culture medium and involve multiple and time-consuming analytical steps. Taking into account the low isolation success rates observed for *T. b. gambiense,* and the fact that during primary isolation, and subsequent expansion, initially mixed-infection populations can be lost due to selection for the best growing one, the need for simplified techniques able to discriminate between the three *T. brucei* subspecies within the animal reservoir and the vector is obvious.[4]

The purpose of this study was to develop a simple PCR test capable of specific detection of the DNA of *T. b. gambiense.* The recently published sequence of the *T. b. gambiense*-specific glycoprotein (TgsGP) served as the base for the primer design.[43] When tested on 73 different trypanosome populations belonging to various taxonomic groups and originating from different hosts and geographic locations, the TgsGP-PCR was shown to be specific for *T. b. gambiense*. Furthermore, the TgsGP-PCR was also successfully applied in the detection of *T. b. gambiense* DNA in total DNA extracts from blood samples of patients with confirmed sleeping sickness.

MATERIALS AND METHODS

Parasite populations. The trypanosomes used in this study were derived from 73 different populations representing vari-

ous species and subspecies. The collection contained nine *T. b. brucei,* 15 *T. b. gambiense,* 12 *T. b. rhodesiense*, eight *T. equiperdum*, 10 *T. evansi*, 12 *T. congolense*, five *T. vivax,* and two *T. theileri* parasites. Initial characterization of *T. b. gambiense*, *T. b. rhodesiense,* and *T. b. brucei* populations was based on host specificity, geographic origin, clinical manifestation of the disease, and human serum resistance. They were kindly provided by Dr. D. Le Ray and N. van Meirvenne (Institute for Tropical Medicine [ITM], Antwerp, Belgium); Dr. D. Mehlitz (Institut für Schiffs und Tropenkrankheiten, Berlin, Germany); Dr. T. Baltz (University II, Bordeaux, France[44]); Dr. D. Verloo (ITM[45–47]); Dr. R. Brun (Swiss Tropical Institute, Basel, Switzerland[48]); Dr. M. Carrington (Cambridge University, Cambridge, United Kingdom); Dr. J. R. Stevens (University of Bristol, Bristol, United Kingdom); Dr. P.H. Clausen (University of Berlin, Berlin, Germany); Dr. J. Hagebock (National Veterinary Services Laboratories, U.S. Department of Agriculture Ames, IA[49]); and Dr. P. Barrowman (Onderstepoort Veterinary Institute, Onderstepoort, South Africa[50]). Within the collection of *T. b. gambiense,* four strains (Ousou, Ligo, Abba, and Kobir) have been reported to share molecular characteristics with *T. b. brucei* strains from Nigeria, differentiating them from the classic *T. b. gambiense* group 1.[2,4,35] The human serum resistance phenotype of those strains was analyzed using the serum incubation infectivity test (SIIT).[51]

Trypanosoma congolense, *T. vivax*, and *T. theileri* populations were identified based on differences in morphology. They were kindly provided by Dr. S. Geerts and Dr. D. Verloo (ITM); Dr. M. Desquesnes (Centre de Cooperation Internationale et Recherche Agronomique Pour le Developpment-Department Elevage et Medicine Veterinaire (CIRAD-EMVT), Centre International de Recmerche Developpment sur L'Elevage en Zone Subhumide (CIRDES), Burkina Faso); Dr. H. Tabel (University of Saskatchewan, Regina, Saskatchewan, Canada).

All trypanosomes were kept as cryostabilates in liquid nitrogen. The bloodstream form trypanosomes were first expanded in OF1 mice (IFFA CREDO, Charles River Laboratories, Brussels, Belgium) and subsequently in Wistar rats (Harlan, Horst, The Netherlands). The parasites were purified from a blood using a DAE52 column (Whatman, Maidstone, Kent, UK) according to the procedure of Lanham and Godfrey.[52] The eluted parasites were centrifuged at 1,200 × *g* for 30 min at 2°C. The sediment was washed three times with phosphate saline glucose buffer (PSG buffer, 38 mM Na_2HPO_4, 2 mM NaH_2PO4, 29 mM NaCl, 83 mM glucose, pH 8) by resuspension and centrifugation. The procyclic forms (four isolates of *T. b. gambiense:* Bage, Nabe, Pakwa, and Seka, one of *T. b. rhodesiense:* O4O4, one of *T. b. brucei:* Ketri, and one of *T. theileri:* Melsele; Table 1) were grown in a kit for *in vitro* isolation or in Cunningham culture medium.[53] They were separated from the culture media by centrifugation at 1,200 × *g* for 30 min at 2°C. The sediment was washed three times with phosphate glucose sacharose buffer (38 mM Na_2HPO_4, 2 mM $NaHPO_4$, 29 mM NaCl, 83 mM glucose, 100 mM sacharose, pH 8) by resuspension and centrifugation. All trypanosome sediments were stored at -70 C°.

Plasmodium falciparum parasites were obtained from the Institute for Tropical Medicine (Antwerp, Belgium). They were grown in BALB/c mice (Harlan). Infected red blood cells were obtained from blood collected by heart puncture into tubes containing heparin at a parasitemia of 30%.

Extraction of DNA from human blood, purified trypanosomes, and *P. falciparum.* A total of 92 human blood samples from individuals in Côte d'Ivoire were analyzed by a PCR. They were collected during a medical survey that was carried out in April-May 2000, where, for regular diagnostic purposes, 2 mL of venous blood was collected into tubes containing heparin. Among them were 41 samples that were found positive in the CATT, from which 14 samples were confirmed positive by a miniature anion exchange centrifugation technique (mAECT). The other 51 samples were collected during the same survey from the CATT/mAECT-negative persons. After serologic and parasitologic tests were performed, 180 μL of the remaining blood was mixed with 180 μL of AS1 storage buffer (Qiagen, Westburg, Leusden, The Netherlands). All blood samples were stored for one month in the dark at -20°C. In addition, 10 control blood samples were collected from healthy Belgian volunteers. Total DNA was extracted using the Qiamp blood DNA extraction protocol (Qiagen). The DNA was collected into 200 μL of AE elution buffer (Qiagen) and precipitated using 20 μL of 3 M sodium acetate (pH 5.2) and 400 μL of pre-chilled 100% ethanol. Samples were then centrifuged (for five minutes at 8,000 × *g*), and the pellet was rinsed once with pre-chilled 70% ethanol and centrifuged for three minutes at 8,000 × g. The concentrated DNA was resuspended in 20 μL of water. The typical DNA yield corresponded to 10 μg/ml as determined by spectrophotometric analysis.

To determine the analytical PCR detection limit, purified trypanosomes in PSG buffer were first counted in a counting chamber under a light microscope. A human blood sample from a volunteer was spiked with 100,000 purified trypanosomes/ml of blood. From this spiked blood, 10-fold serial dilutions to one trypanosome/ml were prepared. These dilutions were processed immediately with proteinase K solution (20 mg/ml) and AL lysis buffer according to the protocol of Qiagen. The DNA was extracted using a blood DNA extraction kit (Qiagen) and precipitated as described earlier.

The extraction of DNA purified trypanosomes was performed as follows: 20 μL of purified trypanosome sediment (2×10^7cells) was resuspended in 200 μL of PSG buffer, and the trypanosome DNA was extracted using a blood DNA mini kit (Qiagen). The DNA was eluted in 200 μL of the AE buffer (Qiagen). Its typical yield was 30 μg/ml as determined by spectrophotometric analysis.

For extraction of *P. falciparum* DNA, 180 μL of infected mouse blood was treated with 20 μL of proteinase K solution (20 mg/ml) and 200 μL of AL lysing buffer (Qiagen). The DNA was extracted using the blood DNA extraction kit, and was eluted in 200 μL of AE buffer at a concentration of 20 μg/ml.

TgsGP-PCR. The primers were derived from the sequence of the *T. b. gambiense*-specific glycoprotein (TgsGP; accession number AJ277951).[43] They were designed using the GenBank homology search program to target the region lacking significant similarity with already known DNA sequences. The following sequences were selected: sense primer: 5′ GCTGCTGTGTTCGGAGAGC-3′ and anti-sense primer: 5′-GCCATCGTGCTTGCCGCTC-3′. The presence of amplifiable DNA in the extracted human samples was checked by PCR with human β-actin-specific primers. The β-actin

TABLE 1

Origin of the trypanosome populations used in this study and corresponding results in the *Trypanosoma brucei gambiense*—specific glycoprotein—polymerase chain reaction (PCR)*

Species/Subspecies	Trypanosome population/Identification code/References†	Parasite form	Origin	Original host	PCR
T. brucei	EATRO/1125,AnTAR:AnTat 1.8 121296A[A,38]	Bloodstream	Uganda	Bushbuck	Neg
T. brucei	NITR40/12,AnTAR2: AnTat 2.2 ITMAS100297B[A,43]	Bloodstream	Nigeria	Tsetsefly	Neg
T. brucei	GUTAR22, AnTAR5: AnTat 5.2 ITMAS220197 A[43]	Bloodstream	Gambia	Bovine	Neg
T. brucei	ITMAV 051078, AnTat 17.1 ITMAS210596[A]	Bloodstream	D.R. of the Congo	Sheep	Neg
T. brucei	Ketri 2494 ITMAS270881 28	Procyclic	Kenya	Tsetsefly	Neg
T. brucei	STIB 348 ITMAS250500B[34,B]	Bloodstream	Tanzania	Hartebeest	Neg
T. brucei	MCRO/ZM/73/J10 ITMAS250500A[28,38]	Bloodstream	Zambia	Hyena	Neg
T. brucei	TSW 196 ITMAS300500A[38]	Bloodstream	Côte d'Ivoire	Pig	Neg
T. brucei	MiTAR1, MiTat 1.1[C]	Bloodstream	Uganda	NA	Neg
T. gambiense	AYL, AnTAR9: AnTat 9.1 ITMAD010399A[43]	Bloodstream	Cameroon	Human	Pos
T. gambiense	LiTAR1,LiTat 1.3; Paris/52/-/-/(ELIANE) ITMAS100500 A,[43]	Bloodstream	Côte d'Ivoire	Human	Pos
T. gambiense	KEMLO,; AnTAR 13; Bwamanda/74/ITMAS280584[38]	Bloodstream	D.R. of the Congo	Human	Pos
T. gambiense	MBA/KINKOLE/74, AnTAR11: AnTat 11.17 ITMAS120484[38]	Bloodstream	D.R. of the Congo	Human	Pos
T. gambiense	PA, AnTAR22: AnTat 22.1 ITMAS110280[43]	Bloodstream	D.R. of the Congo	Human	Pos
T. gambiense	JUA:FONTEM/-/ITMAS 010799[38]	Bloodstream	Cameroon	Human	Pos
T. gambiense	LiTAR1, LiTat 1.6; Paris/52/-/-/(ELIANE) ITMAS121296[A,43]	Bloodstream	Côte d'Ivoire	Human	Pos
T. gambiense	BAGE ITMAP2569[A]	Procyclic	D.R. of the Congo	Human	Pos
T. gambiense	NABE ITMAP2569[A]	Procyclic	D.R. of the Congo	Human	Pos
T. gambiense	PAKWE ITMAP2570[A]	Procyclic	D.R. of the Congo	Human	Pos
T. gambiense	SEKA ITMAP2568[A]	Procyclic	D.R. of the Congo	Human	Pos
T. gambiense	MHOM/CI/82/DAL503/KOBIR ITMAS260600[35]	Bloodstream	Côte d'Ivoire	Human	Pos
T. gambiense	MHOM/CI/82/DAL494/OUSOU ITMAS220600[35]	Bloodstream	Côte d'Ivoire	Human	Pos
T. gambiense	MHOM/CI/83/DAL626/.ABBA ITMAS190600A[35]	Bloodstream	Côte d'Ivoire	Human	Neg
T. gambiense	MHOM/CI/84/DAL655/LIGO/ITMAS190600B[35]	Bloodstream	Côte d'Ivoire	Human	Neg
T. rhodesiense	AnTAR12, AnTat 12.1 ITMAS140476A[A,42]	Bloodstream	Rwanda	Human	Neg
T. rhodesiense	AnTAR25, AnTat 25.1 ITMAS300381A[A,42]	Bloodstream	Rwanda	Human	Neg
T. rhodesiense	ETat 1.2 (TREU164)[A,42]	Bloodstream	Uganda	Tsetsefly	Neg
T. rhodesiense	0404 ITMAS[a]	Procyclic	Uganda	Tsetsefly	Neg
T. rhodesiense	LIRI/UTRO/STIB 847 ITMAS050399A[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 848 ITMAS190399[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 849 ITMAS050399B[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 850 ITMAS050399C[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 851 ITMAS080399C[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 851 ITMAS080399C[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 882 ITMAS080399A[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 883 ITMAS080399B[B]	Bloodstream	Uganda	Human	Neg
T. rhodesiense	LIRI/UTRO/STIB 884 ITMAS150399A[B]	Bloodstream	Uganda	Human	Neg
T. evansi	CAN 86K ITMAS 140799B[A,45]	Bloodstream	Brazil	Dog	Neg
T. evansi	RoTat 1.2 ITMAS020289[A,45]	Bloodstream	Indonesia	Buffalo	Neg
T. evansi	AnTat 3.1 ITMAS070799[A,45]	Bloodstream	South America	Capybara	Neg
T. evansi	MERZOUGA56 ITMAS120399D[A]	Bloodstream	Marocco	Camel	Neg
T. evansi	STIB 816 ITMAS140799C[A,45]	Bloodstream	China	Camel	Neg
T. evansi	Columbia ITMAS150799[A,45]	Bloodstream	Colombia	Horse	Neg
T. evansi	ZAGORA 1.17 ITMAS150799[A,45]	Bloodstream	Marocco	Camel	Neg
T. evansi	Vietnam WH ITMAS101298[A,47]	Bloodstream	Vietnam	Buffalo	Neg
T. evansi	Philipines ITMAS060297[A,45]	Bloodstream	Phillipines	Buffalo	Neg
T. evansi	Ketri 2480 ITMASB110297[34]	Bloodstream	Kenya	Camel	Neg
T. equiperdum	AnTat 4.1 ITMAS210983A[A]	Bloodstream	NA	NA	Neg
T. equiperdum	BoTAR:BoTat 1.1 ITMAS240922A[34,44]	Bloodstream	France	Horse	Neg
T. equiperdum	OVI ITMAS 241199C[A,50]	Bloodstream	South Africa	Horse	Neg
T. equiperdum	Hamburg ITMAS 251199C[A,C]	Bloodstream	NA	NA	Neg
T. equiperdum	Alfort ITMAS241199A[A,C]	Bloodstream	NA	NA	Neg
T. equiperdum	SVP ITMAS241199B[A,C]	Bloodstream	NA	NA	Neg
T. equiperdum	STIB 818 ITMAS01099[A,48]	Bloodstream	China	Horse	Neg
T. equiperdum	Am.Strain ITMS220101	Bloodstream	NA	NA	Neg
T. congolense	TRT 17 ITMAS020699[A]	Bloodstream	Zambia	Bovine	Neg
T. congolense	TRT 57 ITMAS070199[A]	Bloodstream	Zambia	Bovine	Neg
T. congolense	Kilifi K60/1A/Kenya[D]	Bloodstream	Kenya	Bovine	Neg
T. congolense	Kilifi K45/1A/Kenya	Bloodstream	Kenya	Bovine	Neg
T. congolense	Savannah ILRAD3000[D]	Bloodstream	Kenha	Bovine	Neg
T. congolense	Forè Dinder80/CRTA/3[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. congolense	Forè Komoè87/CRTA/153[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. congolense	Savannah ILRAD1180[D]	Bloodstream	Zambia	Bovine	Neg
T. congolense	TC13[E]	Bloodstream	NA	NA	Neg
T. congolense	STIB68[A,B]	Bloodstream	NA	NA	Neg
T. congolense	J4/23[A]	Bloodstream	NA	NA	Neg
T. congolense	TRT55[A]	Bloodstream	NA	NA	Neg
T. vivax	ILRAD 700 ITMAS190199[A]	Bloodstream	Nigeria	Zebu	Neg
T. vivax	Banan83/CRTA/73[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. vivax	Noronin80/CRTA/16[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. vivax	Nyarafo96/CIRDES/1[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. vivax	Sarfaloa83/CRTA/54[D]	Bloodstream	Burkina Faso	Bovine	Neg
T. theileri	Melsele ITMAS020299[A,46]	Procyclic	Belgium	Bovine	Neg
T. theileri	T.th.BF[D]	Bloodstream	Burkina Faso	Bovine	Neg

* Neg = negative; D.R. = Democratic Republic; NA = (information) not available; Pos = positive.

† Superscript A = Institute for Topical Medicine, Antwerp, Belgium; superscript B = Swiss Tropical Institute, Basel; superscript C = Dr. Mark Carrington (Cambridge, United Kingdom) and Dr. P. H. Clausen; superscript D = M. Desquesnes (CIRAD-EMVT, CIRDES, Burkina Faso); superscript E = Dr. H. Table (University of Saskatchewan, Regina, Saskatchewan, Canada). Superscript numbers indicate references.

PCR was performed according to the protocol described by Kinoshita and others.[54]

All PCR amplifications were performed using 10–100 ng of the DNA extracted from purified parasites, or 10 μL of the DNA extracted from human blood samples. The DNA template was amplified in 50 μL of PCR mixture containing 1× PCR buffer (20 mM Tris-HCl pH 8.7, 100 mM KCl, 50 mM $(NH_4)_2SO_4$, Q solution), 1.5 mM $MgCl_2$, 200 μM each of the four dNTPs, 0.5 μM of each primer, and 2.5 units of HotStar *Taq* DNA polymerase (Qiagen). All PCRs were performed using a T3 Thermocycler (Biometra, Westburg, Leusden, The Netherlands). The TgsGP-PCR conditions were as follows: sample incubation for 15 minutes at 95°C followed by 45 cycles of one minute at 94°C, one minute at 63°C, and one minute at 72°C, and a final extension for 10 minutes at 72°C. A 20-μL sample of each PCR product was analyzed by electrophoresis in a 2% agarose gel. The gels were stained with ethidium bromide (1 μg/ml) (Sigma, St. Louis, MO) and analyzed on an Imagemaster Video Detection System (Pharmacia, Bucks, United Kingdom). To increase the analytical PCR detection limit, a second TgsGP-PCR was performed with one microliter of the first PCR product. As negative PCR controls, human DNA sample extracted from blood samples of healthy volunteers and a PCR mixture without the DNA template were run along with the other samples. Samples were collected as a part of routine diagnosis of patients, as prescribed by the World Health Organization (WHO). The study was approved by the ministry of health from Côte d'Ivoire and oral consent was systematically collected from all patients.

RESULTS

Specificity and detection limit of the TgsGP-PCR. Using primers that recognize the *T. gambiense* TgsGP gene, we developed a PCR protocol in which a 308-base pair (bp) PCR product was expected for specific amplification. Results obtained with this PCR are shown in Table 1. Seventy-three different trypanosome populations have been analyzed by the TgsGP-PCR. A specific PCR product was obtained with 13 of 15 *T. b. gambiense* populations. The other 58 non-*T. b. gambiense* populations tested negative, thus confirming the specificity of the TgsGP-PCR for *T. b. gambiense* within the collection of tested *Trypanosoma sp*. (Table 1). Unexpectedly, the *T. b. gambiense* Abba and Ligo strains were TgsGP-PCR negative. When subjected to the SIIT, all 13 TgsGP-positive populations were found to be fully resistant to NHS, while the Abba and Ligo strains were initially found to be sensitive, although they reverted to a resistant phenotype in mice after repeated injection with NHS.

The detection limit of the TgsGP-PCR was evaluated on the series of human blood samples spiked with purified trypanosomes. After a single PCR, the detection limit reached 1,000 trypanosomes/ml of blood. This detection limit increased to 10 trypanosomes/ml of blood when the TgsGP-PCR was repeated using an aliquot of the first PCR product.

Detection of *T. b. gambiense* DNA in total extracts from blood samples of patients with confirmed sleeping sickness by the TgsGP-PCR. Figure 1 shows representative results of the TgsGP-PCR obtained with total DNA extracts from human blood samples. As expected, only one PCR product of 308 bp was amplified in the DNA extracts of parasitologically confirmed patients with sleeping sickness, and in a *T. b. gambiense*-positive control (Figure 1, lanes 1–4 and 9) while DNA extracts from the CATT/mAECT-negative African individuals and healthy Belgian volunteers remained TgsGP-PCR negative (Figure 1, lanes 5–8). Since malaria is very common in regions where sleeping sickness is present, the TgsGP-PCR should not amplify *Plasmodium* DNA. No amplification product was obtained with DNA extracted from *P. falciparum* (Figure 1, lane 10).

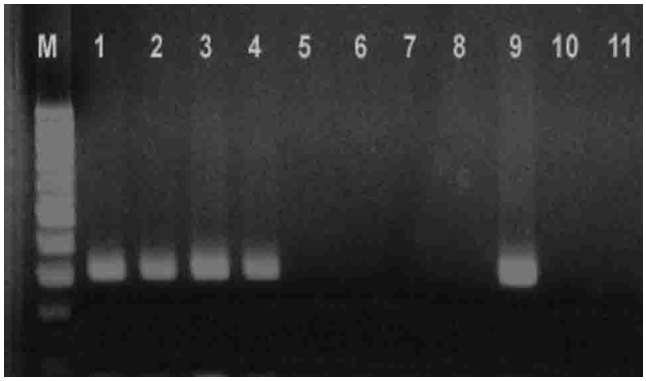

FIGURE 1. Agarose gel electrophoresis showing the representative results obtained with the *Trypanosoma brucei gambiense*–specific glycoprotein–polymerase chain reaction (TgsGP-PCR) on human blood samples from Côte d'Ivoire. The 308-base pair PCR product corresponds to the fragment amplified from the TgsGP gene. Lanes 1–4, samples from patients with confirmed sleeping sickness due to *T. b. gambiense;* lanes 5–8, samples from the CATT/mAECT-negative African individuals and a healthy volunteer; lane 9, positive control (*T. b. gambiense* DNA); lane 10, *Plasmodium falciparum* DNA; lane 11: control PCR without DNA template; lane M, molecular weight marker (1-kb DNA ladder).

The diagnostic potential of the TgsGP-PCR was evaluated on a collection of human blood samples from Côte d'Ivoire and Belgium. All samples were subjected to an initial PCR, followed by a second PCR on an aliquot of the first reaction product. The results are shown in Table 2. All 51 CATT/mAECT-negative samples from Côte d'Ivoire were TgsGP-PCR negative. Of 41 CATT-positive samples from Côte d'Ivoire, 14 were TgsGP-PCR positive, which corresponded exactly to the 14 samples that were parasitologically confirmed by the mAECT. The remaining 27 CATT-positive and mAECT-negative samples from Côte d'Ivoire were negative by the TgsGP-PCR, as were 10 samples from healthy Belgian volunteers.

TABLE 2

Results obtained with the card agglutination test for trypanosomiasis (CATT), the miniature anion exchange centrifugation technique (mAECT), and the *Trypanosoma brucei gambiense*—specific glycoprotein—polymerase chain reaction (TgsGP-PCR) with total RNA extracted from 92 human blood samples from Côte d'Ivoire

Test		CATT	
		Positive	Negative
mAECT	Positive	14	0
	Negative	27	51
TgsGP-PCR	Positive	14	0
	Negative	27	51

DISCUSSION

The purpose of this study was to develop a new PCR test for specific detection of *T. b. gambiense* DNA. The PCR primers used were derived from the sequence of the recently identified TgsGP gene, which encodes a *T. b. gambiense*-specific flagellar pocket glycoprotein.[43] Since this gene shares certain similarities with the 5′-terminal half of the *T. b. brucei* IlTat 1.23 VSG gene, the primer sequences were selected from regions of low similarity. To avoid primer-dimer formation, both 3′ ends did not contain any complementary base pairs and were devoid of any long stretches of guanidine and cytosine repeats. A hot start step was included to avoid generation of non-specific PCR products. The TgsGP-PCR specificity was evaluated with 73 different trypanosome populations. An expected 308-bp specific PCR product was generated solely when DNA from *T. b. gambiense* was tested. Interestingly, two alleged *T. b. gambiense* strains, i.e., Abba and Ligo, that were isolated from patients with sleeping sickness in Côte d'Ivoire remained negative in the TgsGP-PCR. These strains were previously identified as being distinct from the conventional *T. b. gambiense* group 1, sharing molecular characteristics with Nigerian strains of *T. b. brucei*.[4,35,38,55] This initial characterization was based on cluster analysis of the RFLP pattern derived from the ribosomal non-transcribed spacer region.[35] Since the TgsGP-PCR targets a gene that is associated with NHS resistance, we subjected the Abba and Ligo strains to the SIIT.[43,51] Initially, both strains were sensitive to lysis by NHS. When grown in mice under NHS pressure, they converted to an NHS-resistant phenotype, confirming earlier reports that some alleged *T. b. gambiense* strains from humans in Côte d'Ivoire can exert different inducible levels of NHS resistance.[5,6,56,57] Together, these results indicate that the TgsGP-PCR specifically identifies conventional *T. b. gambiense* strains that are persistently resistant to NHS.

Previous reports have described other molecular techniques for the specific detection of *T. b. gambiense*. Mathieu-Daudé and others[40] and Schares and others[41] reported on methods involving a first PCR step or nested PCR, followed by hybridization with a *T. b. gambiense*-specific probe that was derived from the sequence of the kinetoplast minicircle DNA variable regions. A risk associated with targeting kinetoplast minicircle DNA for diagnostic purposes is connected with the high rate of genetic evolution within this sequences, as it is for VSG genes. Indeed, other groups have developed PCR tests based on *T. b. gambiense* VSG sequences (AnTat 11.17 and LiTat 1.3), but observed that some *T. b. gambiense* strains from northwestern Uganda and Cameroon remained negative in these PCRs due to the absence of the corresponding VSG genes within their genome.[40,58–60] Although the TgsGP gene is located on a telomere, it does not belong to a VSG expression site (ES), and as such is not subjected to ES-associated antigenic variation.[43,60] Therefore, the TgsGP-PCR may prove to be a more reliable tool in the detection of *T. b. gambiense*, e.g., for studying its animal reservoir and its transmission dynamics.

Although the geographic distribution of *T. b. gambiense* and *T. b. rhodesiense* is classically confined to west and central Africa, and eastern Africa, respectively, recent reports indicate the presence of *T. b. rhodesiense* outside the traditional foci in southeastern Uganda.[7,61] Isolates from the Masindi district in midwestern Uganda were shown to be *T. b. gambiense* as well as *T. b. rhodesiense*.[61] Since both trypanosomes show differential drug sensitivity, a correct diagnosis between *T. b. gambiense* and *T. b. rhodesiense* is essential for successful drug treatment and may be facilitated by a simple molecular technique, such as the TgsGP-PCR.

One advantage of the TgsGP-PCR is its simplicity compared with other techniques used for differentiation of the three *T. brucei* subspecies, such as isoenzyme analysis, karyotype analysis, RFLP, RAPD, which usually require high numbers of parasites or involve a comparative analysis of genomic patterns.[28–42] By running two subsequent TgsGP-PCRs on the same sample of spiked blood, a detection limit of 10 trypanosomes/ml of blood could be obtained. This is comparable with that reported with a two-run ESAG6/7 PCR or a repetitive nuclear DNA-based PCR used with blood samples from patients with sleeping sickness.[18,21]

Whether the detection limit of the TgsGP-PCR will be low enough to identify all patients with sleeping sickness caused by *T. b. gambiense* in a specific population remains to be investigated. Indeed, in the present study, only the CATT-positive cases that were confirmed by the mAECT (34% of the samples) were positive in the TgsGP-PCR after two amplification reactions, while all the parasitologically unconfirmed CATT-positive samples remained negative. Similar results were obtained by Kyambadde and others, who found that 60% of the CATT-positive samples were negative when tested by both the hematocrit centrifugation technique and the repetitive nuclear DNA-based PCR, while all the parasitologically confirmed CATT-positive samples were also positive in their PCR.[23] Since there is evidence that some of the unconfirmed CATT-positive individuals are indeed infected, lowering the detection limit of the PCR methods, e.g., by analyzing a larger volume of blood, may be necessary.[12]

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