IDENTIFICATION OF *TRYPANOSOMA BRUCEI GAMBIENSE* GROUP I BY A SPECIFIC KINETOPLAST DNA PROBE

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Abstract. A set of 26 *Trypanosoma brucei* stocks from various African countries, previously characterized by multilocus enzyme electrophoresis (MLEE) for 18 polymorphic loci, have been selected to be representative of the three *T. brucei* classic subspecies. The kinetoplast DNA minicircle variable regions from these stocks have been amplified using the polymerase chain reaction (PCR) technique, and hybridized with the amplified variable regions of three *T. brucei* reference stocks, previously identified as *T. brucei brucei, T. brucei gambiense,* and *T. brucei rhodesiense,* respectively. Both *T. b. brucei* and *T. b. rhodesiense* probes hybridized only with their own stocks, but the *T. b. gambiense* probe specifically hybridized with a group of 12 stocks that represented most of the human stocks from West and Central Africa in our sample. These stocks, which appeared as a clearly separable cluster based on previous MLEE analysis, probably correspond to *T. brucei gambiense* group I. No other stock hybridized with this amplified fragment. Since the *T. b. gambiense* probe obtained is specific for many isolates that are pathogenic for humans in Central and West Africa, it appears to be a promising tool for epidemiologic and medical surveys.

The kinetoplast DNA of *Trypanosoma brucei s.l.,* the agent of human sleeping sickness and of a cattle disease in Africa, is composed of a network of maxicircles and minicircles. The one-kilobase minicircles (approximately 10,000 copies in a single cell), contain a 122-basepair (bp) sequence that appears to be conserved within the whole taxon *T. brucei.*[1] These minicircles also contain a nonconserved sequence (hypervariable region or HVR), which seems to be very heterogeneous within a single organism. Indeed, 200–300 different sequence classes of variable frequencies can be found within the same cell.[2] The HVR of *T. cruzi* was successfully used as a target sequence to design probes[3, 4] that are specific for the natural clones of this parasite previously demonstrated by a population genetics approach.[5] The purpose of the present study was to use a similar approach to design specific markers for the *T. brucei s.l.* genotypes and clusters of genotypes characterized by a previous population genetics study.[6]

MATERIALS AND METHODS

Parasite sampling

Twenty-six *T. brucei* stocks isolated from various hosts (humans, wild and domestic mam-

mals, tsetse flies) in Central, West, and East Africa were analyzed. The origin of these stocks is summarized in Table 1. They were selected to represent the three classic subspecies of *T. brucei,* namely *T. b. brucei, T. b. gambiense,* and *T. b. rhodesiense.* This subspecific identification was performed by other investigators (see references in Table 1).

Parasite culturing

Trypanosomes were grown by culturing procyclic forms in Cunningham's medium containing 20% fetal calf serum and 20 μg/ml of gentamicin.[7]

Polymerase chain reaction

The primers, obtained from Centre de Recherche en Biologie et Genetique Cellulaire (Toulouse, France), were selected to anneal DNA sequence at both ends of the 122-bp conserved region of the kinetoplast DNA (kDNA) minicircle described by Chen and Donelson.[1] A restriction site (*Msp* I) was artificially introduced at the 3′-end of each oligonucleotide to facilitate the subsequent isolation of the amplified variable sequence. The sequences of the primers were as

TABLE 1

Trypanosoma brucei *stocks used in the study*

Stock (World Health organization identification)	Host	Country	Year	References[*]	Subspecies identification and references[†]
MKOK/BF/80/KK 39	Waterbuck	Burkina Faso	1980	20, 21	
MHOM/CM/74/MOS	Human	Cameroon	1974	21–25	Tbg; 21–24
MHOM/CM/75/BIM	Human	Cameroon	1975	22–25	Tbg; 22–24
MHOM/CM/88/A 004	Human	Cameroon	1988	21	Tbg; 21
MHOM/CM/88/B 014	Human	Cameroon	1988	21	Tbg; 21
MHOM/CM/88/M 001	Human	Cameroon	1988	21	Tbg; 21
MHOM/CG/80/PEYA	Human	Congo	1980	21, 25	Tbg; 21
MHOM/ET/67/GAMBELA I	Human	Ethiopia	1967	26	
MHOM/CI/52/ELIANE	Human	Cote d'Ivoire	1952	22–24	Tbg; 22–24
MHOM/CI/78/DAL 069	Human	Cote d'Ivoire	1978	20–22, 27	Tbg; 21, 22, 27
MHOM/CI/78/TH 112	Human	Cote d'Ivoire	1978	21	
MHOM/CI/78/TH 149	Human	Cote d'Ivoire	1978	20, 21, 27	Tbg; 21, 27
MHOM/CI/78/TH 2	Human	Cote d'Ivoire	1978	20, 27	
MHOM/CI/86/DAL 967	Human	Cote d'Ivoire	1986	21	Tbg; 21
MHOM/CI/??/NANA	Human	Cote d'Ivoire	19??	21, 28	Tbg; 21, 28
GPAL/KE/69/EATRO 1535	Fly	Kenya	1969		
GPAL/KE/69/S 29	Fly	Kenya	1969		
GMOS/NG/70/NITR 40.12	Fly	Nigeria	1970	22–24	Non-g; 22, 23 Tbb; 24
MBOT/NG/77/IBADAN 22 cl.A	Cattle	Nigeria	1977	21, 26	Tbb; 26
MHOM/RW/70/0404	Human	Rwanda	1970		
MHOM/SD/82/BIYAMINA cl.B	Human	Sudan	1982	28	Tbg; 28
MTRG/UG/66/EATRO 1125	Bushbuck	Uganda	1966	22, 24, 25	Non-g; 22 Tbb; 24
MHOM/ZR/71/C 126	Human	Zaire	1971	21, 25, 26	Tbg; 21, 26
MBOI/ZM/82/TRPZ 166	Zebu	Zambia	1982		
MHOM/ZM/74/058 cl.A3	Human	Zambia	1974	26	Tbr; 26
MHOM/ZM/82/TRPZ 227	Human	Zambia	1982	21	

[*] References of previous studies on the stock.

[†] Tbg = *Trypanosoma brucei gambiense*; non-g = *non-gambiense*; Tbb = *T. brucei brucei*; Tbr = *T. brucei rhodesiense*.

follows: 5'-CGCCCATAAGATTTCCGGTT-3' and 5'-GGTGTAATACTCACCCGGTT-3'. Buffer and incubation conditions were performed according to Breniere and others.[4] Amplification was performed using 1 IU of *Thermus aquaticus* DNA polymerase (Genofit, Geneva, Switzerland) and a PHC-2 apparatus (Techne, Cambridge, UK). Parasite cells (10^4) were harvested by centrifugation, boiled in 100 μl of distilled water for 5 min, and centrifuged in an Eppendorf (Madison, WI) microfuge at 5,000 × g for approximately 30 sec. Ten microliters of the supernatant obtained by this procedure was used as template.

Probe purification

The PCR-amplified fragments were purified from a 1% preparative gel (low-melting point ultrapure agarose in Tris-acetate-EDTA [TAE] buffer) using the enzymatic preparation GE-Lase® (Epicentre Technologies Corporation, Madison, WI) according to the manufacturer's instructions. The extracts were then digested with *Msp* I restriction endonuclease to eliminate part of the oligonucleotide primers selected in the conserved region of the minicircle. The DNA was precipitated with ethanol and resuspended in 100 μl of distilled water. The DNA concentration was assessed with a DU 70 spectrophotometer (Beckman, Palo Alto, CA).

Southern blotting

The PCR products were electrophoresed in a 0.8% grade agarose gel (Bethesda Research Laboratories, Uxbridge, UK) in TAE buffer for 40 min at 100 volts. The gels were stained with ethidium bromide and were then transferred onto charged nylon membranes (Hybond-N[+]; Amersham, Buckinghamshire, UK) after alkaline denaturation (15 min twice in 0.5 M NaOH, 1.5 M NaCl) by the pocket-blotting procedure.[8] Subsequently, the membranes were washed in 2× SSPE (1 × SSPE = 0.15 M NaCl, 0.01 M sodium diphosphate, 0.001 M EDTA), dried, and stored in a protective film until the hybridization stage.

Labeling and hybridization conditions

The ECL® (Amersham) gene detection system based on enhanced chemiluminescence was used according to the manufacturer's instructions. Si-

multaneously, the membranes were incubated at 42°C in hybridization buffer supplied by the manufacturer (0.25 ml/cm^2 of blot) for 15 min, and the purified probe (20 ng/ml of hybridization buffer) was labeled for 10 min at 37°C. The hybridization was performed at 42°C overnight in a rotary oven (Appligene, Illkirch, France). Before the detection step, the membranes were washed twice in a highly stringent solution (6 M urea, 0.1% sodium dodecyl sulfate, 0.1 × SSC [SSC = 0.15 M NaCl, 0.015 M sodium citrate]) at 42°C, and twice in a 2× SSC at room temperature. Serial exposures were performed (1, 5, and 20 min) on autoradiography films (Hyperfilm®; Amersham).

RESULTS

All 26 *T. brucei* stocks showed positive amplification. The amplified band, when analyzed by electrophoresis, had a length of approximately 930 bp, which corresponds to the expected molecular weight of the minicircle HVR. Figure 1A shows the amplification results for some of these stocks. As a control, amplification was tested on stocks belonging to the following other species: *T. evansi*, *T. cruzi*, and *Leishmania mexicana* (one stock each). *Trypanosoma cruzi* and *L. mexicana* both showed no amplification, while *T. evansi* exhibited a slight signal at the same molecular weight as the *T. brucei* band. These results suggest that the sequence homology observed by Chen and Donelson actually involves a conserved sequence that is specific for the whole taxon *T. brucei*.[1] This sequence would be different from the minicircle conserved regions characterized in *T. cruzi* and *Leishmania*,[9, 10] while it would be very similar to the *T. evansi* sequence, as indicated by Bajyana Songa and others.[11]

Three *T. brucei* PCR products were selected to be representative of the three classic subspecies and were used as probes. The stocks from which these amplified products were obtained are DAL 967 (*T. b. gambiense*), NITR 40.12 (*T. b. brucei*), and 058 cl.A3 (*T. b. rhodesiense*) (Table 1). After purification, the amplified variable regions of these stocks were hybridized with the membrane-blotted PCR products of the whole set of 26 stocks. Two radically different kinds of hybridization patterns were observed (Table 2). The two probes obtained from the NITR 40.12 and 058 cl.A3 stocks hybridized only to their own stock. The DAL 967 probe showed a pos-

A

955 —   — 910

1 2 3 4 5 6 7 8 9 10 11 12 13 14
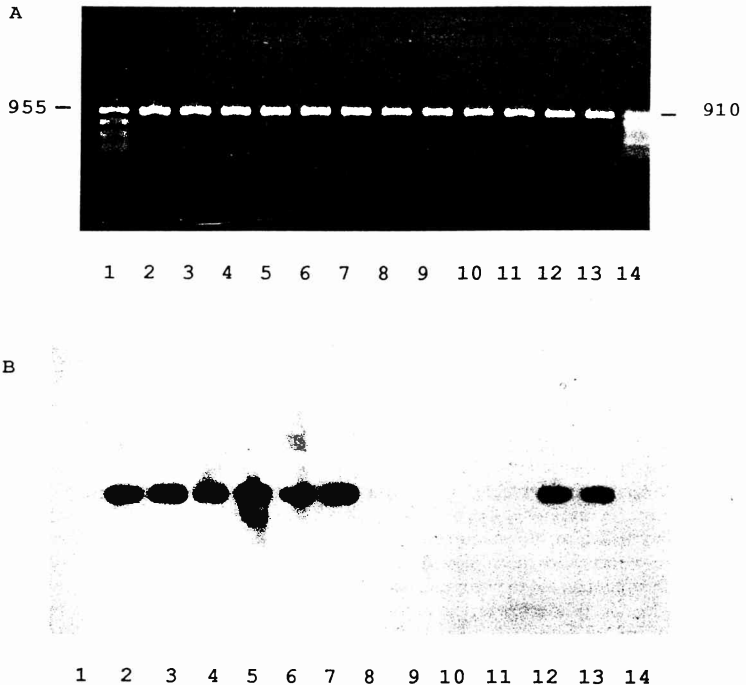
B

1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIGURE 1.   A, polymerase chain reaction (PCR) amplification of the kinetoplast DNA minicircle variable region of *Trypanosoma brucei*. The PCR products were analyzed by electrophoresis on a 0.8% agarose gel run in Tris-acetate-EDTA buffer and stained with ethidium bromide. The amplified fragment had a length of approximately 930 basepairs. Lanes 1 and 14, DNA size markers (*Sau* 3A I digest of pUC18 and *Alu* I digest of pBR322, respectively); lanes 2–13, PCR products of *T. brucei* stocks DAL 069, DAL 967, ELIANE, TH 149, PEYA, MOS, TH 112, BIYAMINA, EATRO 1125, 058 cl.A3, A 004, and M 001, respectively. Molecular weight standards (in basepairs) are shown on both sides of the gel. B, Southern blot of the agarose gel in A, showing hybridization with the DAL 967 kinetoplast DNA probe and chemiluminescent detection. No bands were detectable in lanes 8–11, while strong signals were exhibited in lanes 2–7 and 12 and 13.

itive hybridization, not only with its own stock, but also with 11 other stocks. Figure 1B shows the hybridization of the DAL 967 probe with seven of these stocks.

When previous MLEE phylogenetic results based on 18 polymorphic loci are considered, it is interesting to note that those 12 stocks that hybridize with the DAL 967 probe can be assigned to a clearly separate cluster.[6] Moreover, it is worth emphazing that this cluster involves most of the human stocks from Central and West Africa studied here. Figure 2 shows a dendrogram constructed using the unweighted pair group method with arithmetic mean (UPGMA)[12] from the Jaccard distance matrix obtained from the

MLEE data among the 26 stocks studied, which corresponds to 22 zymodemes. The group of zymodemes indicated by the brace in Figure 2 corresponds to this separate cluster of human stocks. This specific clustering pattern was confirmed for 78 *T. brucei s.l.* stocks,[6] not only by UPGMA dendrograms as shown here, but also by both Wagner networking and principal component analysis.[13, 14]

DISCUSSION

It is quite probable that this cluster of human stocks corresponds to *T. brucei gambiense* group I.[15] Since this group of trypanosomes involves
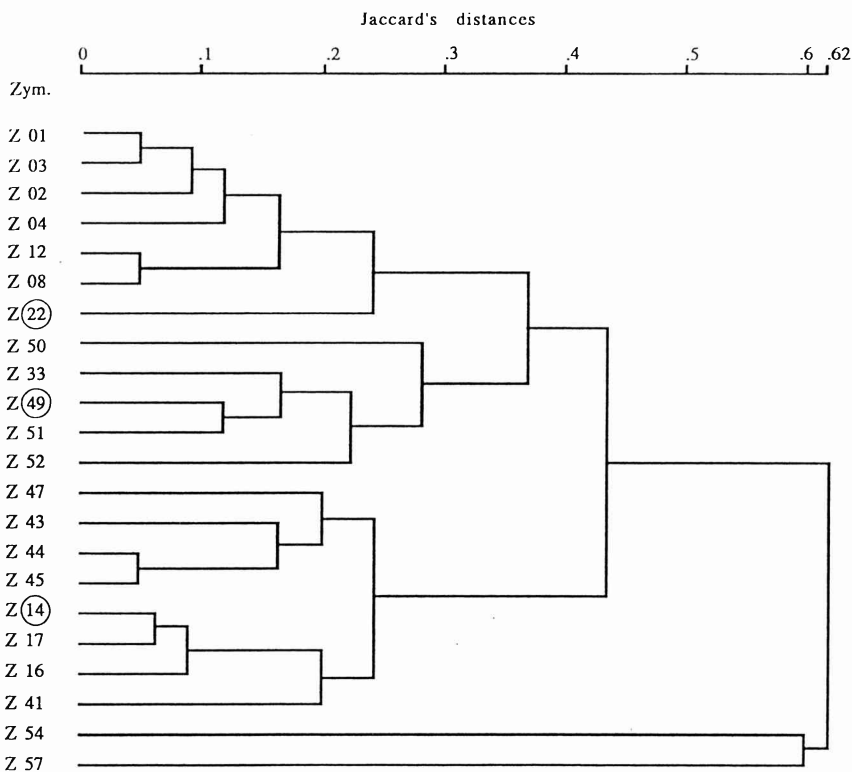
Jaccard's distances

FIGURE 2.   Dendrogram constructed using the unweighted pair group method with arithmetic mean from a Jaccard distance matrix, based on a multilocus enzyme electrophoresis study on 18 polymorphic loci.[6] Zymodemes (Zym.) Z22, Z49, and Z14 **(circled)** indicate the three stocks used as probes (NITR 40.12, 058 cl.A3, and DAL 967, respectively). The brace delimits the zymodemes of the 12 stocks that hybridize with the DAL 967 probe. This cluster corresponds to *Trypanosoma brucei gambiense* group I.[15]

most of the human isolates from Central and West Africa, its medical and epidemiologic relevance is considerable. Thus, the kDNA probe designated in the present study appears to be a promising tool that can be used to specifically identify the stocks belonging to this group of trypanosomes in tsetse flies, as well as in patients and potential mammalian reservoirs.

From a population genetics point of view, successful designing of this probe is consistent with two hypotheses that are not incompatible with each other. First, it can be considered that the cluster identified by the probe is genetically isolated from any other stock studied here. If not, there would be no reason to observe this strong

linkage disequilibrium between the kDNA sequence of the probe and nuclear genes that cause the isoenzyme variability, i.e., the probe should randomly hybridize with any stock of the present sample. Second, this result is consistent (although not definitive evidence by itself) with the more general hypothesis that natural populations of *T. brucei s.l.* have a basically clonal evolutionary pattern.[6, 16, 17] Such a hypothesis does not mean that genetic recombination is absent in *T. brucei*, but only that it does not occur frequently enough to alter a prevalent pattern of clonal population structure.

These results also have implications regarding the question of kDNA evolution. It has been

TABLE 2

Hybridization results for the Trypanosoma brucei *probes tested**

Stock	Zymodeme†	Probe		
		DAL 967	NITR 4012	058 cl.A3
BIYAMINA	1	−	−	−
IBADAN 22	2	−	−	−
TH 112	3	−	−	−
TH 2	4	−	−	−
KK 39	8	−	−	−
GAMBELA 1	12	−	−	−
DAL 069	14	+	−	−
DAL 967	14	+	−	−
NANA	14	+	−	−
TH 149	16	+	−	−
ELIANE	17	+	−	−
NITR 40.12	22	−	+	−
EATRO 1125	33	−	−	−
MOS 84	41	+	−	−
C 126	43	+	−	−
A 004	44	+	−	−
B 014	44	+	−	−
M 001	44	+	−	−
PEYA	45	+	−	−
BIM	47	+	−	−
058 cl.A3	49	−	−	+
TRPZ 227	50	−	−	−
TRPZ 166	51	−	−	−
0404	52	−	−	−
S 29	54	−	−	−
EATRO 1535	57	−	−	−

* − = negative; + = positive.
† Identified using 18 polymorphic loci.[6]

postulated that *T. brucei* minicircle HVR sequences had a very high rate of evolution due to both nucleotide substitutions and segmental rearrangements.[2] This rapid sequence evolution of the minicircle has up to now precluded its use for taxonomic purposes.[18, 19] The results obtained with both the NITR 40.12 and 058 cl.A3 probes are consistent with this hypothesis, but the result for the Dal 967 probe at least shows that this hypothesis is not constantly verified, since it indicates limited evolution of an HVR sequence within a whole group of parasites that exhibit non-negligible genetic variability (Figure 2). On the other hand, since several classes of minicircle sequences coexist within a single cell,[2] the present result suggests that within the group of trypanosomes specifically labeled by the DAL 967 probe, a dominant class of minicircles exists that shares an identical or closely related HVR sequence.

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