MOLECULAR MARKERS FOR THE DIFFERENT (SUB)-SPECIES OF THE TRYPANOZOON SUBGENUS

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Abstract

Recently developed PCRs based on the internal transcribed spacer 1 (ITS-1) allow differentiation of the Trypanozoon subgenus, *T.vivax* and *T.congolense* based on differences in length of the amplification products [1]; [2]. These assays also allow the detection of mixed infections by one single PCR assay.

PCR assays for differentiating the different members of the Trypanozoon subgenus, however, remained a challenging issue. During the past years, species specific PCR's for identifying the different taxa within the Trypanozoon subgenus have been developed. For the detection of the two human pathogenic Trypanosomes, PCR-SRA for T.b.rhodesiense [3; 4] and PCR-TgsGP gene for *T.b.gambiense* [5] were developed. For animal Trypanosomiasis, a *T.evansi* specific PCR based on the RoTat 1.2 VSG was developed [6]. Only for *T.b.brucei* and *T.equiperdum*, no specific markers could be identified. However, the results do point out that *T.equiperdum* is more closely related to *T.b.brucei* than to *T.evansi* and even might be a particular strain of *T.b.brucei* [7].

1. INTRODUCTION

Diagnosis of Trypanosomosis usually starts with clinical suspicion or with the detection of antibodies in the blood of the examined mammalian host. Conclusive evidence of infection however relies on detection of the parasite in the blood or tissue fluids of infected humans or animals. Unfortunately, parasitological techniques cannot always detect ongoing infections as the level of parasitaemia is often low and fluctuating, particularly during the chronic stage of the diseases [8].

As an alternative to parasitological tests, DNA detection based on PCR is being investigated. *Trypanozoon* specific primers have been designed previously: TBR primers which target a 177 bp repeat [9]; pMUTEC primers targeting a retrotransposon [10], ORPHON primers that target the spliced leader sequence [11], and the PCR-ESAG6/7 that target the transferrin receptor [12]; [13]. PCR tests for diagnosis of *T.congolense* and *T.vivax* infections exist as well [14]; [15].

Hence, to differentiate between the three major pathogenic Salivarian Trypanosomes at least three sets of primers were necessary. To obtain the same result with one single PCR, Desquesnes and colleagues developed an assay based on the ribosomal region [2]. In parallel, [1] developed a PCR for amplification of the ITS-1 region, with primers and conditions different from those used by Desquesnes *et al.* [2]. Both these PCRs allow the differentiation of the *Trypanozoon* subgenus from *T.congolense* and *T.vivax* and might lead to a pan-

Trypanosoma assay in those regions where concomitant infections of different pathogenic Salivarian Trypanosomes occur.

PCR assays for differentiating the different members of the *Trypanozoon* subgenus, however, remained a challenging issue. In this mini-review we will give an overview of the work carried out during the last few years at the Institute of Tropical Medicine Antwerp and collaborating institutes, in this field.

2. MATERIALS AND METHODS

2.1. Trypanosome populations

A large collection of Trypanosomes, including clones and stocks from *T. b. gambiense, T.b.rhodesiense, T.b.brucei, T.evansi* and *T.equiperdum, T. congolense, T.theileri* and *T.vivax* was used in the different experiments. They are isolated from different host and regions, all over world (Table I). All populations were kept as cryostabilates in liquid nitrogen.

2.2. DNA preparation

Bloodstream form Trypanosome populations were grown in mice and rats and subsequently purified according to Lanham and Godfrey [16]. Pure Trypanosome pellets were stored at -80°C. Twenty µL of Trypanosome pellet (approximately 2.10^7 cells) were resuspended in 200 µL of Phosphate Buffered Saline (PBS) (8.1m*M* $Na_2HPO_4.2H_20$, 1.4m*M* $NaHPO_4$, 140 m*M* NaCl, pH 7.4) and the Trypanosome DNA was extracted using the commercially available QIAamp DNA mini kit (Westburg, Leusden, The Netherlands), resulting in pure DNA in 200 µL of TE buffer. The typical yield of DNA extracted from a 20 µL pellet was 150 ng/µL or 30 µg total DNA. Obtained extracts were diluted 200 times in water and divided into aliquots of 2 mL in microcentrifuge tubes for storage at -20°C.

2.3. Polymerase chain reactions (PCRs)

2.3.1. PCR EGAG 6/7 [13]

Twenty µL of sample was mixed with 30 µL of a PCR-mix consisting of: 1,5 U Taq DNA recombinant polymerase (Gibco BRL, UK), 10X PCR buffer (Gibco BRL, UK), 3,0 m*M* $MgCl_2$ (Gibco BRL, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.5 µM of each ESAG 6/7 primer. The ESAG 6/7 primers were used for amplification of a 237 bp fragment from *T.evansi* genomic DNA, ESAG 6/7 Forward ACA TTC CAG CAG GAG TTG GAG and ESAG 6/7 Reverse CAC GTG AAT CCT CAA TTT TGT [17]. Cycling conditions were as follows: a first denaturation step of 4 min at 94°C was followed by 35 cycles consisting of 1 min denaturation at 94°C, 1 min primer-template annealing at 55°C and 1 min polymerization at 72°C. The last extension step of 5 min at 72°C was performed to polymerize all remaining single strand DNA fragments (ssDNA).

Twenty µL of the PCR product were electrophoresed on a 2% agarose gel (25 min at 100V) with a 100 bp marker (Gibco BRL, UK) as size marker. The gels were stained with ethidium bromide (2 µL/50mL gel) and analysed on a U.V. transilluminator.

2.3.2. PCR ORPHON [11]

The sequence of the ORPHON primers is as follows:

ORPHON5J-F 5' GAT CCC TCT CCA CCA ATC GAC CG 3'

ORPHON5J-R 5' AAC TGC CCC GAC CTC CGC AGT 3'

To each 20 µL of sample, 30 µL of a PCR cocktail was added, consisting of 25 µL of 2x Goldstar PCR-mix (Eurogentec) enriched with 400 µ*M* of each of the four deoxynucleotides and 3.0 m*M* $MgCl_2$, 0.5 µL 1U/µL Goldstar DNA polymerase (Eurogentec), and 20 p*M* of each primer.

The amplification programme was as follows: 50 cycles of 30 s at 94°C, 90 s at 68°C; 1 cycle of 120 s at 72°C. PCR products were electrophoresed (30 min at 180-200 V) in 2% agarose gels (Biozym) previously stained by submersion in an ethidium bromide solution (1.5 mg/l Tris acetate EDTA buffer, pH 8.0). Signals were made visible by UV illumination and photographed with a Polaroid camera.

2.3.3. PCR ITS-1 [1]

Primers were derived from the *T.evansi* 18S DNA sequence (D89527). Primer sequences were identified within the end region of the 18S ribosomal subunit and the beginning of the 5.8 small subunit. Primer sequences were as follows;

ITS-1 Forward 5' TGT AGG TGA ACC TGC AGC TGG ATC 3'

ITS-1 Reverse 5' CCA AGT CAT CCA TCG CGA CAC GTT 3'

For amplification, 20 µL of extracted DNA were mixed with 30 µL of a PCR-mix containing: 1 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 2.5 m*M* $MgCl_2$ (Promega, UK), 200 µ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.8 µ*M* of each primer (Gibco BRL, UK). All amplifications were carried out in a Biometra® T3 thermocycler. Cycling conditions were as follows: denaturation for 4 min at 94°C, followed by 35 amplification cycles of 1 min denaturation at 94°C, 1 min primer-template annealing at 54°C and 1 min polymerization at 72°C. A final elongation step was carried out for 5 min. at 72°C. Finally, 20 µL of the PCR product and 10 µL of a 100 bp size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (25 min. at 100V). Gels were stained with ethidium bromide (0.5 µg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK).

2.3.4. PCR-SRA [3]

Primers were derived from the sequence of the serum resistance associated gene (SRA) (accession number Z37159). Primer sequences were as follows:

PCR-SRA-*f* 5'ATA GTG ACA AGA TGC GTA CTC AAC GC

and

PCR-SRA-*r* 5'AAT GTG TTC GAG TAC TTC GGT CAC GCT.

They amplify a 284 bp fragment between nucleotides 383-667 of the *SRA* gene. All PCR amplifications were performed using 10 ng of the DNA extracted from purified parasites. The DNA templates were amplified in 50 µL of PCR reaction mixture containing 1

x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$), 1.5 m*M* $MgCl_2$, 200 μ*M* of each of the four dNTPs, 1 μ*M* of each of the primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen). All PCR amplifications were performed on a T3 Thermocycler (Biometra). PCR conditions were as follows: sample incubation for 15 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, and a final extension at 72°C for 10 min. A 20 μL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. The gels were stained with ethidium bromide (1 $\mu g.mL^{-1}$) (Sigma) and analysed on an Imagemaster Video Detection System (Pharmacia).

2.3.5. PCR-TgsGP [5]

The primers were derived from the sequence of the *T.b.gambiense* specific glycoprotein (TgsGP) [18]. Using the Genbank homology search program, the primer sequence was derived from the region lacking any significant similarity with already known DNA sequences. Primer sequences were as follows:

PCR-TgsGP-*f* 5' GCT GCT GTG TTC GGA GAG C

and

PCR-TgsGP-*r* GCC ATC GTG CTT GCC GCT C.

All PCR amplifications were performed using 10 ng of extracted DNA. Amplification was performed using 50 μL of a reaction mixture containing 1x PCR buffer (20 m*M* Tris-HCl pH 8.7, 100 m*M* KCl, 50 m*M* $(NH_4)_2SO_4$, Q solution), 1.5 m*M* $MgCl_2$, 200 μ*M* each of the four dNTPs, 1 μ*M* each of the primers and 2.5 units of HotStar Taq DNA polymerase (Qiagen, Westburg, Leusden, The Netherlands). All PCR amplifications were performed using T3 Thermocycler supplied by Biometra (Westbur, Leusden, Netherlands). The PCR amplification using TgsGP derived primers was performed by incubating the samples for 15 min at 95°C followed by 45 cycles of 1 min at 94°C, 1 min at 63°C and 1 min at 72°C, and a final extension at 72°C for 10 min. A 20 μL sample of each PCR product was analysed by electrophoresis in a 2% agarose gel. Additionally, the detection limit was increased by a second PCR amplification that was performed using 1 μL of the first one.

2.3.6. PCR-RoTat 1.2 [6]

Primers were derived from the RoTat 1.2 VSG sequence (AF317914), recently cloned and sequenced by [19]. Primer sequences were identified within the region (608-812 bp) lacking homology with any other known VSG sequences. Primer sequences (in 5'-3' direction) and annealing temperatures were as follows:

RoTat 1.2 Forward GCG GGG TGT TTA AAG CAA TA, $T_{ann.}$ 59°C and

RoTat 1.2 Reverse ATT AGT GCT GCG TGT GTT CG, $T_{ann.}$ 59°C.

Twenty μL of extracted DNA were mixed with 30 μL of a PCR-mix containing: 1 U Taq DNA recombinant polymerase (Promega, UK), PCR buffer (Promega, UK), 2.5 m*M* $MgCl_2$ (Promega, UK), 200 μ*M* of each of the four dNTPs (Roche, Mannheim, Germany) and 0.8 μ*M* of each primer (Gibco BRL, UK). All amplifications were carried out in a Biometra® Trio-block thermocycler. Cycling conditions were as follows: denaturation for 4 min. at 94°C, followed by 40 amplification cycles of 1 min denaturation at 94°C, 1 min primer-template annealing at 59°C and 1 min. polymerization at 72°C. A final elongation step was carried out for 5 min. at 72°C. Twenty μL of the PCR product and 10 μL of a 100 bp size marker (MBI Fermentas, Germany) were subjected to electrophoresis in a 2% agarose gel (25 min.

at 100V). Gels were stained with ethidium bromide (0.5 μg/mL) (Sigma, USA) and analyzed on an Imagemaster Video Detection System (Pharmacia, UK).

2.3.7. Multiple-endonuclease genotyping approach (MEGA) [7]

A fine-scale genotyping approach involving multiplex endonucleases in combination with a pair of cognate adapters was used according to Agbo and colleagues [20]. Briefly, 100-250 ng genomic DNA were digested for 4 h using 10 U each of *Bgl*II, *Bcl*I, *Acs*I and *Mun*I endonucleases in two successive double digestion reactions. The final digestion products were precipitated and reconstituted in 10 μL distilled water. Ten μL of a buffer containing 660 m*M* Tris HCl, 50 m*M* $MgCl_2$, 10 m*M* Dithiothreitol, 10 m*M* ATP, pH7.5, and 20 p*M* of each *Bgl*II and *Mun*I adapters were added. The *Bgl*II adapter also ligated to the overhang sites created by *Bcl*I, while *Mun*I adapter also ligated to the *Acs*I site. One μL (400U) of T4 DNA ligase (New England Biolabs) was added and the mixture incubated for 2 h at 25°C. Pre-selective amplification was performed in a total volume of 20 μL containing 4 μL of 1:1-diluted ligation product, 1 U of Taq polymerase (Roche Molecular Biochemicals, Almere, The Netherlands), 10X PCR buffer (100 m*M* Tris HCl pH 9.0, 50 m*M* KCl, 1% triton X-100, 0.1% w/v gelatin), 2.5 m*M* $MgCl_2$, 200 μ*M* of each dNTP and 5 p*M* of each *Bgl*II ($^{5'}$-GAGTACACTGTCGATCT) and *Mun*I ($^{5'}$-GAGAGCTCTTGGAATTG) primers. The reaction mix was incubated for 2 min at 95°C, and subjected to 20 cycles of PCR (30 s at 95°C, 30 s at 56°C and 2 min at 72°C). Four μL of 1:20-diluted pre-selective products were used as template for selective reaction with *Mun*-0/*Bgl*-A selective primer combination (in which the *Mun* primer was fluorescently labelled). The PCR program was essentially the same as for pre-selective amplification, except that the last cycling step was followed by 30 min incubation at 60°C. The final products were diluted 1:1 with TE, and Genescan-500 internal lane standard (PE Applied Biosystems) was added. One μL of the mix was resolved in a 7.3% denaturing sequencing gel using a model ABI 373A automated DNA sequencer. Gels were routinely prepared by using ABI protocols and electrophoresed for 5 h. Gel patterns were collected with GenScan software (PE Applied Biosystems) and sample files were transferred to GelCompar II software (Applied Maths, Kortrijk, Belgium).

3. RESULTS

3.1. PCR ESAG 6/7 and PCR ORPHON

Both PCRs are specific for the *Trypanozoon* group: *T. brucei*, *T.evansi* and *T.equiperdum* DNA can be detected while *T.congolense*, *T.vivax* and *T.theileri* samples remained negative. Detection limit of the PCR ESAG6/7 was determined at 100 Trypanosomes/mL using spiked mouse blood, while 200 Trypanosomes/mL was the detection limits when spiked water buffalo blood was used, i.e. five Trypanosomes per PCR reaction (data not shown). The detection limit of the ORPHON PCR was determined at fifty Trypanosomes per reaction, which corresponds to an analytical sensitivity of 2,000 parasites/mL.

3.2. ITS-1

As shown in Fig. 1, all Trypanosomes from the subgenus *Trypanozoon* yielded a band of approximately 450 bp (lane 1-15); the *T.vivax* yielded a 150 bp band (lane 16), while the *Trypanosoma congolense* Savannah yielded a larger band of approximately 650 bp (lane 17).

Double and triple mixed infections were simulated by mixing 10 μL (approximately 7.5 ng) of *T.b.brucei* (AnTat 1.8), *T.congolense* (TRT 17, Savannah) and *T.vivax*

(ILRAD 700) DNA. For each preparation, the obtained PCR pattern reflected the species composition of the mixture Fig. 2).

3.3. PCR-SRA

The expected 284 bp *SRA*-PCR product was obtained with the DNA of the 24 different populations considered as *T.b.rhodesiense*. All other 72 non- *T.b.rhodesiense* populations were negative, thus confirming the specificity of the *SRA*-PCR for the *T.b.rhodesiense* subspecies. An overview of the PCR-SRA results with different human African Trypanosomiasis strains (*T.b.rhodesiense* and *T b.gambiense*) is shown in Fig. 3.

3.4. PCR-TgsGP

In total, 73 different Trypanosome populations have been analysed by the TgsGP-PCR. A specific PCR product was obtained with 13 out of 15 *T b.gambiense* populations. All other 58 non-*T.b.gambiense* populations remained negative, thus confirming the specificity of the TgsGP-PCR for *T.b.gambiense* within the collection of tested *Trypanosoma sp.* After a single PCR reaction, the detection limit reached 1000 Trypanosomes/mL blood. This detection limit was lowered to 10 Trypanosomes/mL blood when the TgsGP-PCR was repeated using an aliquot of the first PCR reaction product. An overview of the PCR-TgsGP results with different human African Trypanosomiasis strains is shown in Fig. 4.

3.5. PCR RoTat 1.2

As shown in Fig. 5, the RoTat 1.2 PCR yielded a 205 bp amplicon in the positive control (lane 1) as well as in all other *T.evansi* populations (lanes 3-8). Moreover, the same fragment was found in seven out of the nine *T.equiperdum* populations tested. Only the *T.equiperdum* BoTat 1.1 (lane 10) and the *T.equiperdum* OVI strain (lane 11) were PCR negative. All other tested Trypanosome populations, including six *T.b.brucei*, eight *T.b.gambiense*, five *T.b.rhodesiense*, two *T. congolense*, one *T.vivax* and one *T.theileri*, were negative. (lanes 18-40). As a negative control, a PCR-mix without template DNA was included (lane 2). The PCR was able to detect as few as 10 Trypanosomes per PCR reaction, which corresponds with a lower detection limit of 50 Trypanosomes per mL. In principal, this limit can still be lowered if a blood sample of 200 µL extracted with the QIAamp DNA mini kit is eluted in less than 200 µL.

3.6. MEGA, a modified AFLP

In the UPGMA clustering data obtained from the modified AFLP analysis, all *T.evansi* are grouped in one cluster with a similarity of 85-95%, together with eight out of ten *T.equiperdum* strains. The *T.b.brucei* group appeared as a heterogeneous cluster, including the *T.equiperdum* BoTat 1.1 and OVI strains. Based on the modified AFLP data, the level of similarity of these two latter strains was calculated at 74%. In this analysis, OVI seems closely related to *T.b.brucei* KETRI 2494, while BoTat 1.1 shares more homology with *T.b.brucei* AnTat 2.2 (Fig. 6).

4. DISCUSSION AND CONCLUSION

PCR ITS-1, a pan-*Trypanosoma* assay. A single PCR was developed that detected all pathogenic *Trypanosoma* spp. Unfortunately, this pan-*Trypanosoma* test cannot discriminate within the subgenus *Trypanozoon.* As well as other pan-*Trypanosoma* tests, such

as the PCR-ITS [2] and PCR-RFLP [21], this PCR targets conserved DNA sequences within the genome. So far, these techniques seem to work for discriminating species within the *Trypanosoma* genus except for the closely related taxa within the *Trypanozoon* subgenus. Apparently, *T. brucei*, *T.evansi* and *T.equiperdum* are too similar to observe any differences within the 18S and ITS regions. Pan-*Trypanosoma* assays may have a potential for distinguishing *T. congolense*, *T.vivax* and *T.brucei* and to detect mixed infections in bovine or small ruminants.

A *T.b.rhodesiense* specific marker. The *SRA*-based PCR was shown to be specific for *T.b.rhodesiense*, as a 284 bp specific PCR product was generated with 24 of the 25 *T.b.rhodesiense* used in this study, whereas this fragment was never detected in other subspecies and species. Other recently used analytical techniques such as isoenzyme analysis and RFLP failed to identify an unequivocal criterion to differentiate *T.b.rhodesiense* from *T.b.brucei* [22-24]. As a large variety of game and domestic animals serve as a reservoir for both subspecies, our PCR test could be used for identification of human infective and non-human infective Trypanosomes within the animal reservoir and the vector [22]; [24-26].

A *T.b.gambiense* specific marker. The obtained results showed that the expected 308 bp specific PCR product was generated solely with *T.b.gambiense* parasites, and that no cross-reactivity occurred with any other DNA templates used in this study. Interestingly, two *T.b.gambiense* strains i.e. ABBA and LIGO scored negative in PCR amplification. These strains however had previously already been classified as being distinct from the conventional *T.b.gambiense* parasites, and were grouped together with the Nigerian *T.b.brucei* subspecies [27]. This classification had been made based on the cluster analysis of the restriction enzyme polymorphism pattern using the ribosomal non-transcribed spacer region. As both strains were negative by TgsGP-based PCR but were previously characterized as human infective, we have re-tested their resistance to normal human serum. Both strains appeared to be completely human serum sensitive and as such were presumed to be non-infective to human, possibly representing a *T.b.brucei* subspecies. On the other hand, two other *T. b. gambiense* strains used in this study i.e. OUSOU and KOBIR that were previously classified together with the ABBA and LIGO strains, did contain the TgsGP gene and were confirmed to be completely resistant to normal human serum. Besides the diagnostic value, the developed TgsGP based PCR test can also serve as a useful tool for disease, vector and reservoir control, indeed rendering possible the differentiation of *T.b.gambiense* from *T.b.rhodesiense* and *T.b.brucei.* As such, the TgsGP-based PCR can be used for epidemiological purposes as well.

A new marker for *T.evansi*. Species-specific markers for *T.evansi* have been previously developed, based on kDNA mini-circle sequences [28-30]. These assays however could not detect dyskinetoplastic *T.evansi* strains since their kDNA is severely reduced or even absent. Bayana Songa and Hamers [31]; and Verloo and colleagues [32]; already identified the RoTat 1.2 VSG as an interesting candidate for species-specific diagnosis of *T.evansi.* Several serological antibody detection tests have been developed and tested both on camels [33]; and water buffaloes [34]. Results indicated the usefulness of the RoTat 1.2 VSG for diagnostic purposes of classic *T.evansi* strains. Only for *T.evansi* type B a problem of diagnosis may appear since this type does not contain the RoTat 1.2 VSG gene [35]; [36]. However, so far this *T.evansi* type B has only been reported in one locality in Kenya. All other isolates in our collection, originating from all over the globe, are from the classic type A and contain the RoTat 1.2 VSG gene. Hence, in general, the RoTat 1.2 VSG gene may be considered as a molecular marker for classic *T.evansi* type A.

Regarding the results of this study, another problem with species-specificity appears unless we accept that the *T.equiperdum* reacting with RoTat 1.2 VSG (gene) are misclassified *T.evansi*. In that case, the RoTat 1.2 VSG (gene) is a potential marker for all *T.evansi* strains, including the dyskinetoplastic strains.

Fitting in *Trypanosoma equiperdum.* Data provided in this study indicate that the *T.equiperdum* collection is not as homogenous as previously believed and the generally followed concept that *T.equiperdum* is very closely related to *T.evansi* and more distant from *T.b.brucei* seems to be incorrect. If we accept the presence of RoTat 1.2 gene to be a specific *T.evansi* marker, only two strains of *T.equiperdum, in casu* the BoTat 1 and the OVI strain, are non *T.evansi* and are more closely related to *T.b.brucei* than to *T.evansi*. Thus the problem of differentiating *T.equiperdum* from the rest of the *Trypanozoon* species shifts from *T.evansi* (the general belief) to *T.b.brucei* (the new concept).

TABLE I. DIFFERENT TRYPANOSOME POPULATIONS, PRESENT IN THE INSTITUTE OF TROPICAL MEDICINE, ANTWERP

(sub)-species	Trypanosome populations	Origin	Isolation year	Original host
T.b.brucei	AnTat 1.8	Uganda	1966	bushbuck
T.b.brucei	AnTat 2.2	Nigeria	1970	tsetse
T.b.brucei	AnTat 5.2	Gambia	1975	bovine
T.b.brucei	AnTat 17.1	D.R.Congo	1978	sheep
T.b.brucei	Ketri 2494 ITMAS 270881	Kenya	1980	tsetse
T. b .brucei	J10 ITMAS 250500A	Zambia	1973	Hyena
T.b.brucei	TSW 196 ITMAS 300500A	Côte d'Ivoire	1978	Pig
T. b. gambiense	AnTat 9.1 ITMAP 1788	Cameroon	1976	man
T. b. gambiense	LiTat 1.3	D.R.Congo	1952	man
T. b. gambiense	AnTat 11.6	D.R Congo	1974	man
T. b. gambiense	AnTat 22.1	Congo/Brazza.	1975	man
T. b. gambiense	JUA ITMAS 010799	Cameroon	1979	man
T. b. gambiense	BAGE ITMAP 2569	D.R.Congo	1995	man
T. b. gambiense	NABE ITMAP 2569	D.R.Congo	1995	man
T. b. gambiense	PAKWE ITMAP 2570	D.R.Congo	1995	man
T. b. gambiense	SEKA ITMAP 2568	D.R.Congo	1995	man
T. b. gambiense II	KOBIR ITMAS 260600	Côte d'Ivoire		man
T. b. gambiense II	OUSOU ITMAS 220600	Côte d'Ivoire		man
T. b. gambiense II	ABBA ITMAS 190600A	Côte d'Ivoire		man
T. b. gambiense II	LIGO ITMAS 190600B	Côte d'Ivoire		man
T.b.rhodesiense	AnTat 25.1	Rwanda	1971	man
T.b.rhodesiense	0404	Rwanda	1970	man
T.b.rhodesiense	STIB 847 ITMAS 050399A	Uganda	1990	man
T.b.rhodesiense	STIB 848 ITMAS 190399	Uganda	1990	man
T.b.rhodesiense	STIB 849 ITMAS 050399B	Uganda	1991	man
T.b.rhodesiense	STIB 850 ITMAS 050399C	Uganda	1990	man
T.b.rhodesiense	STIB 851 ITMAS 080399C	Uganda	1990	man
T.b.rhodesiense	STIB 882 ITMAS 080399A	Uganda	1993	man
T.b.rhodesiense	STIB 883 ITMAS 080399B	Uganda	1994	man

T. congolense	TRT 17 ITMAS 020699	Zambia	1997	bovine
T. congolense	IL 1180			
T.vivax	ILRAD 700	Nigeria		bovine
T.evansi	AnTAR 3 ITMAS 180274A	South America	1969	Capybara
T.evansi	AnTat 3.1 ITMAS 070799	South America	1969	Capybara
T.evansi	AnTat 3.2 ITMAS 270280A	South America	1969	Capybara
T.evansi	AnTat 3.3 ITMAS 161189A	South America	1969	Capybara
T.evansi	AnTat 3.4 ITMAS 301189A	South America	1969	Capybara
T.evansi	AnTat 3.5 ITMAS 310180A	South America	1969	Capybara
T.evansi	RoTat 1.2 ITMAS 020298	Indonesia	1982	Buffalo
T.evansi	MHRYD/BR/86/E18 020297	Brazil	1986	capybara
T.evansi	CAN 86 K ITMAS 140799B	Brazil	1986	Dog
T.evansi	Colombia ITMAS 150799	Colombia	1973	Horse
T.evansi	Vietnam WH ITMAS 101298	Vietnam	1998	Buffalo
T.evansi	STIB 816 ITMAS 140799A	China	1978	Camel
T.evansi	KETRI 2479 ITMAS 100883A	Kenya	1980	Camel
T.evansi	KETRI 2480 ITMAS 110297	Kenya	1980	Camel
T.evansi	KETRI 2481 ITMAS 010883C	Kenya	?	?
T.evansi	KETRI 2485 ITMAS 080981B	Kenya	?	Camel
T.evansi	Philippines ITMAS 060297	Philippines	1996	Buffalo
T.evansi	Kazakstan ITMAS 060297	Kazachstan	1995	Camel
T.evansi	Merzouga56 ITMAS 120399D	Morocco	1998	Camel
T.evansi	Merzouga 94 original stab.	Morocco	1998	Camel
T.evansi	Merzouga 93 ITMAS 150399C	Morocco	1998	Camel
T.evansi	Zagora I.3 ITMAS 010399B	Morroco	1997	Camel
T.evansi	Zagora I.5 ITMAS 040399A	Morroco	1997	Camel
T.evansi	Zagora I.10 ITMAS 220299	Morroco	1997	Camel
T.evansi	Zagora I.17 ITMAS 040399B	Morroco	1997	Camel
T.evansi	Zagora I.28 ITMAS 040399	Morroco	1997	Camel
T.evansi	Zagora I.31 ITMAS 120399A	Morroco	1997	Camel
T.evansi	Zagora I.75 ITMAS 010399C	Morroco	1999	Camel
T.evansi	Zagora I.81 ITMAS 010399D	Morroco	1998	Camel
T.evansi	Zagora I.86 original stab.	Morroco	1997	Camel
T.evansi	Zagora II.28 ITMAS 150399B	Morroco	1997	Camel
T.evansi	Zagora II.42 original stab	Morroco	1998	Camel
T.evansi	Zagora II.52 ITMAS 120399B	Morroco	1998	Camel
T.evansi	Zagora II.111 original stab	Morroco	1998	Camel
T.evansi	Zagora II.114 original stab	Morroco	1998	Camel
T.evansi	Zagora II.115 original stab	Morroco	1998	Camel
T.evansi	Zagora III.25 ITMAS 120399C	Morroco	1998	Camel
T.equiperdum	AnTat 4.1 ITMAS 210983A	unknown	unknown	unknown
T.equiperdum	Alfort ITMAS 241199A	unknown	unknown	unknown
T.equiperdum	SVP ITMAS 241199B	unknown	unknown	unknown
T.equiperdum	Hamburg ITMAS 251199A	unknown	unknown	unknown
T.equiperdum	ATCC 30019 ITMAS 020301	France	1903 ?	Horse
T.equiperdum	ATCC 30023 ITMAS 280201	France	1903 ?	Horse
T.equiperdum	STIB 818 ITMAS 010999	P. R. China	1979	Horse
T.equiperdum	American ITMAS 220101	unknown	unknown	unknown
T.equiperdum	Canadian ITMAS 290101	unknown	unknown	unknown
T.equiperdum	OVI ITMAS 241199C	South Africa	1975	Horse
T.equiperdum	BoTat 1.1 ITMAS 240982A	Morocco	1924	Horse

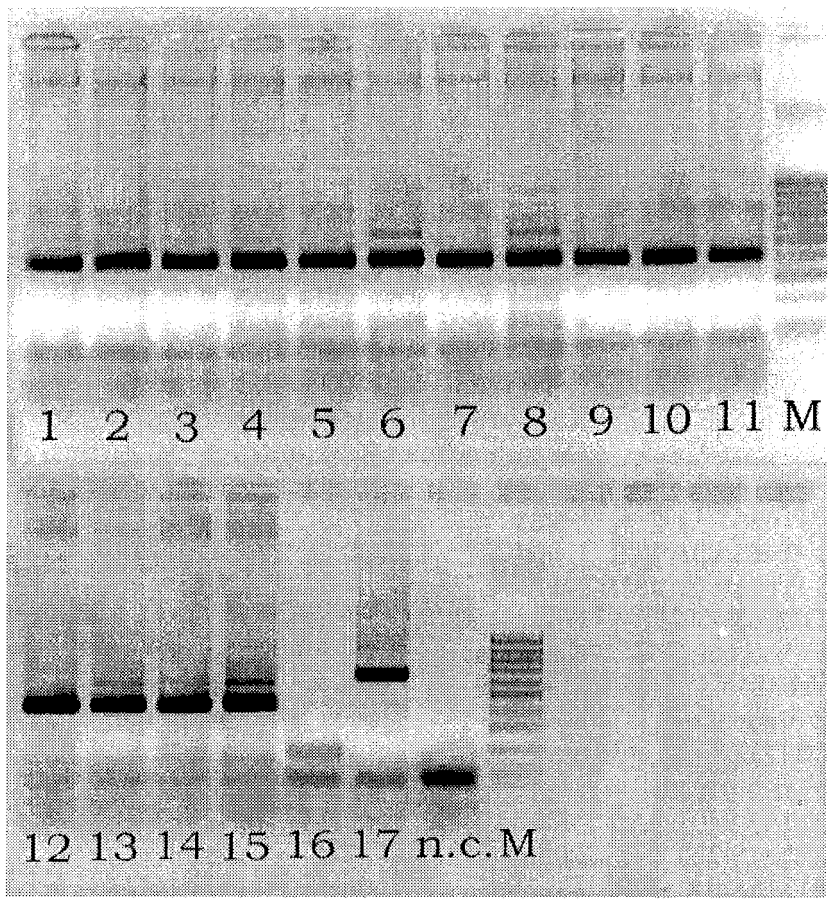

FIG. 1. PCR results obtained with the different Trypanosome populations.

Lanes 1-5, *T.evansi* strains respectively RoTat 1.2, AnTat 3.1, STIB 816, CAN 86K, Merzouga; Lanes 6-12 *T.equiperdum* strains respectively BoTat 1.1, AnTat 4.1, OVI, STIB 818, SVP, Hamburg, Alfort, lane 13 *T.b.brucei* KETRI 2494, lane 14, *T. b. gambiense* AnTat 9.1, lane 15 *T.b.rhodesiense* STIB 850, lane 16 *T.vivax* ILRAD 700, lane 17 *T.congolense* Savannah TRT 17.

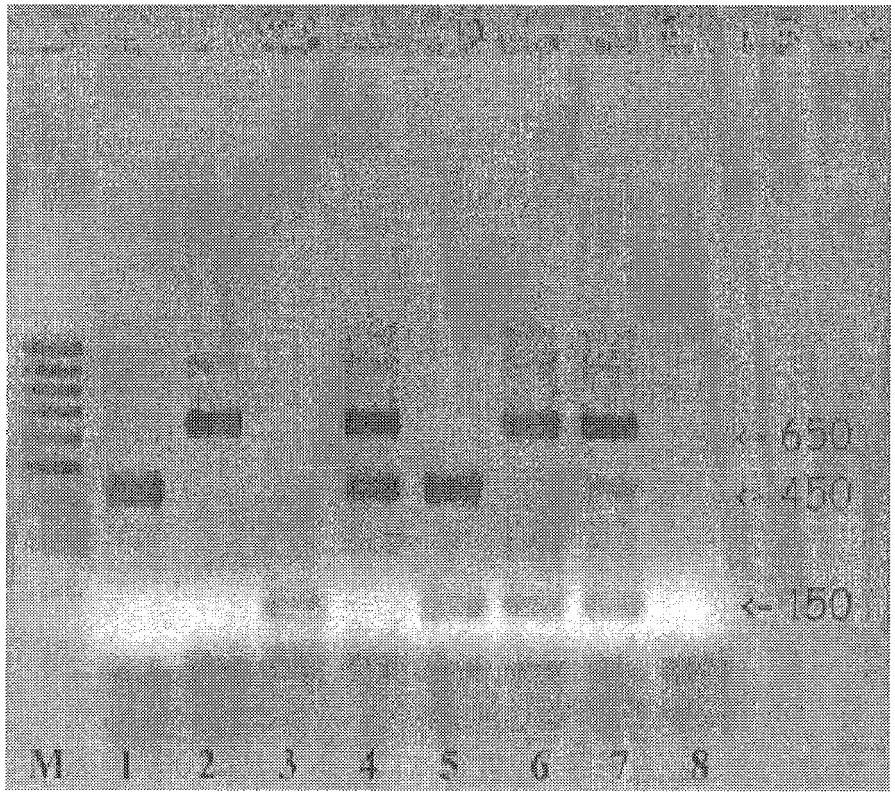

FIG. 2. PCR results for mixed preparations of T.b.brucei, T.vivax and T.congolense Savannah DNA.

Lane 1: *T.b. brucei* AnTat 1.8; lane 2: *T.congolense* TRT 17; lane 3: *T.vivax* ILRAD 700; lane 4: *T.congolense + T.b. brucei*; lane 5: *T.b.brucei* + ***T.vivax***; lane 6*: T.congolense + T.vivax*; lane 7: *T.congolense + T.b.brucei + T.vivax;* lane 8: neg. control; M: 100 bp molecular marker (MBI Fermentas, Germany)

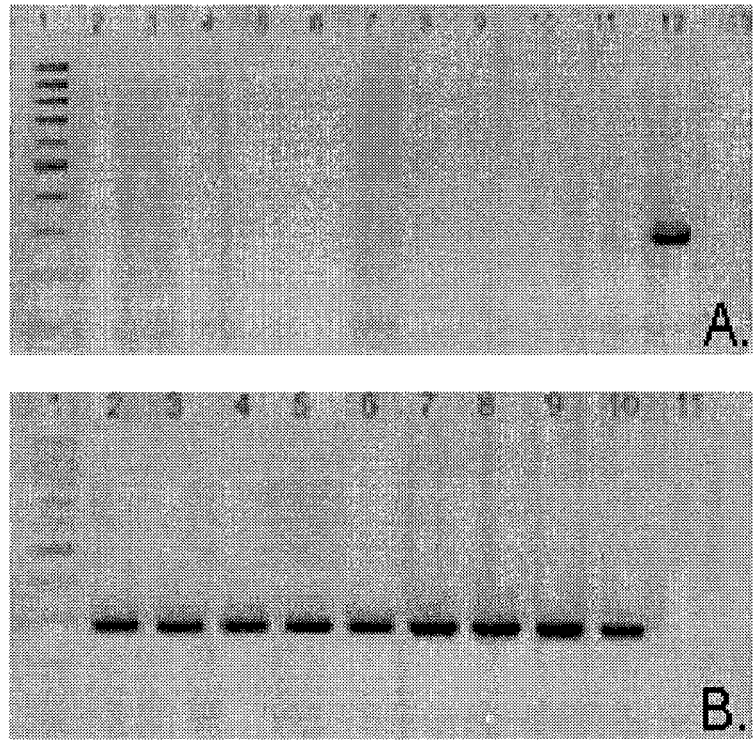

FIG. 3. PCR-SRA, a T.b.rhodesiense specific PCR. Part A.

Lane 1 Marker, lanes 2-11 are *T.b.gambiense*, respectively JUA, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, AnTat 11.6, LiTat 1.6, LiTat 1.3, BAGE, NABE, lane 12 *T.b.rhodesiense* STIB 850, lane 13 white blood cells. ***Part B***. lane 1 Marker, lanes 2-11 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850, STIB 849, JUA

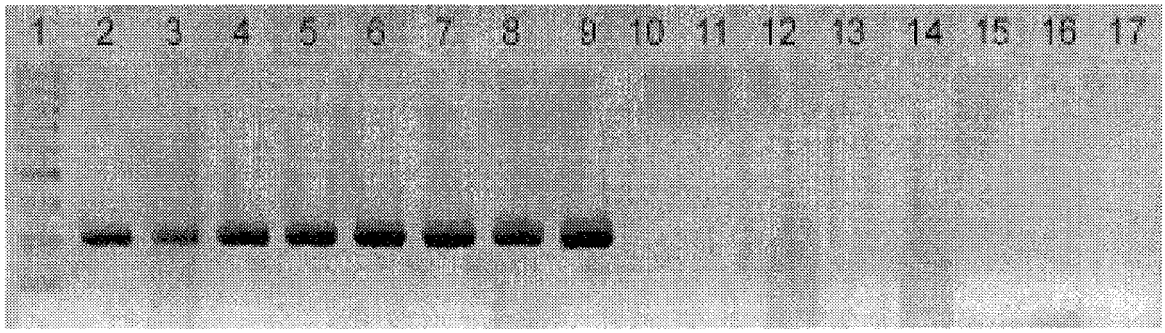

FIG. 4. PCR-TgsGP, a T.b. gambiense specific PCR..

Lane 1 Marker, lanes 2-9 are *T.b.gambiense*, respectively JUA, LiTat 1.3, AnTat 11.17, AnTat 22.1, SEKA, AnTat 9.1, BAGE, LiTat 1.6; lanes 10-17 are *T.b.rhodesiense*, respectively STIB 883, STIB 882, AnTat 12.1, AnTat 25.1, STIB 847, STIB 884, STIB 851, STIB 850, STIB 849, JUA

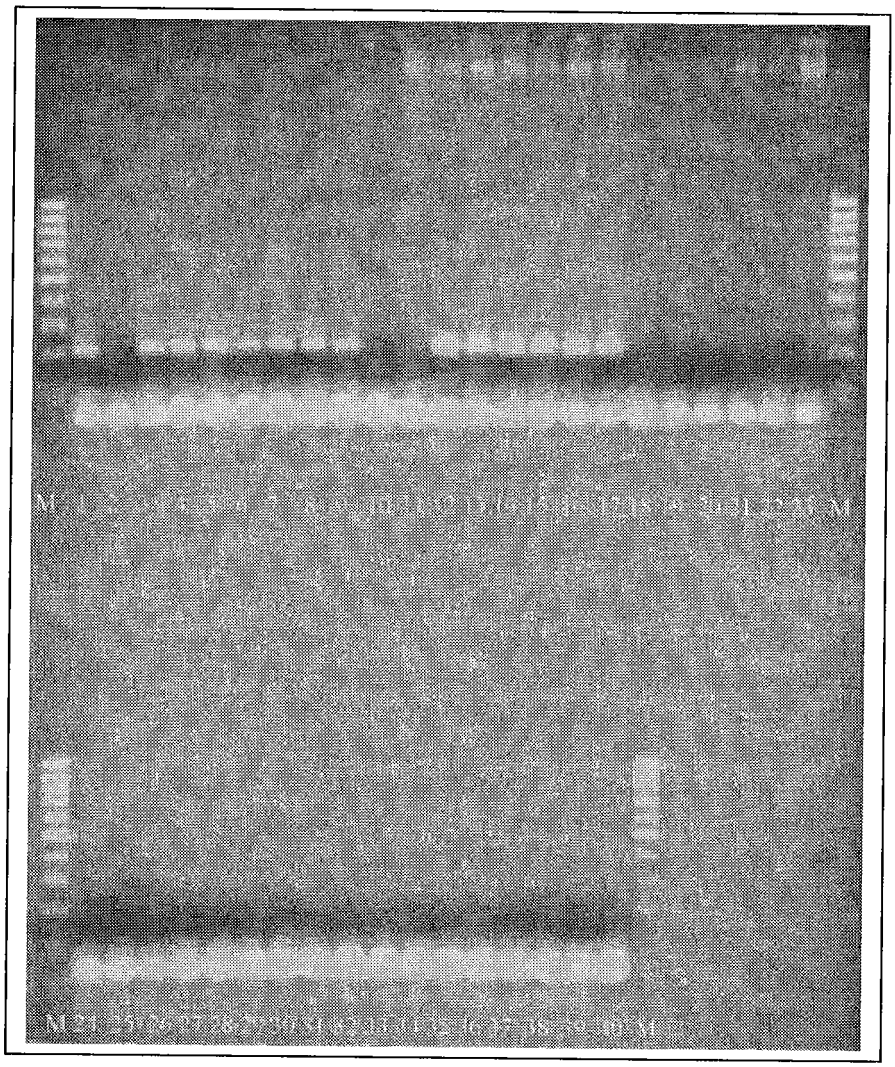

FIG. 5. PCR RoTat 1.2 specificity results for the different Trypanosoma (T.) species and subspecies in this study.

Lane 1 pos. control RoTat 1.2, Lane 2 neg. control, Lanes 3-8 (*T.evansi*) are, respectively, AnTat 3.1, STIB 816, Zagora I.17, Colombia, Merzouga 56, CAN 86K; Lanes 9-17 (*T.equiperdum*) are, respectively, AnTat 4.1, BoTat 1.1, OVI, STIB 818, Alfort, Hamburg, SVP, Am. Strain, Can. Strain ; Lanes 18-23 (*T.b.brucei*) are, AnTat 1.8, AnTat 2.2, AnTat 5.5, KETRI 2494, TSW 196, STIB 348; Lanes 24-31 (*T. b. gambiense*) are, respectively, AnTat 9.1, AnTat 11.6, AnTat 22.1, NABE, SEKA, ABBA, LIGO, LiTat 1.6; Lanes 32-36 (*T.b.rhodesiense*) are STIB 884, STIB 850, AnTat 25.1/S, Etat 1.2/S, AnTat 12.1/S ; Lanes 37-38 (*T. congolense*) are IL1180, TRT 17; Lane 39 (*T.vivax*) is ILRAD 700 and Lane 40 (*T.theileri*) is MELSELE ; Lanes M 100 bp molecular marker (MBI Fermentas, Germany)

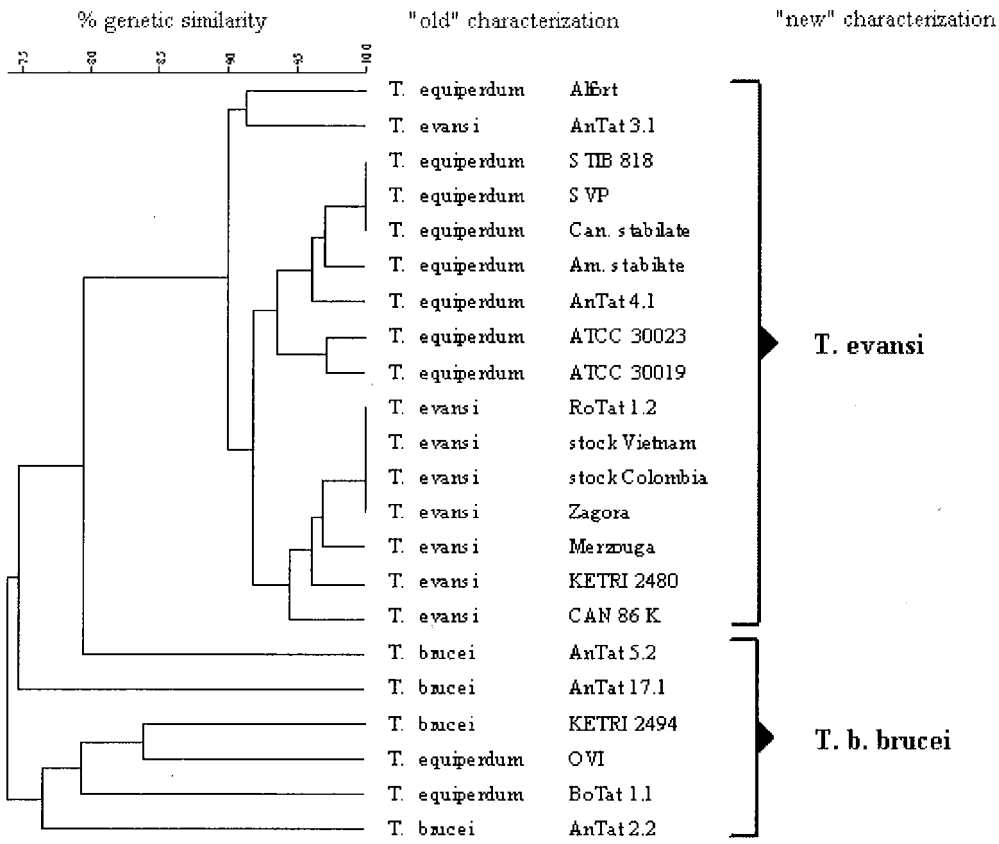

FIG. 6. UPGMA cluster analysis based on the MEGA results.
All *T.evansi* and 8 out of 10 *T.equiperdum* cluster out in one group with a 90-100% genetic similarity. All these strains also contain the RoTat 1.2 VSG gene. Thus, in our newly proposed characterization, this group are *T.evansi* while the other strains can be characterized as *T.b.brucei.*

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