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Full Length Research Paper

Characterization of *Trypanosoma brucei gambiense* stocks isolated from humans by RAPD fingerprinting in Côte d'Ivoire: another evidence for multiple infections

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***Trypanosoma brucei gambiense* was isolated twice from each of 23 patients in Côte d'Ivoire. Genetic characterization using RAPD (Random Primed Amplified Polymorphic DNA) showed additional variability within a given isoenzyme profile (zymodeme), confirming that this fingerprinting method has a higher discriminative power (faster molecular clock) than isoenzymes. RAPD confirmed also the evidence of multiple infections by different genotypes in the same patient despite a low genetic variability among *Trypanosoma brucei gambiense* stocks. The involvement of this phenomenon in treatment failure is discussed.**

Key words: Human African Trypanosomiasis, *Trypanosoma brucei gambiense*, RAPD, multiple infections.

INTRODUCTION

Human African Trypanosomiasis (HAT) is a serious health problem in the sub-Saharan part of Africa. It is estimated that about 600 000 people are infected in 2003 (W.H.O., personal communication). In West Africa, the causative agent of the chronic form of the disease is *Trypanosoma brucei gambiense*. However, diversified clinical evolutions were recorded in Côte d'Ivoire including a suspicion of an acute form (Jamonneau et al., 2000 a, Truc et al., 1997). The taxonomic status of the subspecies described within the complex *T. brucei* is still under debate, mainly because geographical distribution and pathogenicity of the subspecies are difficult to link with clear genetic categories (Jamonneau et al., 2002). Indeed, the only clear-cut genetic subdivision remains *T. brucei gambiense* group 1 (Gibson, 1986), while it is unclear whether *T. b. brucei* and *T. b. rhodesiense* correspond to distinct phylogenetic subdivisions (Mathieu-Daudé et al., 1994). Trypanosomes were genetically characterized using different methods, including Multilocus Enzyme Electrophoresis (MLEE) and several molecular methods (Gibson et al., 1999), such as

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Table 1. Patient code number, stocks isolated twice from each patient (A and B), corresponding location and focus and year of isolation in Côte d'Ivoire.

Patient/DNA	Location	Focus	Year
611 A and B	Yaokro	Sinfra	1997
622 A and B	Yaokro	Sinfra	1997
2499 A and B	Sinfra	Sinfra	1996
2562 A and B	Dioulabougou	Sinfra	1997
5/7A and B	Sinfra	Sinfra	1999
93/5 A and B	Sinfra	Sinfra	1997
634 A and B	Konéfla	Sinfra	1997
614 A and B	Yaokro	Sinfra	1997
659 A and B	Bonon	Bonon	1998
51/11 A and B	Sinfra	Sinfra	1997
664 A and B	Bonon	Bonon	1998
666 A and B	Bonon	Bonon	1998
4/ 5 A and B	Sinfra	Sinfra	1999
687 A and B	Bouaflé	Bonon	1998
806/9 A and B	Aboisso	Aboisso	1997
B120/9 A and B	Aboisso	Aboisso	1997
654 A and B	Bouaflé	Bonon	1998
662 A and B	Bonon	Bonon	1998
668 A and B	Bonon	Bonon	1998
669 A and B	Bonon	Bonon	1998
694 A and B	Bouaflé	Bonon	1998
2508 A and B	Bonon	Bonon	1997
T21/4 A and B	Grand Zathry	Sinfra	1999

Random Primed Amplified Polymorphic DNA (RAPD) (Tibayrenc et al., 1993), ribosomal gene 18S sequencing (Stevens, 1999) or microsatellites (Biteau et al., 2000, Truc et al., 2002).

This study has been conducted in Côte d'Ivoire where parasites were isolated twice from each patient at different time, first during the medical survey in the field, and second when arriving at the hospital before treatment (Truc et al., 2002). Isolates were cultivated *in vitro*, then genetically characterised by RAPD. The aim of this study was first, to record the diversity of *T. brucei* genotypes circulating in this area, and then, to confirm the evidence of multiple infections as previously described by Truc et al. (2002) using another PCR based method.

MATERIALS AND METHODS

Population surveyed

Twenty three patients were diagnosed according to standard procedures (WHO, 1998) between 1996 and 1999 by the Ivoirian National Control Program in three foci of Côte d'Ivoire: Sinfra and Bonon in central-western part and Aboisso in the eastern part of the country, on the border with Ghana (Table 1). Consenting patients were bled twice by venepuncture with a minimum interval of 3 days.

Parasite collection

Trypanosomes were isolated using the Kit for In Vitro Isolation of trypanosomes (KIVI, Aerts et al., 1992). Reference stocks were included: JUA and PEYA (*T. b. gambiense* group 1), TSW65 and KP465 (*T. brucei* " bouaflé " group), 058Cl.A3 (*T. b. rhodesiense*), EATRO 1125 (*T. b. brucei*) and TRPZ105 (*T. congolense*, savannah group). These reference stocks were previously studied by Truc et al. (1993, 1997) and Mathieu-Daudé et al. (1994). Isolates and reference stocks were cultivated in semi-defined medium (Cunningham, 1977). Parasite pellets were collected, prepared and stored at -20°C according to Truc et al. (1991, 1993, 2002). For each patient, two pellets labelled A and B corresponded respectively to the cultures initiated from each of the two isolates (Truc et al., 2002).

RAPD (Random Primed Amplified Polymorphic DNA)

DNAs were extracted according to the method described by Oury et al. (1997). RAPD amplifications were performed according to the protocol described by Welsh and McClelland (1990) and Williams et al. (1990) and modified by Tibayrenc et al. (1993). Genomic DNA samples (20 ng) were amplified in 60 µl of specific buffer (10 mM

Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), supplied by Roche Diagnostics (Mannheim, Germany), in presence of 0.2 µM of primer, 4 x 100 µM dNTP and 0.9 U of Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany). The characterization was based on the use of ten base-long primers from Operon Technologies (USA). One hundred and twenty primers were first screened on the set of the seven reference stocks. Twenty primers were selected on the basis of two criteria, namely the readability and the reproducibility of the patterns obtained: A2, A4, A8, A10, A11, A15, A16, A18, A19, A20, B1, B8, B9, B18, F3, F4, F10, N20, U3 and U18 (see primer sequences through Operon Technologies, USA). The amplification program was performed on a PTC 100-60 thermocycler (MJ Research Inc., Watertown, Massachusetts, USA) and consisted of 45 cycles at 94°C for 1 min, 36°C for 1 min and 72°C for 2 min and a final elongation step at 72°C for 7 min. Amplified DNA was kept at +4°C until analysis. Amplifications were repeated three times for each primer.

Amplification products were separated by electrophoresis on 1.6% agarose gels with TAE buffer (40 mM Tris-acetate pH 7.5, 1 mM EDTA) at 5 V/cm. DNA fragments were visualized by ethidium bromide staining.

Data analysis

The Jaccard distance was calculated to estimate the genetic differences among the stocks (Jaccard, 1908). DNA bands were coded starting from the slowest band. All genotypes were computed thanks to the Genetics software designed and optimized in our laboratory by S. Noël and performed using Sun stations. The UPGMA (Unweighted pair-group method with arithmetic average) dendogram was built from the Jaccard distance matrix according to Sneath and Sokal (1973).

RESULTS AND DISCUSSION

46 stocks were isolated from 23 patients (i.e. 2 stocks from each patient). Amplification patterns showed a maximum of seven bands for a given primer. Because of the high number of primers used for 46 stocks, observed bands are not detailed in a table. All 46 newly isolated stocks fell into only one cluster, which was genetically fairly homogeneous (Figure 1). When compared with JUA and PEYA reference stocks, results confirmed that the 46 stocks under study belong to the *T. b. gambiense* group 1 identified as zymodeme 3 (Z3, Jamonneau et al, 2000 b). Out of 23 pairs of stocks, only 13 pairs were strictly identical and showed no detectable genetic difference. The remaining ten pairs showed slight genetic differences ($d<0.1$). Out of twenty primers, sixteen showed no variability for all 46 stocks. Using the four remaining primers (A2, B18, F10 and N20), most part of the bands was shared by all 46 stocks: the differences observed between patterns involved only one band.

The additional genetic variability evidenced in the present study by RAPD analysis within a given zymodeme (Z3) confirms that RAPD has a higher discriminatory power than MLEE, at least in the case of *T. brucei* spp.. This is due to the fact that the various genomic regions randomly surveyed by RAPD have an overall faster molecular clock than MLEE genes. This still limited genetic variability corroborates the pattern previously found in *T. b. gambiense* group 1 (see Gibson et al., 1999). A similar result was observed for some lower genetic subdivisions of *T. cruzi* (Tarrieu, 1993, Tibayrenc et al., 1993, Barnabé et al., 2000). For a given primer, a difference of only one band when comparing two stocks isolated from the same patient could be considered either as an artefact, a mutation or a limited genomic rearrangement. However the occurrence of artefact bands in our results is improbable because the reproducibility of all patterns has been checked for. Thus it can be considered that these different patterns correspond to different, although closely related genotypes.

While ten pairs of stocks were different when using RAPD analysis, only two were detected using TBDAC 1/2 and TRBPA 1/2 microsatellite markers (Truc et al., 2002). Only the 614A and B pair of stocks were different by using both microsatellite and RAPD typing methods. The 634A and B pair of stocks were different for TBDAC 1/2, but not for RAPD.

The present results suggest the occurrence of mixtures of *T. brucei* genotypes within a given HAT patient. However, (i) it is interesting to note that all double infections involve very closely related genotypes. A totally different result was observed in *T. cruzi*, since mixed infections with drastically divergent clonal genotypes are commonplace in a given chagasic patient (Tibayrenc et al., 1986); (ii) one cannot rule out the hypothesis that such slight differences are explainable, not by original infection by two different genotypes, but rather, by point mutation or limited genomic rearrangement occurring after infection by only one genotype. Mixtures of genotypes in HAT patients were already suspected through the double isolation of stocks from the same patient, the *in vitro* isolation of stocks using KIVI, the proportion of stumpy and slender forms at the time of blood inoculation into KIVI (Truc et al., 2002).

The existence of mixtures of genotypes might play a role on HAT pathogenicity, and on several researches such as treatment failure, since different *T. brucei* genotypes may have different virulence and different drug sensitivities. It is probable that the frequency of mixtures of genotypes has been until now underestimated, since the process of isolation and culturing introduces a selection, which could eliminate one genotype and not the other. Evaluation of drug sensitivity of a stock isolated from a given patient might therefore be not reliable. Further investigations are required to determine accurately the potential impact of mixtures of *T. brucei* genotypes on HAT clinical diversity and treatment failure.

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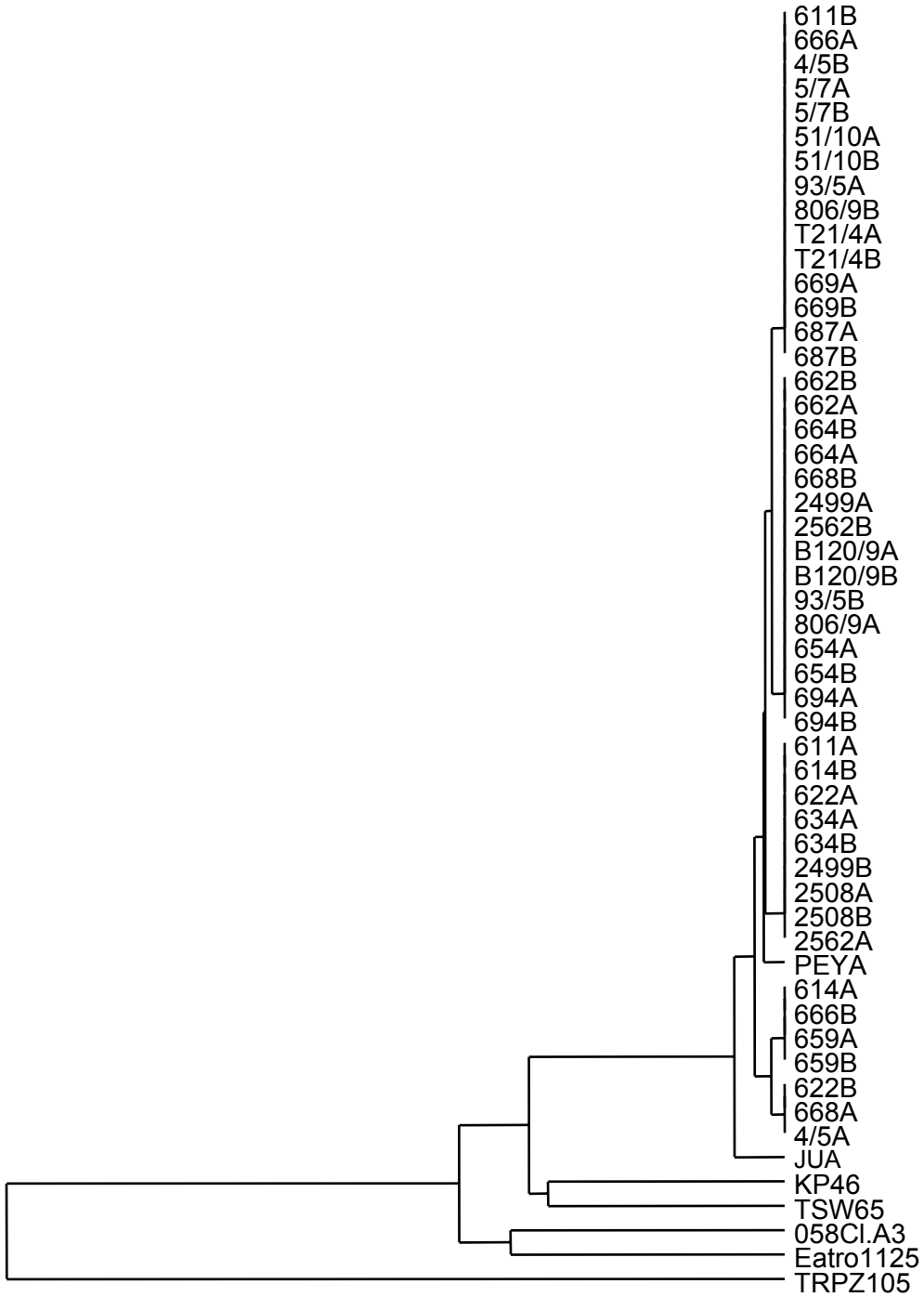

Figure 1. UPGMA dendrogram built using Jaccard distances for each pair of stocks (A and B) isolated from the same patient (code number). For reference stocks JUA and PEYA (*T. b .gambiense* group 1), TSW65 and KP465 (*T. brucei* " bouaflé " group), 058Cl.A3 (*T. b. rhodesiense*), EATRO 1125 (*T. b. brucei*) and TRPZ105 (*T. congolense*, savannah group), see text.

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