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Aparasitemic serological suspects in *Trypanosoma brucei gambiense* human African trypanosomiasis: A potential human reservoir of parasites?

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Abstract

The serological and parasitological tests used for *Trypanosoma brucei gambiense* human African trypanosomiasis (HAT) diagnosis have low specificity and sensitivity, respectively, and in the field, control program teams are faced with subjects with positive serology but negative parasitology who remain untreated. The aim of this work was to explore, using PCR tool, the significance of these aparasitemic serological suspects. Since discordant PCR results have been observed earlier with different extraction methods, two DNA extraction methods were compared (the Chelex 100® resin and the DNeasy® Tissue kit). The study was conducted on 604 blood samples: 574 from parasitologically confirmed patients, aparasitemic serological suspects and endemic controls collected in Côte d'Ivoire and 30 from healthy volunteers collected in France. No significant differences were observed between the PCR results obtained with the two extraction methods. Concerning PCR, problems of reproducibility and discordances with both serological and parasitological test results were observed, mainly for the aparasitemic serological suspects. In addition to previous results that pointed to the existence of non-virulent or non-pathogenic trypanosome strains and of individual susceptibility leading to long term seropositivity without detectable parasitaemia but positive PCR, the results of this study support the notion of a long lasting human reservoir that may contribute to the maintenance or periodic resurgences of HAT in endemic foci.
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1. Introduction

Human African trypanosomiasis (HAT) or sleeping sickness remains an important public health problem in Sub-Saharan Africa. About 55 million people are exposed to the risk of infection and it is estimated that there are about 300,000–500,000 infected but untreated

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people (WHO, 1998). Hence, as control heavily relies on the detection and treatment of patients especially for the chronic form of the disease caused by *Trypanosoma brucei gambiense* (*T. b. gambiense*) and occurring in West and Central Africa, a reliable diagnosis is of key importance.

A broad clinical diversity (features and evolutions) is observed in *T. b. gambiense* HAT (Jamonneau et al., 2000, 2002; Sternberg, 2004), thus rendering difficult accurate diagnosis only based on clinical investigation (Jannin et al., 1993). Diagnosis is therefore generally based on direct (parasitological) or indirect (serological) evidence of blood, lymph or cerebrospinal fluid (CSF) invasion by trypanosomes (reviewed in Lejon et al., 2003 and Chappuis et al., 2005). Because the drugs used for treatment may have potentially side effects (Legros et al., 2002), demonstration of the parasite in tissue fluids of a patient is required before the initiation of chemotherapy (WHO, 1998).

Mass screening of the population at risk for *T. b. gambiense* infection is currently performed using the serological tests in order to select individuals with positive response to trypanosome antigens on whom parasitological examinations are carried out. Thanks to its simplicity and low price, the most commonly serological test used in the field is the Card Agglutination Test for Trypanosomiasis (CATT/*T. b. gambiense*, Magnus et al., 1978). The parasitological tests are based on the detection of trypanosomes in lymph node aspirates, blood and CSF. Currently, the most sensitive parasitological technique is the miniature anion-exchange/centrifugation technique (mAECT, Lumsden et al., 1979; Chappuis et al., 2005).

In the field, control program teams are faced with three categories of individuals: apparently healthy subjects with negative serological tests; sleeping sickness cases with positive serological and parasitological tests and subjects with positive serology but negative parasitology. This phenomenon of apparently aparasitemic serological suspects may be due to cross reactivity of CATT with animal trypanosomes (Noireau et al., 1986a) or other infectious diseases (Diallo et al., 1996), or due to failing parasite detection tests due to weak or fluctuating parasitaemia (Dukes et al., 1984; Truc et al., 1994). Finally, for some subjects, a phenomenon of control of infection by means of an appropriate immune system response is suspected (Garcia et al., 2000). In these two latter cases, subjects who remain untreated may represent a potential parasite reservoir that could be responsible for the persistence of transmission and re-emergence of historical sleeping sickness foci.

Polymerase chain reaction (PCR) has been increasingly applied in diseases for which early and reliable diagnosis is required while asymptomatic carriers and/or fluctuating parasitaemia are frequent (reviewed in Yera et al., 2003). When applied to HAT diagnosis, higher sensitivity and specificity have been reported compared to parasitological methods and serological ones, respectively (Kanmogne et al., 1996; Penchenier et al., 1996, 2000; Kabiri et al., 1999; Kyambadde et al., 2000; Solano et al., 2002; Radwanska et al., 2002; Jamonneau et al., 2003; Becker et al., 2004). However, some problems of reproducibility and discordances with serological tests have been reported mainly on aparasitemic serological suspects (Kyambadde et al., 2000; Garcia et al., 2000; Solano et al., 2002). The aim of this work was to explore, using PCR, the significance of this complex phenomenon of positive serology without parasitological confirmation. The TBR1/2 primers (Moser et al., 1989) which were shown to be highly sensitive for HAT diagnosis (Penchenier et al., 2000; Solano et al., 2002; Jamonneau et al., 2003) were used in this study, and two simple DNA extraction methods were compared: the Chelex 100® resin method, an easy procedure, but which was suspected to give problems of sensitivity and reproducibility of PCR attributed to incomplete DNA purification (Ravel et al., 2004; Becker et al., 2004), and the DNeasy® Tissue kit, predicted to yield better purification of DNA thus avoiding the problems of PCR sensitivity and reproducibility.

2. Materials and methods

2.1. Study area and collection of samples

Samples from patients were collected in 2003 and 2004 in the active sleeping sickness focus in Central-West Côte d'Ivoire, during active surveillance (medical survey) carried out by the National Control Program (NCP) in collaboration with Institut Pierre Richet (IPR) and Institut de Recherche Pour le Développement (IRD). Blood was also collected from French volunteers (30) who had never been travelled to Africa (healthy subjects, H).

The HAT diagnostic procedure in the field was performed according to HAT National Control Program recommendations. Briefly, people were first screened using CATT on whole blood (Magnus et al., 1978). CATT whole blood seropositives were tested subsequently in CATT on plasma (Magnus et al., 1978). Subjects positive with CATT on whole blood and negative with CATT on plasma were called "serological suspects" or C+ and did not undergo further microscopic examinations. Parasitological tests (mAECT and direct examination of lymph node aspirate) were only performed on the sub-

jects positive in CATT on blood and on plasma (i.e. not performed on C+ subjects). Positive ones (trypanosomes seen by microscopy) were considered as sleeping sickness cases or "patients" (T+) and negative ones were called "seropositive" or Pl+. In each area, some subjects negative in CATT on whole blood (parasitological tests not performed according to the diagnostic procedure) living in the focus (C−) were invited for giving blood as endemic negative controls. Signed or oral consent was obtained from each subject included in this study. Sampling consisted of 574 blood samples collected in heparinized vacutainers: 38 T+, 250 Pl+, 213 C+ and 73 C−. For each sample, 1 ml blood was aliquoted in 1.5 ml microcentrifuge tube. All samples were stored at −20 °C for subsequent PCR analysis.

2.2. DNA extraction procedure

A first DNA extraction (Chelex) from thawed blood samples was based on the procedure described in Penchenier et al. (1996) using an anion chelating resin (Chelex 100® Resin, Bio-Rad Laboratories, CA, USA). Briefly, 500 μl of blood were transferred into a 1.5 ml microcentrifuge tube containing 500 μl of pure water. To lyse the cells, the mixture was vortexed every other 2 min at room temperature for 10 min. The mixture was then centrifuged at 13,000 × *g* for 4 min, the supernatant gently removed and discarded and the pellet resuspended into 400 μl of a 5% (w/v) Chelex suspension in sterile purified water. The tubes were then vortexed for 1 min, incubated at 56 °C for 1 h and at 95 °C for 30 min and centrifuged at 13,000 × *g* for 5 min. The supernatant was used as a template in PCR essays.

A second DNA extraction was performed using DNeasy® Tissue kit (Qiagen). For the preparation of samples, 500 μl of blood were transferred into a 1.5 ml microcentrifuge tube containing 500 μl of pure water. The mixture was vortexed every other 2 min at room temperature for 10 min. The mixture was then centrifuged at 13,000 × *g* for 4 min, the supernatant gently removed and discarded and the pellet resuspended into 200 μl of PBS. The prepared samples were processed according to the instructions provided by the manufacturer. The last step of the protocol consisted in the elution of DNA in 400 μl AE buffer.

2.3. PCR conditions

PCR was performed according to Penchenier et al. (1996) using TBR1–2 primers (TBR1: 5′-CGA-ATG-AAT-ATT-AAA-CAA-TGC-GCA-G-3′; TBR2: 5′-AGA-ACC-ATT-TAT-TAG-CTT-TGT-TGC-3′) targeting a 177 bp satellite DNA in *Trypanosoma brucei sensu lato* (Moser et al., 1989). PCR was carried out in a final volume of 50 μl containing 10 pmol of each primer, 0.2 mM of each desoxyribonucleotide, 1× incubation buffer with 1.5 mM $MgCl_2$, 0.5 units of Taq polymerase (QBIOgene, Ilkirch, France) and 10 μl DNA sample. PCR amplification was performed in triplicate in three different essays to test the reproducibility. When the expected 177 bp product was visible, the PCR was considered positive.

3. Results

When triplicate PCR gave three positive or three negative results, the sample was considered as + or −, respectively. When a discordance was observed (for example: two PCR positive results and one PCR negative result for the same sample), the sample was considered as doubtful (±).

A comparison between Chelex and DNeasy® extraction methods was first performed on 255 blood samples: 12 T+, 82 Pl+, 99 C+, 32 C− and 30 H. The results are given in Table 1. Results are clearly similar concerning T+, C− and H subjects. For Pl+ and C+ individuals, no significant difference ($p > 0.10$) was observed whatever the extraction method. Then, taking into account this result, all the remaining samples were processed only by the Chelex method which is cheaper and easier from a technical point of view.

The PCR results obtained with the 574 blood samples from Côte d'Ivoire and the 30 H subjects processed by the Chelex method are given in Table 2. All 30 H subjects did not show any amplification signal whereas one posi-

Table 1
PCR results for the 255 blood samples processed with the two DNA extraction methods

PCR	Chelex			DNeasy®		
	+	±	−	+	±	−
12 T+	11 (92)	1 (8)	0	12 (100)	0	0
82 Pl+	7 (9)	14 (17)	61 (74)	5 (6)	20 (24)	57 (70)
99 C+	1 (1)	8 (8)	90 (91)	5 (5)	14 (14)	80 (81)
32 C−	1 (3)	0	31 (97)	0	3 (9)	29 (91)
30 H	0	0	30	0	0	30

Percents are given in parentheses; T+: positive in CATT on whole blood, positive in CATT on plasma and positive in parasitological tests (mAECT and/or direct examination of lymph node aspirate); Pl+: positive in CATT on whole blood, positive in CATT on plasma but negative in parasitological tests; C+: positive in CATT on whole blood but negative in CATT on plasma and in parasitological tests; C−: endemic controls, negative in CATT on whole blood (CATT on plasma and parasitological tests not performed according to the diagnostic procedure); H: healthy subjects who had never been in Africa.

Table 2
PCR results for the 574 blood samples from Côte d'Ivoire and the 30 H processed with the Chelex method

	PCR		
	+	±	–
38 T+	36 (95)	2 (5)	0
250 Pl+	38 (15)	55 (22)	157 (63)
213 C+	9 (4)	32 (15)	172 (81)
73 C−	1 (1)	3 (4)	69 (95)
30 H	0	0	30 (100)

Percents are given in parentheses; T+: positive in CATT on whole blood, positive in CATT on plasma and positive in parasitological tests (mAECT and/or direct examination of lymph node aspirate); Pl+: positive in CATT on whole blood, positive in CATT on plasma but negative in parasitological tests; C+: positive in CATT on whole blood but negative in CATT on plasma and in parasitological tests; C−: endemic controls, negative in CATT on whole blood (CATT on plasma and parasitological tests not performed according to the diagnostic procedure); H: healthy subjects who had never been in Africa.

tive and three doubtful results were observed within the 73 C−. Concordant results were noticed between parasitological tests and PCR results on the patients, since 36 T+ out of 38 gave a positive result by PCR. The two remaining T+ gave a doubtful PCR result.

PCR gave a positive or a doubtful result for 93 (38 + 55) out of 250 Pl+ (37%) and 41 (32 + 9) out of 213 C+ (19%). A total of 92 doubtful results out of 574 (16%) were observed, for which 32 out of the 213 C+ subjects (15%, representing 35% of the doubtful results), and 55 out of the 250 Pl+ (22%, representing 60% of the doubtful results).

4. Discussion

The aim of this work was, using PCR, to explore the significance of aparasitemic but serologically positive subjects (Pl+ and C+) in HAT. PCR was performed using TBR1/2 primers on blood samples from Côte d'Ivoire (C−, C+, Pl+ and T+) and France (H), from which DNA was extracted by two methods (Chelex and DNeasy®).

The Chelex method has been questioned because of possible incomplete purification of DNA leaving inhibiting factors in the PCR reaction mixture (Ravel et al., 2004; Becker et al., 2004). However, when compared to the DNeasy® method which offers a total purification of DNA (see DNeasy® Tissue Handbook provided with the kit), no significant differences were observed between the PCR results obtained with these two methods. This confirms the potential of the easily applicable Chelex method for DNA purification, as also recently reported by Becker et al. (2004) in real-time PCR assays, and shows that the problem of reproducibility of PCR in serological suspects is not due to the DNA extraction method used.

Our results confirm the high specificity and sensitivity of PCR since all healthy subjects (H) and 95% of C− subjects were negative and since all parasitological confirmed cases (T+) gave a positive or a doubtful result. The 5% positive or doubtful cases within the C− group (such cases had already been observed by Solano et al., 2002) can be explained by limited sensitivity of the CATT (in case of early infection or absence of the LiTaT 1.3 gene in circulating trypanosome strains for example, Truc et al., 1994; Enyaru et al., 1998) or, more probably, by a cross reaction of the PCR with a transient infection with *T. b. brucei*, as already suspected by Garcia et al. (2000). Indeed, the here used TBR1/2 primers amplify DNA of *T. brucei* s.l. species comprising *T. b. gambiense*, *T. b. rhodesiense* but also *T. b. brucei* which is non-pathogenic for humans. Unfortunately, PCRs that are specific for *T. b. gambiense* (i.e. *T. b. gambiense* group 1) are targeting a single copy gene (Radwanska et al., 2002) thus limiting the sensitivity of these tests in case of low parasitaemia, which is commonly observed in *T. b. gambiense* HAT.

On the C+ and Pl+ subjects, our study confirms both the earlier observed discordances between PCR and CATT (Kyambadde et al., 2000; Garcia et al., 2000) and between PCR and parasitological tests (Penchenier et al., 2000; Solano et al., 2002). All these discordances may be due to cross reactions of CATT with antibodies induced by other parasitic diseases (Noireau et al., 1986a, 1987; Penchenier et al., 1991; Diallo et al., 1996), cross reactions of PCR with transient infection with *T. b. brucei* mentioned above or extremely low *T. b. gambiense* parasitaemia.

Indeed, problems of both (i) PCR reproducibility and (ii) discordances between PCR and CATT on one side, and PCR and parasitology on the other side, occur in the same group of C+ and Pl+ subjects. Then another hypothesis can be put forward: these persons are actually infected with *T. b. gambiense* but their parasitaemia is so low that parasitological tests will remain negative and that PCR will randomly be positive or negative since the target DNA concentration is at the very detection limit of the test.

In a 8-year follow-up (from 1995, year of HAT diagnostic, to 2002) of six asymptomatic patients positive in serology and parasitology in 1995, but refusing treatment (Sinfra focus, Côte d'Ivoire), it was observed that these patients remained always positive or doubtful in CATT but none of them showed any parasite from 1998 to 2002. However, in 2002, trypanosome DNA was consistently detectable in their blood and, moreover, mixed

infections with two different trypanosome stocks, *T. b. gambiense* group 1 and another stock belonging to *T. brucei s.l.*, was strongly suspected (Jamonneau et al., 2000, 2004). Without PCR, these asymptomatic patients with detectable parasites in 1995 would have been considered as seropositive Pl+ (i.e. never diagnosed as HAT cases). In another longitudinal follow-up of 77 Pl+ individuals from the same Sinfra focus, observations on parasitologically negative but serologically positive persons with non-consistent PCR results suggested the existence of an individual susceptibility to HAT infection, i.e. "human trypanoresistance" (Garcia et al., 2000). The existence of non-virulent or non-pathogenic trypanosome strains and of human individual susceptibility leading to long-term seropositivity without detectable parasitaemia but positive PCR strongly suggests the existence of a human reservoir of trypanosomes. This human reservoir may contribute to the maintenance or periodic resurgences of human African trypanosomiasis in endemic foci, often attributed to an animal reservoir (Mehlitz et al., 1982; Noireau et al., 1986b; Njiokou et al., 2006). The respective roles of host and parasite in this complex phenomenon of positive serology but negative parasitology must be further investigated.

A complementary study of the role of the host and of the parasite (and of their potential interactions) on the variability of response to infections is essential (see Lambrechts et al., 2006). The information generated from this area will advance knowledge of host and parasite mechanisms which influence the pathogenesis and manifestation of diseases and then in HAT will have a major impact on drug, diagnostic tool and vaccine strategy development but also on the investigation of the mechanisms implied in the re-emergence phenomenon.

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