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First evidence that parasite infecting apparent aparasitemic serological suspects in human African trypanosomiasis are *Trypanosoma brucei gambiense* and are similar to those found in patients

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ABSTRACT

Thanks to its sensitivity and its ease of use in the field, the card agglutination test for trypanosomiasis (CATT) is widely used for serological screening of *Trypanosoma brucei gambiense* human African trypanosomiasis (HAT). Positive subjects are then examined by microscopy to confirm the disease. However, the CATT exhibits false-positive results raising the question of whether CATT-positive subjects who are not confirmed by microscopic detection of trypanosomes (SERO) are truly exposed to *T.b. gambiense* infection. For this purpose, we applied microsatellite genotyping on DNA extracted from blood of both HAT confirmed patients and SERO subjects in Guinea and Côte d'Ivoire since microsatellite genotyping has proved useful for the study of *T.b. gambiense* genetic diversity. Problems of amplification failures raise the question of the sensitivity of microsatellite markers when applied on biological samples especially from SERO subjects for who low blood parasitaemia are suspected. Nevertheless, we have shown that the trypanosomes from SERO individuals that have been genotyped belong to *T.b. gambiense* group 1 and were identical to those found in HAT patients. These results constitute the first evidences that at least some SERO are indeed infected by *T.b. gambiense* group 1 and that they may constitute a human reservoir of parasite in HAT foci. Whether these individuals should undergo treatment remains an open question as long as their role in HAT transmission is unknown. Our results strongly recommend the follow-up of such subjects to improve control strategies.

1. Introduction

Human African trypanosomiasis (HAT) caused by *Trypanosoma brucei* (*T.b.*) *gambiense* in West and Central Africa is usually diagnosed using two sequential steps: first the Card Agglutination Test for Trypanosomiasis CATT, (Magnus et al., 1978) is used for serological screening, followed by microscopy to confirm the disease and initiate treatment (Chappuis et al., 2005; WHO, 1998). Currently, CATT continues to be used as a test for mass screening because of its simplicity and high sensitivity. However, the CATT can often exhibit false-positive results raising the question of whether CATT-positive subjects who are negative by microscopy (SERO) are exposed to *T.b. gambiense* infection (Garcia et al., 2006). Indeed, some of these subjects may harbor low blood parasitaemia undetected by microscopy even when the mini Anion Exchange Centrifugation Technique (mAECT), the most sensitive (detection threshold around 10 parasite/ml) parasitological method to date (Büscher et al., 2009; Camara et al., 2010), is used.

Because of the low sensitivity of parasitological methods, molecular methods that amplify parasite specific DNA sequences have been developed for HAT diagnostic (Deborggraeve and Büscher, 2010). These methods were partly used to address

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the question of the parasitological status of unconfirmed CATT-positive subjects (Garcia et al., 2000; Kabiri et al., 1999; Kanmogne et al., 1996; Koffi et al., 2006; Kyambadde et al., 2000; Solano et al., 2002). Unfortunately, the most sensitive PCR methods targeting sequences occurring at multiple copies in the parasite genome are *Trypanozoon* specific and cannot differentiate between *T. brucei* subpecies, i.e. the human pathogenic *T.b. gambiense* and *T.b. rhodesiense* from *T.b. brucei*, *T. evansi*, and *T. equiperdum* that cause animal African trypanosomiasis (AAT). In HAT endemic areas, where animal trypanosomiasis are commonly encountered, humans are exposed to the bites of tsetse flies infected by *T.b. brucei*. While *T.b. brucei* is currently considered as non-infective to humans (Pays et al., 2006), it is possible that *T.b. brucei* DNA remains detectable in blood of such subjects thus resulting in positive PCR. It is then difficult to say if positive PCR results are due to *T.b. gambiense* infections or repeated exposure to *T.b. brucei*. (Garcia et al., 2000; Koffi et al., 2006). Unfortunately, PCRs that are specific for *T.b. gambiense* (i.e. *T.b. gambiense* group 1) are targeting single copy genes (Mathieu-Daude and Tibayrenc, 1994; Radwanska et al., 2002) thus limiting the sensitivity of these tests in case of low parasitaemia. Another hypothesis is that such SERO subjects could be infected by particular non-virulent strains of *T.b. gambiense*.

Microsatellite markers were shown to be polymorphic enough to study the genetic diversity among *T.b. gambiense* group 1 (Balmer et al., 2006; Biteau et al., 2000; Koffi et al., 2007, 2009; MacLeod et al., 2005a; Morrison et al., 2008). Such markers were confirmed to be sensitive enough to be applied to *T. brucei* profiling directly from biological samples (Koffi et al., 2007) thus avoiding the need to isolate and amplify the parasites in laboratory rodents or axenic medium before analysis. In this study, we applied microsatellite genotyping to address the following questions: are SERO subjects infected with *T.b. gambiense* strains and if yes, are these strains the same as those found in HAT confirmed patients?

2. Materials and methods

2.1. Ethical consideration

All samples used in the frame of the present study (excluding reference stocks) were collected during medical surveys conducted by the National Control Programs (NCP) of Guinea and Côte d'Ivoire according to the respective national HAT diagnostic procedures. No samples other than those collected for routine screening and diagnostic procedures were collected for the purpose of the study. All participants were informed about the objective of the study in their own language and signed an informed consent. Children less than 12 years old were excluded. For participants between 12 and 18 years old, informed consent was obtained from their parents. This study is part of a larger project aiming at improving HAT diagnosis for which ethical clearance was obtained from WHO and IRD ethical committees.

2.2. Study subjects

All study subjects were identified in Guinea and Côte d'Ivoire during HAT surveillance activities. Subjects positive for the *T.b. gambiense* specific CATT (CATT-B) performed on blood collected by finger prick, blood was collected in heparinised tubes and twofold plasma dilution series in CATT buffer were tested to assess the end titer, i.e. the highest dilution still positive (CATT-P). All CATT-P positive subjects underwent parasitological examinations by direct examination of lymph node aspirate when swollen lymph nodes were present and/or by mAECT. Thus, two categories of study participants were defined for the purposes of the study:

HAT (patients): CATT-P end titer $\geq$1/8 and parasitologically confirmed;

SERO (seropositives): CATT-P end titer $\geq$1/8 but no parasites detected.

Specimens were collected:

i) in 2004 in the Bonon focus situated in the Western central part of Côte d'Ivoire, between the savannah and the mesophilic forest where HAT prevalence is about 0.1% (Kaba et al., 2006). Stocks isolated from HAT patients from this area had already been genotyped (Koffi et al., 2007) and were included in this study ($N = 13$) as reference stocks (see below). For the SERO sample, we selected blood extracted DNA samples from subjects already described in Koffi et al. (2006) for whom diagnostic PCR (see below) had already been performed. From the 38 PCR-positive SEROs, we selected those diagnosed in 2004 and for whom extracted DNA was still available ($N = 24$).
ii) in 2008 in the Dubreka mangrove focus of coastal Guinea that is currently the most active West African focus with a prevalence of about 1% (Camara et al., 2005).

2.3. PCR/diagnostic

For all participants, 1 ml blood was aliquoted in 1.5 ml microcentrifuge tube and stored at −20 °C until use. DNA extraction was performed using the DNeasy® Tissue kit (Qiagen) as described in Koffi et al. (2006). A PCR using the highly sensitive TBR1/2 primers, specific to *Trypanozoon* (Moser et al., 1989) was performed with the DNA samples (as described in Koffi et al., 2006). Only TBR1/2 positive DNA was used for subsequent microsatellite analysis.

2.4. Microsatellite genotyping

Reference stocks including those isolated in 2004 in the Bonon focus used for this study have been genotyped using the method described in Koffi et al. (2007). We then used the same method to genotype SERO that were from the same study site. Since microsatellite genotyping technology has been improved these last years, we used more recent methods to characterize Guinean samples.

For Côte d'Ivoire samples, microsatellite loci were amplified using seven primer pairs: M6C8-CA (M6C8) and MT30/33-AC/TC (MT30/33, Biteau et al., 2000), Micbg1, Micbg5, Micbg6, Misatg4, and Misatg9 (Koffi et al., 2007). Primer sequences are given in Supplementary Table S1. The amplification conditions were identical to those previously described in the corresponding articles. Allele bands were resolved and band size determined in 10% non-denaturing acrylamide gels. Bands were visualized by ethidium bromide staining under UV illumination.

For Guinean samples, microsatellite loci were amplified using six primer pairs: M6C8-CA (M6C8, Biteau et al., 2000), Micbg5, Micbg6 and Misatg4 (Koffi et al., 2007) and CH5JS2 and CH118 (MacLeod et al., 2005b). One μl of blood-extracted DNA was used as template for PCR, in a volume of 10 μl. Nested PCR amplifications were carried out using the following conditions, PCR buffer (45 mM Tris–HCl pH 8.8, 11 mM $(NH_4)_2SO_4$, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 μM EDTA, 113 μg per ml BSA, 1 mM of each of the four deoxyribonucleotide triphosphates), 1 μM of each primer, and 0.1 unit of Taq polymerase (ABgene) per 10 μL reaction. Nested PCR conditions for all markers for both rounds were 28 cycles of 50 s at 95 °C, 50 s at 55 °C and 1 min at 65 °C. One μl of a 1/280 dilution of the first round product was used as template in the second round PCR. One primer out of every