1 **Molecular analysis of archived blood slides reveals an atypical human *Trypanosoma* infection**

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4 Running head: Molecular analysis of *Trypanosoma* infection

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Abstract

In 2003, a 10-months old Ghanaian boy recovered from a *Trypanosoma brucei* infection although the patient was not treated with anti-trypanosomal drugs. Only *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are able to infect humans causing human African trypanosomiasis (HAT). The disease is considered 100% fatal if left untreated. The identity of the trypanosome was determined by DNA extraction from the archived stained blood slides followed by sequential application of PCRs that are specific for the order, subgenus, species and subspecies followed by genotyping with microsatellite PCR. Molecular analysis indicated that the parasites observed in the patient's blood in 2003 belong to the *Trypanosoma brucei* subspecies *brucei*, which is normally not infectious to humans. Next to the clinical message, this article provides technical information to extract successfully DNA from archived blood slides for subsequent molecular analysis and to identify a trypanosome by taxon specific PCRs and microsatellite genotyping.

Introduction

The genus *Trypanosoma* contains a large number of parasitic species which infect humans, domesticated and wild animals (Hoare, 1972). The subgenus *Trypanozoon* includes *Trypanosoma brucei*, *Trypanosoma evansi* and *Trypanosoma equiperdum* of which the latter two cause surra and dourine in animals respectively. The salivarian species *Trypanosoma brucei* (*T.b.*) is widely distributed in sub-Saharan Africa and consists of three morphologically indistinguishable subspecies, *T.b. brucei*, *T.b. gambiense* and *T.b. rhodesiense*. Only the latter two are able to infect humans causing the lethal disease human African trypanosomiasis (HAT) or sleeping sickness. *T.b. gambiense* gives rise to the chronic form of HAT in West and Central Africa, while *T.b. rhodesiense* is associated with the acute form in South-East Africa. *T.b. brucei* is only infective to animals causing nagana in domestic livestock throughout sub-Saharan Africa. However, some particular strains from *T.b. brucei* have been isolated from humans in Burkina Faso and Côte d'Ivoire and are subsequently called *T.b. gambiense* group 2 (Gibson, 1986; Truc et al., 1997; Gibson, 2007). No recent isolates of this group have been described. Most of the molecular characterization methods using cladistic analysis evidence a monophyletic genetic taxonomic unit, *T.b. gambiense* group 1, and a very heterogeneous group gathering *T.b. brucei*, *T.b. rhodesiense* and *T.b. gambiense* group 2 (Gibson, 1986). *T.b. rhodesiense* and *T.b. gambiense* group 2 are considered as variants of *T.b. brucei* that could acquire resistance to human serum (Gibson, 2007).

The management of a suspected HAT case is based on accurate diagnosis and effective treatment. The diagnosis of *T.b. gambiense* group 1 HAT relies mainly on initial serological screening with the card agglutination test for trypanosomiasis (CATT/*T.b. gambiense*) followed by demonstration of the parasites in blood, lymph and/or cerebrospinal fluid (CSF). Patients inevitably die after a period of grave illness if not treated. From 1993 till today two HAT cases have been reported in Ghana although no active case-finding surveys have been conducted in the last ten years. The last HAT case was reported in 2000 from Takoradi (Western region) (anonymus, 2006).

On 27 December 2003 a 10-months old boy was admitted at the Effia Nkwanta Regional Hospital in Takoradi city, Ghana, with fever, severe dyspnoeic and very pale. The boy was born in Annoe (Takoradi, Ghana), a peri-urban farmer's community of about 1500 inhabitants situated 10 km south-east from Takoradi city. Tsetse flies of the species *Glossina palpalis* and *Glossina tachinoides* have been reported in this area by the Tsetse and Trypanosomiasis Unit from the Ministry of Food and Agriculture. The boy's blood was examined in the laboratory by thick blood film and was negative for *Plasmodium* but showed trypanosomes at very high parasitaemia. Consequently, the boy was diagnosed with HAT but no lumbar puncture to determine the disease stage was done. On 30 December 2003 severe anaemia was observed next to high fever and dyspnoeic. As no HAT specific treatment was available the patient received a blood transfusion before being sent home to wait for arrival of the drugs to treat HAT. A request for HAT drugs was sent to the World Health Organisation (WHO) on 12 January 2004 by the WHO country office in Accra, Ghana. Pentamidine and melarsoprol were sent to Accra on 14

1 January 2004 but for unknown reasons the drugs didn't arrive at the Public Health
2 Regional Service in Takoradi. Due to the patient's malnutrition and the poverty of the
3 family, the Regional Public Health Division provided food support for the patient and
4 performed regular visits for follow-up of his physical condition. Since the patient
5 apparently recovered without any HAT treatment, his blood was checked again for
6 trypanosomes in February 2004 and proved to be negative. The patient was sent back
7 home for further follow-up.
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9 Although reports from Annoe Health Centre indicated that the patient was healthy, the
10 mother came with the child to the Efia Nkwanta Regional Hospital in June 2005 upon
11 request of the health staff. On examination the child looked completely healthy and blood
12 examination remained negative for trypanosomes. Therefore the health staff decided not
13 to treat for HAT and to send the child back home for further follow-up.
14
15 Upon invitation of the Ghana Health Services, WHO carried out a mission to assess the
16 situation of sleeping sickness in the Takoradi area in December 2005. During the visit at
17 the laboratory of the Efia Nkwanta Regional Hospital, the blood slides prepared in
18 December 2003 were re-examined and the presence of trypanosomes at very high
19 parasitaemia was confirmed. The WHO representatives visited the child and his family at
20 home in Annoe village. The mother confirmed that the family had never traveled outside
21 Takoradi area and that the whole family was healthy. The CATT was performed on blood
22 from the boy, his sister and his mother. All CATT results were negative. Five thick blood
23 films per person were prepared and examined. The blood films of the child showed

Plasmodium falciparum gametocytes but no trypanosomes. The child was referred to the health centre for appropriate treatment of malaria. In the active survey 2,630 people were screened with the CATT in eleven selected villages. No HAT case was detected. In the Annoe village 296 individuals were tested with the CATT. One 17 years old girl was positive in CATT on whole blood and on serum diluted ¼. Since no trypanosomes were detected by micro-haematocrit centrifugation technique during the parasitological confirmation test and since the girl didn't show any clinical sign that might be associated with HAT, a false positive reaction in CATT was concluded.

The results of these investigations raise the following questions. Which trypanosome infected the child in 2003? Are we dealing with a spontaneous cured HAT case, which is unusual? In this article we describe how we identified the parasite through molecular analysis of the archived blood slides.

1 **Materials and Methods**

2

3 ***Microscopic analysis***

4 One giemsa stained thick drop blood slide and two giemsa stained thin blood smears from
5 the child in 2003 and two giemsa stained thick drop blood slides from the child in 2005
6 were transferred via WHO to the Institute of Tropical Medicine in Antwerp (Belgium) for
7 analysis. The blood slides were analysed using an Olympus BX41 microscope (Olympus,
8 Aartselaar, Belgium).

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10 ***DNA extraction***

11 DNA was extracted from one thin blood smear from the child in 2003, one thick drop
12 blood slide from the child in 2005 and one control thick drop blood slide from a healthy
13 Belgian person. Thick drop blood slides from a HAT patient in Angola were used as
14 positive control slides to optimize the DNA extraction method. DNA extraction of the
15 archived blood slides was based on the methods described by Schönian et al. (2003),
16 Meredith et al. (1993) and El Tai et al. (2000). Briefly, the slides were soaked in xylene
17 for 24 hours at room temperature to remove the immersion oil. The cellular material was
18 hydrated in water for 5 minutes and overlaid with 267 µl lysis buffer (50 mM NaCl, 10
19 mM EDTA pH 8.0, 50 mM Tris.HCl pH 7.4). The softened cellular material were
20 carefully scraped off with a pipette tip and collected in a 1.5 ml microcentrifuge tube.
21 Proteinase K (200 µg/ml final concentration) and SDS (1% final concentration) was
22 added to the solution and incubated overnight at 60°C. The sample was then subjected to
23 the classical phenol-chloroform extraction, ethanol and sodium acetate precipitation (van

Eys et al., 1989). DNA pellets were dried using a DNA SpeedVac (Savant Instruments, Farmingdale, NY) for 5 minutes and dissolved in 50 µl PCR grade molecular biology water (Sigma, Bornem, Belgium). The DNA samples were kept at 4°C until use.

Taxon specific PCRs

Success of DNA extraction from the archived blood slides was checked by amplifying a part of the human β-globin gene. In the next steps, several specific PCR assays were applied to determine if the parasite belongs to the *Trypanosomatidae* order, the *Trypanozoon* subgenus, the *T. evansi* species, the *T.b. gambiense* group 1 subspecies or the *T.b. rhodesiense* subspecies. An overview of the applied PCR protocols is given in table 1. For each PCR the 25-µl reaction mixture contained 1x Qiagen PCR buffer (Qiagen, Hilden, Germany), 2.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 200 µM of each deoxynucleotide triphosphate (Roche, Mannheim, Germany), 0.8 µM forward primer (Sigma, Bornem, Belgium), 0.8 µM reverse primer (Sigma, Bornem, Belgium), 0.1 mg/ml acetylated bovine serum albumin (Promega, Madison, Wis.), 0.5 U of HotStar *Taq* polymerase (Qiagen, Hilden, Germany) and 2.5 µl of sample DNA. Cycling conditions were: 94°C for 15 min to activate the HotStar *Taq* polymerase followed by 40 cycles of 94°C for 30 s, Ta for 30°C, 72°C for 30s and a final extension at 72°C for 5 min except for the *T.b. rhodesiense* specific SRA PCR (94°C for 15 min followed by 45 cycles of 94°C for 20 sec, Ta for 45 sec, 72°C for 1 minute and a final extension at 72°C for 5 minutes). Amplification was conducted in 200-µl thin-wall PCR tubes (Abgene, Epsom, United Kingdom) in a T3 thermocycler 48 (Biometra, Göttingen, Germany). Amplified products were analysed by electrophoresis in a 2 % agarose gel (Eurogentec, Seraing,

Belgium) and U.V. illumination (Syngene, Cambridge, U.K.) after ethidium bromide staining (Sigma, Bornem, Belgium).

Microsatellite PCR

The DNA extracts were sent to Institut de Recherche pour le Développement in Montpellier (France) to be typed by microsatellite PCR as described by Koffi et al. (Koffi et al., 2007). Microsatellite PCR using eight primer pairs was performed on the DNA samples from the three blood slides together with DNA extracted from 7 *T.b. gambiense* group 1 reference stocks, 15 *T.b. gambiense* isolates from patients in Côte d'Ivoire, 7 *T.b. gambiense* group 2 reference stocks, 3 *T.b. brucei* reference stocks, 2 *T.b. rhodesiense* reference stocks.

Results

Micropscopic analysis

The extremely high parasitaemia with long slender trypomastigotes was confirmed in the blood slides from 2003 (Figure 1). The parasites contained a well-developed undulating membrane, free flagellum and clearly visible nucleus and kinetoplast. Morphology indicated that the parasites belong to the genus *Trypanosoma* and subgenus *Trypanozoon*. The thick drop blood slides from the child in 2005 were confirmed to be negative.

Taxon specific PCRs

The human β-globin PCR showed that DNA extraction on the archived blood slides was successful (Figure 2a). The results of the different specific PCRs indicate that the observed parasites belong to the *Trypanosomatidae* order (Figure 2b) and the *Trypanozoon* subgenus (Figure 2c) but not to the *T. evansi* species (Figure 2d), *T.b. gambiense* group 1 subspecies (Figure 2e) nor the *T.b. rhodesiense* subspecies (Figure 2f). The other members of the *Trypanozoon* group are *T. equiperdum*, *T. brucei brucei* and *T.b. gambiense* group 2 for which no specific molecular marker is available. Infection of the child with *Trypanosoma equiperdum* seems very unlikely since this parasite is considered infectious only for equines and transmitted only by sexual intercourse.

In conclusion, the molecular diagnosis results bear evidence that *T. brucei brucei* or *T.b. gambiense* group 2 may be the cause of the clinical presentation of the child in 2003.

1 ***Microsatellite PCR***

2 DNA of the blood slide from the child in 2005 and the negative control blood slide
3 yielded no amplification with any microsatellite primer pair used, thus confirming the
4 absence of trypanosome DNA. The DNA sample from the child in 2003 (patient03)
5 showed amplification only with four of the eight primer pairs (Micbg1, M6C8, MT30/33
6 and TRBPA1/2), probably due to low trypanosome DNA concentration combined with
7 low sensitivity of the primers. An unweighted pair group method with arithmetic mean
8 (UPGMA) dendrogram was built using the Jaccard's genetic distances (Sneath and Sokal,
9 1973) to visualize the genetic relationships between the different reference stocks and the
10 patient03 DNA sample (Figure 3). The UPGMA dendrogram confirms that the parasite
11 detected in the child in 2003 does not belong to the *T.b. gambiense* group 1. The
12 patient03 DNA sample fits into the very heterogeneous group comprising *T.b.*
13 *rhodesiense*, *T.b. brucei* and *T.b. gambiense group 2*, which cannot be subdivided in
14 clearly divided genetic entities based on these microsatellite results.

Discussion

Successful DNA extraction of the archived stained blood slides allowed us to identify the trypanosome that caused the transient infection of the child in 2003. The DNA extracts were subjected to sequentially applied PCRs that are specific for the order, subgenus, species and subspecies followed by genotyping with microsatellite PCRs. The molecular analysis results indicated that the child was infected with *T.b. brucei* or *T.b. gambiense* group 2. However, the hypothesis that the described infection is caused by *T.b. gambiense* group 2 is to our opinion unlikely since (i) this pathogenic variant of *T.b. brucei* has never been isolated from humans outside Burkina Faso and Côte d'Ivoire (Gibson, 1986; Truc et al., 1997), (ii) no new cases have been reported in the last fifteen years, (iii) a spontaneous cure of a *T.b. gambiense* group 2 infection in humans has never been described and finally (iv) since *T.b. gambiense* group 2 is normal human serum resistant in the blood incubation infectivity tests (BIIT) (Van Meirvenne et al., 1976) (unpublished results). We may conclude that the child showed a transient infection with *T. b. brucei*.

Human infections with non-pathogenic trypanosomes have been reported earlier. *T. lewisi* infection in humans was recently reported in The Gambia (Howie et al., 2006). In 2005, Truc et al. described a human infection with *T. evansi* (Joshi et al., 2005). A mixed human infection of *Trypansoma brucei* ssp. and *T. congolense* in Côte d'Ivoire was reported in 1998 (Truc et al., 1998). In 1988, BIIT results on an autochthonous sleeping

sickness case in Western Ethiopia indicated that the person was infected with *T.b. brucei* (Abebe et al.,1988).

From 1994 till 2000, 120 animal infections with *T.b. brucei* have been reported by the Veterinary Service Unit (Ministry of Agriculture) in the Takoradi area, mainly in dogs, goats, sheep, pigs and cattle. The presence of *T.b. brucei* in domestic animals and tsetse flies in the region together with the malnutrition of the 10 months old child may explain this transient infection. We cannot tell precisely how long the infection remained in the child neither if some of the symptoms observed at admission in December 2003 were due to the *T.b. brucei* infection. Nevertheless it is evident that the infection was cleared by the child without specific treatment for trypanosomes. The *T.b. brucei* parasite is normally not infectious to humans since it undergoes immediate lysis through the trypanolytic activity of the human-specific apolipoprotein L-1 (ApoL1) (Vanhamme et al., 2003; Vanhamme and Pays, 2004). The subspecies *T.b. gambiense* and *T.b. rhodesiense* can resist this trypanocidal activity thus enabling them to cause sleeping sickness in humans. The mechanism of the parasite's resistance to normal human serum is yet only elucidated for *T.b. rhodesiense* with as key factor the *T.b. rhodesiense* specific serum resistance-associated protein (SRA) (Vanhamme et al., 2003; Xong et al., 1998). ApoL1 is naturally present in the human blood and is not a response of the triggered immune system by infection. Vanhollebeke et al. showed that the human infection with *T. evansi* in India was linked to the lack of the trypanolytic ApoL1 protein in the patient's serum (Vanhollebeke et al. 2006). Since the Ghanaian patient recovered from the *T. b. brucei*

infection without HAT treatment, we hypothesize that the ApoL1 level in his blood was transiently reduced due to his general weak condition.

This report demonstrates that (i) HAT spontaneous cure in endemic regions where other *Trypanosoma* (sub)species exist should be reservedly accepted and that (ii) HAT suspected cases in non-endemic regions should be critically diagnosed and managed, mainly in immunosuppressed individuals. Misdiagnosis of non-HAT cases gives rise to the unnecessary administration of toxic HAT drugs and the subsequent discomfort and risks. Off course, in any doubt about the identity of the parasite the patient should be considered as HAT case and should receive appropriate treatment as soon as possible. In this context, a simple and rapid molecular tool to identify and distinguish the different *T. brucei* subspecies is most welcome.

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Table and figure legends

Table 1. Applied PCR protocols for parasite identification.

Figure 1. Microscopy on thin blood smear from the patient in 2003.

Magnification 100x.

Figure 2. Analysis of the archived blood slides with taxon specific PCRs.

Human β-globin PCR (a), *Trypanosomatidae* specific 18S PCR (b), *Trypanozoon* specific TBR PCR (c), *Trypanosoma* (*T.*) *evansi* specific RoTat 1.2. PCR (d), *Trypanosoma brucei* (*T.b.*) *gambiense* group 1 specific TgsGP PCR (e), *T.b. rhodesiense* specific SRA PCR (f) on DNA extracted from one thin blood smear from the child in 2003 (1), one thick drop blood slide from the child in 2005 (2) and one control thick drop blood slide from a healthy Belgian person (3). Positive control DNA (+) and negative control DNA (-) were included in every PCR.

Figure 3. UPGMA dendrogram of the microsatellite PCR results of the reference stocks and the patient03 DNA.