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Parasitological detection of *Trypanosoma brucei gambiense* in serologically negative sleeping-sickness suspects from north–western Uganda

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Forty-five parasitologically confirmed cases of sleeping sickness were diagnosed in north–western Uganda using a combination of two or three techniques. Forty of the cases were positive by the card agglutination test for trypanosomiasis (CATT), four were negative and one was not screened by the CATT. Trypanosomes isolated from the four CATT-negative but parasitologically positive cases were propagated for detailed biochemical genetic analysis. The aim was to demonstrate whether these four stocks lacked the LiTat 1.3 gene which encodes the antigen on which the CATT is based. All the DNA extracts isolated from these CATT-negative stocks and from six CATT-positive stocks of *Trypanosoma brucei gambiense* were targeted for amplification by the three variable-surface-glycoprotein genes thought to be ubiquitous in *T. b. gambiense*. The LiTat 1.3 gene was shown to be present in all 10 stocks. Trypanosome carriers may be CATT-negative because the CATT is not sensitive enough, because their parasites lack the LiTat 1.3 gene, or because their parasites have this gene but do not express it. The four sleeping-sickness cases who gave negative CATT results in the present study have very important implications in the diagnosis of *T. b. gambiense* infections using the CATT. Following treatment of the CATT-positive cases, the CATT-negative carriers of the trypanosomes remain as human reservoir hosts for continuous infection of the population. Because CATT-negative individuals are rarely examined further, the general prevalence of parasitologically positive but CATT-negative cases is unclear. This study demonstrates the value of co-ordinated use of serological and parasitological techniques in the diagnosis of Gambian sleeping sickness.

Epidemics of sleeping sickness caused by *Trypanosoma brucei gambiense* have occurred periodically in north–western Uganda since the beginning of this century. The most affected districts have been Moyo, Arua and Gulu. The return of Ugandan refugees from southern Sudan, where there is an epidemic of Gambian sleeping sickness, to northern Uganda has compounded the problem, leading to an increase in annual incidence of the disease.

Most cases of human trypanosomiasis in

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north–western Uganda are currently detected by screening local populations, using the card agglutination test for trypanosomiasis (CATT) on whole blood and plasma diluted 1:4 (Bailey and Smith, 1992). A subject is investigated parasitologically, by the microhaematocrit centrifugation technique, if the corresponding blood and plasma samples are both found positive by the CATT.

There has been some concern about apparently false-positive CATT results. *Médecins Sans Frontières* (MSF), working in Moyo and Adjumani districts, found that trypanosomes could not be demonstrated in approximately 25% of CATT-positive subjects (Bailey and Smith, 1992). Similarly, data from the National Sleeping Sickness Control Programme indicate that only 897 of the 1670 subjects found CATT-positive among 6910 examined in Moyo district in 1991 apparently had trypanosomes in their blood samples (unpubl. obs.). There has been relatively little interest in subjects who give false-negative CATT results; blood samples which were CATT-negative have not been examined for the presence of trypanosomes by parasitological methods.

The LiTat 1.3 gene, which encodes the antigen chosen for the CATT, was once thought to be expressed by all *T. b. gambiense* (Magnus *et al.*, 1984) but is now known to be absent from some isolates (Dukes *et al.*, 1992; Kanmogne *et al.*, 1996). It is unclear how many cases of sleeping sickness are going undetected in CATT-based surveys because the cases are infected with parasites that do not have or express LiTat 1.3. Such cases would remain sources of trypanosomes for subsequent epidemics. The objectives of the present study were to determine the prevalence of parasitologically positive but CATT-negative individuals among those subjected to routine, CATT-based diagnosis, and to determine the probable cause of the false-negative results.

SUBJECTS AND METHODS

Field Surveys

In May and November 1995, two sleeping-sickness surveys were carried out in Terego county of Arua district, in north–western Uganda. The local people were mobilized, by sleeping-sickness aides and local chiefs, and encouraged to assemble at selected screening centres for examination. Mass screening was done using the CATT (see below). Blood samples from both CATT-positive and CATT-negative subjects were checked for trypanosomes as wet smears (each of about 10 μl blood, examined for 40–50 fields at 400 $\times$) and by the haematocrit centrifugation technique (HCT; Woo, 1970). Blood samples from subjects who were negative by CATT, wet smear and HCT but still suspected to be sleeping-sickness cases on clinical grounds were checked by the miniature anion-exchange centrifugation technique (MAECT; Lumsden *et al.*, 1977). Furthermore, all enlarged cervical lymph glands were punctured and the juice from each gland examined under a microscope like the wet smears of blood. The strategy was to examine, by the two most readily available and less costly methods (wet smear and HCT), as many CATT-negative individuals as possible in order to demonstrate the presence of trypanosomes.

A blood sample from each of the parasitologically confirmed cases was inoculated into a *Mastomys natalensis* rat for isolation and propagation.

Card Agglutination Test for Trypanosomiasis (CATT)

A commercial CATT (Testryp-CATT; Smith-Kline-RIT, Antwerp; Magnus *et al.*, 1978) was used to test blood samples collected in heparinized capillary tubes, according to the manufacturer's instructions. One drop of well homogenized CATT reagent and one drop of blood were mixed on a test area on the card and spread to within 1 mm of the edge of the test area using a stirring rod. The card was then rotated for 5 min on a rotator before the test result was read.

Polymerase Chain Reaction (PCR)

The DNA from 10 stocks—four from CATT-negative subjects and six from CATT-

TABLE 1
Results of card agglutination tests for trypanosomiasis (CATT) in north-western Uganda

Date	No. and (%) of subjects:		No. of subjects found to be parasitologically positive		
	Screened by CATT	*Positive by CATT*	*CATT + ve*	*CATT − ve*	*CATT result not determined*
May 1995	710	218 (30.7)	21	4	1
November 1995	556	113 (20.3)	19	0	0
All	1266	331 (26.1)	40	4	1

positives—were amplified for variant-surface-glycoprotein (VSG) genes by PCR (Bromidge *et al.*, 1993) using AnTat 11.17, LiTat 1.3 and VSG 117 primer sets. Another two PCR primers (TBR1 and TBR2), both specific for *T. brucei* ssp. (Masiga, 1994), were used for amplification of genomic DNA from the 10 isolates. Each PCR amplification was carried out in 25 μl reaction mixture containing final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 200 μM each of dATP, dGTP, dCTP and dTTP, 0.1 μM of each of the 5′ and 3′ primers, 100 ng DNA template, and 1 unit of Taq DNA polymerase. The mixture was overlaid with one drop of mineral oil and then amplified in a Hybaid thermal cycler. Initial denaturation was either at 94°C for 3 min, followed by 30 cycles, each involving denaturation for 1 min at 94°C, annealing at 50°C for 1 min and extension at 74°C for 2 min (AnTat 11.17, LiTat 1.3 and VSG 117), or at 92°C for 3 min, followed by 30 cycles, each involving 30 s at 92°C, 60 s at 60°C and 30 s at 72°C (TBR1 and TBR2). Each DNA sample from the 10 isolates was amplified three times to check on the reproducibility of the PCR results. The absence of contaminants was routinely demonstrated by the inclusion of negative controls in which the DNA samples were replaced with sterile distilled water. The reaction products (15 μl/lane) were subjected to electrophoresis in 1.5% agarose containing 0.5 μg ethidium bromide/ml, and observed under ultra-violet trans-illumination.

RESULTS

Overall, 1266 subjects were screened using the CATT during the two surveys (Table 1). Of the 331 (26.1%) found CATT-positive, 281 appeared to be parasitologically negative. Trypanosomes were detected in blood samples and/or lymph-node aspirates from 44 of those screened by CATT and one other subject. Four of the 44 trypanosome-positives tested by the CATT were CATT-negative, and the rest were positive.

Trypanosome isolates from the four CATT-negative but parasitologically positive cases and six CATT-positive cases were analysed by PCR. The DNA extracts from all 10 of these isolates were amplified by the *Trypanosoma-brucei*-specific primer sets, TBR1 and TBR2. TBR1 and TBR2 were both found to be very sensitive, requiring quite low concentrations of DNA for the amplifications. Primer sets for three VSG genes (LiTat 1.3, AnTat 11.17 and VSG 117) were also used with the 10 isolates, to see which of these genes were present. The LiTat 1.3 gene, which encodes the antigen chosen for the CATT, was shown to be present in all 10 isolates, including the four which were negative in the CATT. However, in all replicates, AnTat 11.17 was not detected in two isolates (NW4 and NW15)

TABLE 2

Results of PCR-based testing of DNA from 10 trypanosome stocks isolated from humans in north-western Uganda

Stock	Result using primer set:*			
	TBR (1 & 2)	*LiTat 1.3 (A & B)*	*AnTat 11.17 (A & B)*	*VSG 117 (A & B)*
MHOM/UG/95/NW4	+	+	−	+
MHOM/UG/95/NW8	+	+	+	−
MHOM/UG/95/NW11	+	+	+	+
MHOM/UG/95/NW12	+	+	+	+
MHOM/UG/95/NW13	+	+	+	+
MHOM/UG/95/NW14	+	+	+	+
MHOM/UG/95/NW15	+	+	−	+
MHOM/UG/95/NW16	+	+	+	+
MHOM/UG/95/NW17	+	+	+	+
MHOM/UG/95/NW18	+	+	+	+

* Following electorophoresis, gels contained the amplified product (+) or no product of the expected size (−).

and VSG 117 was not detected in another (NW8) (Table 2).

DISCUSSION

The main reason why trypanosomes could not be demonstrated in 281 (85%) of the 331 CATT-positive suspects is probably that the parasitaemias were too low for the parasites to be detected in wet smears or even by HCT. Some of these CATT-positives may have been false-positives, the result of cross-reaction between the *T. b. gambiense* LiTat 1.3 antigens on the test cards and antibodies to closely related antigens that were not of trypanosome origin. It is likely that the numbers of CATT false-positives could have been reduced by testing plasmas at a dilution of 1:8 or 1:4, but this was not attempted because the main interest of the present study was in the CATT false-negatives

Although trypanosomes were only detected in four CATT-negative cases, these few cases have very important implications for the CATT-based diagnosis of *T. b. gambiense* infections and in the control of such infections. Trypanosome carriers are being missed by CATT-based screening and, following treatment of the CATT-positives, they remain as human reservoir hosts. The problem may be compounded by the nature of Gambian sleeping sickness, in which those infected may remain asymptomatic and engaged in normal activities for a long time while fly–man contact and transmission are maintained. The proportion of trypanosome-positive subjects found to be CATT-negative in February 1988 (two of 18; Dukes *et al.*, 1992) was comparable to that recorded in the present study (four of 45).

The DNA extracts isolated from all 10 *T. b. gambiense* stocks analysed were amplified by the TBR1 and TBR2 primers that are specific for the *T. brucei* subgroup (Moser *et al.*, 1989; Masiga, 1994), confirming that all the isolates belong to this subgroup. Use of the LiTat 1.3 primer set demonstrated the presence of the LiTat 1.3 gene in all 10 isolates, including those from the four CATT-negative isolates. It is possible that the LiTat 1.3 gene in parasites in some infections is being expressed too late in the infection to induce early production of polyclonal antibodies against the gene product. Alternatively, expression of some versions of the LiTat 1.3 gene may be hampered by their minichromosomal location. A lack of facilities prevented the presence of the LiTat 1.3 genes in the 10 *T. b. gambiense* isolates being confirmed by Southern analysis.

CATT-negative but parasitologically positive cases may therefore have at least two causes: (1) absence of the LiTat 1.3 gene (Dukes *et al.*, 1992; Kanmogne *et al.*, 1996); and (2) non-expression of the LiTat 1.3 gene (present study). It remains to be determined if non-expression is a short-lived phenomenon, such that infection may eventually be detectable by CATT. Subjects suspected of having sleeping sickness should always be subjected to parasitological examination, even when found CATT-negative. Since the present study, more CATT-negative but trypanosome-positive cases have been detected by MSF staff working at Adjumani hospital in north–western Uganda (unpubl. obs.).

The AnTat 11.17 gene is considered to be characteristic of Group-1 *T. b. gambiense*. Its absence from two of the present isolates, NW4 and NW15, shows the difficulty of distinguishing *T. b. gambiense* from other trypanosomes of the *T. brucei* group in Uganda. Although six of eight Ugandan isolates were found not to have this gene in an earlier investigation (Enyaru *et al.*, 1993), absence of the VSG 117 gene, seen in stock NW8 in the present study, has not been seen before in isolates from north–western Uganda. These results are further evidence of the micro-heterogeneic nature of *T. b. gambiense* in this area (Enyaru *et al.*, 1993). VSG 117 could not be detected in some stocks of *T. b. gambiense* Type II from West Africa, *T. b. rhodesiense* and *T. b. brucei* (Bromidge *et al.*, 1993). *Trypanosoma b. gambiense* is not a single, static entity (Bromidge *et al.*, 1993).

The variety of *T. b. gambiense* circulating in Uganda may also have some bearing on the epidemiology of the disease, such as the high rate of relapse following treatment with eflornithine (10%; Maiso *et al.*, 1995). There has recently been a sudden increase in the rate of relapse among patients treated with melarsoprol (MelB) in the Arua district, to about 20% (unpubl. obs.). This sudden increase may be the result of increasing prevalence of infection with resistant trypanosomes or low drug concentrations in the central nervous system.

In conclusion, it is relatively easy to detect trypanosomes in some CATT-negative subjects in north–western Uganda. The proportion of subjects who are falsely deemed non-infected during CATT-based screening, because of absence or non-expression of the LiTat 1.3 genes in their parasites or because of insufficient sensitivity in the CATT, is largely unknown because CATT-negative individuals are rarely examined further. Co-ordinated use of serological and parasitological techniques in the diagnosis of *T. b. gambiense* sleeping sickness is recommended.

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