

Short communication: **Towards saliva-based screening for sleeping sickness?**

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Summary

A pilot study was carried out on the detection of trypanosome-specific antibodies in saliva for diagnosis of sleeping sickness. All twenty-three saliva samples of parasitologically confirmed *Trypanosoma brucei gambiense* patients tested positive in an indirect enzyme-linked immunosorbent assay, whereas all 14 saliva samples of a negative control group remained negative. Trypanosome-specific antibody levels in patient saliva correlated with antibody levels in serum, but were about 250-fold lower. Eight of 23 undiluted saliva samples of sleeping sickness patients tested positive in CATT/*T. b. gambiense* and two of 23 in LATEX/*T. b. gambiense*. All fourteen saliva samples of the negative control group were also positive in CATT/*T. b. gambiense*, as were four of 14 in LATEX/*T. b. gambiense*. CATT and LATEX were thus inappropriate for antibody detection in saliva. These results indicate that trypanosome-specific antibody detection in saliva is possible. This could lead to the development of a simple, non-invasive, reliable saliva field test for diagnosis of sleeping sickness.

keywords diagnosis, sleeping sickness, *Trypanosoma brucei gambiense*, saliva, ELISA, CATT/*T. b. gambiense*, LATEX/*T. b. gambiense*

Introduction

Control of human African trypanosomiasis (HAT) or sleeping sickness relies on the detection and treatment of HAT cases, along with vector control. Diagnosis of HAT is obtained by the microscopic demonstration of trypanosomes, but parasitological techniques are poorly sensitive and laborious. Parasitological examination is therefore preceded by screening of the population at risk for presence of trypanosome-specific antibodies. The current test of choice is the card agglutination test for trypanosomiasis (CATT, Magnus *et al.* 1978), used by virtually all control programmes in *Trypanosoma brucei gambiense* endemic areas. Blood or serum sampling for CATT requires venipuncture or finger-prick, a relatively invasive procedure which is inconvenient for the blood donor and is also a risky procedure for the medical personnel.

Antibodies diffuse from blood into the oral fluid. Saliva collection is non-invasive and painless, thus saliva testing could present a simple and safe alternative for antibody detection in blood or serum. The principle has been applied for antibody detection in HIV, hepatitis, measles and rubella infection (Parry *et al.* 1987; Ettiègne-Traoré *et al.* 1998; Nokes *et al.* 2001), and in parasitic infections such as schistosomiasis and visceral leishmaniasis (Masum & Evans 1994; Garcia Santos *et al.* 2000). To our knowledge,

antibody detection in saliva has not yet been investigated for the diagnosis of HAT.

The objective of our study was to explore the presence of trypanosome-specific antibodies in saliva of *T. b. gambiense* patients by enzyme-linked immunosorbent assay (ELISA) and to examine if these antibodies are detectable by the CATT/*T. b. gambiense* or LATEX/*T. b. gambiense* agglutination tests, which can be performed under field conditions.

Materials and methods

Sample collection and processing

Paired saliva and serum samples from 23 *T. b. gambiense* patients originating from Mbongi (Bandundu Province, D.R. Congo) were collected during routine control activities of the mobile team of the National Sleeping Sickness Control Program (PNLTHA). The population of the village was screened for the presence of antibodies with CATT/*T. b. gambiense*. In CATT seropositives, parasites were searched for in lymph node aspirate, in wet blood film, in thick blood film, by microhaematocrit centrifugation or by mini anion exchange centrifugation. From parasitologically confirmed *T. b. gambiense* patients, oral consent for saliva and serum collection was obtained by the medical

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supervisor of the mobile team. Five millilitres of blood were collected by venipuncture and processed following standard protocols for serum preparation. Saliva was collected with the Salivette device (Sarstedt): patients were asked to keep the cotton plug for 5 min under the tongue and plain saliva was collected from the plug by centrifugation. Serum and saliva samples were transferred into 2-ml storage tubes and stored at -20°C before testing. No information was available on the disease stage of the patients, as samples were collected during control activities by the mobile team, before lumbar puncture, in the treatment centre.

Following the same protocol, negative control saliva was obtained from 14 ITM staff members and students without indication of present or past trypanosomiasis infection.

ELISA

Antibodies in serum and saliva were detected by the ELISA/*T. b. gambiense* protocol (Büscher *et al.* 1995), with some modifications. Briefly, microplates (Maxisorp, Nunc) were coated overnight at 4°C with $150\ \mu\text{l/well}$ of a mixture of purified variable surface glycoproteins of *T. b. gambiense* LiTat 1.3, LiTat 1.5 and LiTat 1.6 (Büscher *et al.* 1999) each at a concentration of $2\ \mu\text{g/ml}$, giving a total protein concentration of $6\ \mu\text{g/ml}$ in phosphate-buffered saline (PBS) ($0.01\ \text{M}$ phosphate, $0.14\ \text{M}$ NaCl, pH 7.4). Antigen-free control wells received $150\ \mu\text{l/well}$ of PBS. Plates were blocked for 1 h at ambient temperature with $350\ \mu\text{l/well}$ of PBS-Blotto ($0.01\ \text{M}$ phosphate, pH 7.15; $0.2\ \text{M}$ NaCl, 0.05% w/v NaN_3 ; 1% w/v skimmed milk powder Régilait). For testing, serum and saliva were diluted, respectively at 1:500 and 1:2 ratio in PBS-Blotto. Antigen-containing and antigen-free wells were filled with $150\ \mu\text{l}$ of serum or saliva dilution and incubated for 1 h at ambient temperature. Plates were rinsed briefly with $350\ \mu\text{l/well}$ of PBS-Tween ($0.01\ \text{M}$ phosphate, $0.14\ \text{M}$ NaCl, 0.05% v/v Tween 20, pH 7.4), and washed manually for $3 \times 5\ \text{min}$ with $350\ \mu\text{l/well}$ of PBS-Tween. Goat anti-human IgG (H + L) peroxidase (Jackson) was diluted at 1:10 000 in PBS-Tween and incubated for 60 min ($150\ \mu\text{l/well}$). After five washes, wells were incubated for 1 h at ambient temperature with $150\ \mu\text{l}$ ABTS [2,2'-azinobis(3-ethylbenzothiazoline)-6-sulphonic acid; Boehringer] substrate-chromogen solution. The latter was prepared from 50 mg ABTS dissolved in 100 ml of ABTS buffer (phosphate-citrate-sodium perborate solution, pH 4.6; Boehringer). The plate was shaken for 10 s and the optical density (OD) read at 415 nm (Multiskan RC Version 6.0; Labsystems). Corrected OD values were obtained by subtracting the OD of the antigen-free control well from the OD of the corresponding antigen-containing well.

Agglutination tests

Serum was titrated in CATT/*T. b. gambiense* and in LATEX/*T. b. gambiense* as described by the manufacturers (Magnus *et al.* 1978; Büscher *et al.* 1999). Saliva samples were tested undiluted, following the quantitative protocol using $25\ \mu\text{l}$ of saliva in CATT/*T. b. gambiense*, and following the cerebrospinal fluid protocol in LATEX/*T. b. gambiense* ($30\ \mu\text{l}$ of saliva + $15\ \mu\text{l}$ of reagent, 10 min of agitation).

Immunofluorescence

In a test-tube, $50\ \mu\text{l}$ of positive control (HAT serum with CATT end titre of 1:32, diluted 1:8 for IgG detection and 1:4 for IgM or IgA detection) and PBS or undiluted saliva (of the negative control group, CATT score ++) were added to two drops of CATT reagent, mixed and agitated gently for 5 min. The reagent was washed three times by centrifugation and resuspension of the pellet in $150\ \mu\text{l}$ of PBS buffer. After the last centrifugation, $150\ \mu\text{l}$ of sheep anti-IgG(Fab')₂-FITC (Bio-Rad), swine anti-IgM(Fc)-FITC (Nordic) or rabbit anti-IgA-FITC (Nordic) diluted, respectively at a ratio of 1:50, 1:50 and 1:20 in PBS were added and incubated for 30 min. The reagent was washed two times, resuspended in a final volume of $50\ \mu\text{l}$ and examined under a fluorescence microscope.

Desorption of test samples with PBS-Blotto

A twofold dilution series of one negative control saliva and two HAT sera was made in PBS-Blotto. Samples were incubated for 30 min at ambient temperature and centrifuged. CATT/*T. b. gambiense* reagent was reconstituted in PBS-Blotto or PBS, and CATT was performed, respectively on the supernatant of the desorbed sample or on similar twofold dilutions of the sample prepared in PBS.

Results**ELISA**

Corrected ODs obtained with saliva of the negative control group and of the patient group are shown in Figure 1. A cut-off was determined using the mean OD (-0.0237) + 3 standard deviations of the negative control group, corresponding to an OD value of 0.052. Applying this cut-off, the ELISA on saliva had 100% sensitivity and 100% specificity for trypanosome infection (95% confidence intervals of 85.2–100% and 76.8–100%, respectively). In HAT patients, a positive relationship between the ODs of saliva and serum was observed (Figure 2; $y = 1.26x - 0.365$, $r^2 = 0.439$).

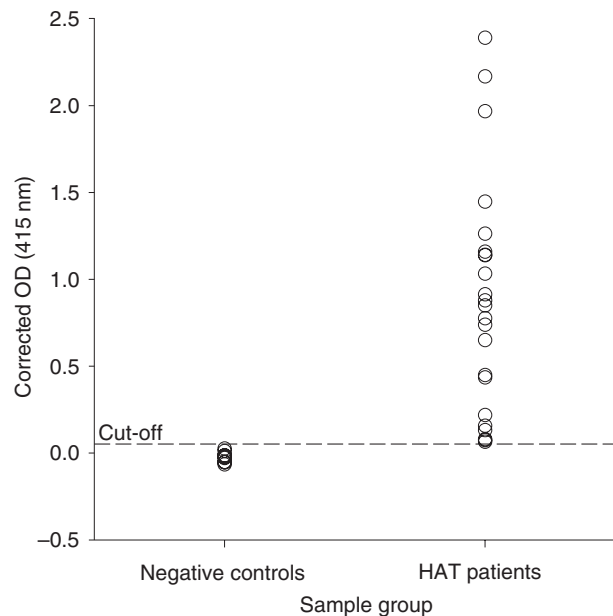
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Figure 1 Corrected optical densities (OD) obtained by an indirect enzyme-linked immunosorbent assay (ELISA) detecting trypanosome-specific antibodies in saliva samples from 14 negative controls and 23 human African trypanosomiasis patients. The cut-off was calculated based on the mean corrected OD + 3 standard deviations of the negative control group.

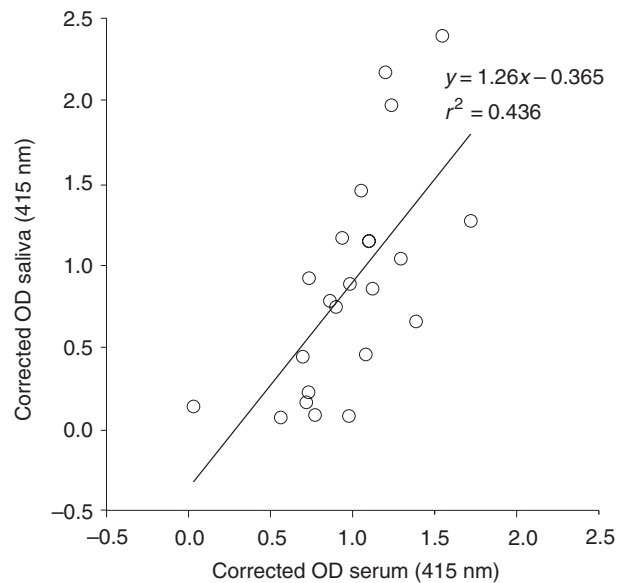


Figure 2 Corrected optical densities (OD) obtained by an indirect enzyme-linked immunosorbent assay (ELISA) detecting trypanosome-specific antibodies in saliva and serum samples from 23 human African trypanosomiasis patients (two patients had almost identical serum and saliva OD values). The linear regression line and its equation are indicated.

Agglutination tests

All HAT serum samples were positive in CATT/*T. b. gambiense* (end titres of 1:4–1:64; median 1:16). Of these, 22 of 23 samples were positive in LATEX/*T. b. gambiense* (end titres of 1:8–1:256; median 1:32). The LATEX negative serum sample (end titre <1:4) had a corrected OD in ELISA of 0.030.

Of 23 HAT patients' saliva samples, eight (35%) showed agglutination in the CATT/*T. b. gambiense* and two (9%) in LATEX/*T. b. gambiense*. However, all 14 saliva samples of the negative control group were positive in CATT and four were also positive in LATEX.

Immunofluorescence

In immunofluorescence, HAT serum reacted strongly positive for IgG, weakly positive for IgM and IgA. All PBS controls remained negative. Using an anti-IgG or anti-IgM conjugate, the undiluted saliva of a negative control remained negative. With the anti-IgA conjugate, a weak immunofluorescence on the CATT antigen was observed with the negative control saliva sample.

Desorption with PBS-Blotto

Desorption of negative control saliva reduced the end titre from 1:32 in PBS dilutions to 1:1 in PBS-Blotto (only agglutination with undiluted saliva). Desorption with PBS-Blotto had no effect on the end titres of the two HAT serum samples. End titres remained 1:32 for the first serum sample and 1:64 for the second serum sample.

Discussion

As a consequence of the selection of seropositives for parasitological examination in the field, all HAT serum samples were positive in CATT/*T. b. gambiense* thus had trypanosome-specific antibodies. Serum immunoglobulins and antibodies transudate via the gingival crevicular fluid into the oral fluid. Our pilot study demonstrates that trypanosome-specific antibodies of the IgG isotype are detectable in saliva of sleeping sickness patients by indirect ELISA. The antibody levels in saliva correlated to a certain degree with the serum levels but were about 250-fold lower.

To profit from the advantages of saliva testing, i.e. ease of collection, high acceptability and reduced biohazard, saliva collection should be combined with low-tech methods for antibody detection, applicable for mass screening activities on sleeping sickness in rural Africa. We therefore tried to detect trypanosome-specific antibodies with the

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CATT/*T. b. gambiense*, which is currently used for screening of the population on sleeping sickness, and LATEX/*T. b. gambiense*, an experimental latex agglutination test which has a slightly lower detection limit (Büscher *et al.* 1999). The observed lack of sensitivity of both tests is explained by the low antibody levels present in saliva. All negative control saliva samples caused agglutination of the CATT reagent. The same phenomenon was observed with some negative control saliva in the LATEX test. Based on our immunofluorescence results, a possible role for saliva secretory IgA cannot be excluded, either by direct binding or as a part of a complex, as in *Streptococcus mutans* and *Salmonella typhimurium* agglutination (Rundegren & Arnold 1987). The aspecific saliva agglutinating activity could be largely abolished with milk, similar to *S. mutans* agglutination (Mitoma *et al.* 2001). Dilution of the samples in PBS-Blotto or detection of IgG alone, may have by-passed these aspecific reactions of saliva with trypanosome components in ELISA. As CATT/*T. b. gambiense* or LATEX/*T. b. gambiense* are not appropriate for antibody detection in saliva because of aspecific agglutination, another field test capable to detect low antibody levels in saliva should be developed.

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