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AN EXPERIMENTAL LATEX AGGLUTINATION TEST FOR ANTIBODY DETECTION IN HUMAN AFRICAN TRYPANOSOMIASIS

by

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Summary — A latex card agglutination test for detection of antibodies in human African trypanosomiasis is presented. The latex was covalently coated with semipurified surface glycoprotein of Variable Antigen Type LiTat 1.6 of *Trypanosoma brucei gambiense*. Sera from 100 patients infected with *T.b. gambiense*, 26 patients infected with *T.b. rhodesiense* and 707 individuals without trypanosomiasis, including 132 malaria seropositives, have been tested. At serum dilution 1:16, sensitivity of the test was 91 % for the *T.b. gambiense* and 42.3 % for the *T.b. rhodesiense* group. Specificity was over 99 %. The reagent remained stable at ±6°C for at least 3 months. Reagent kept at 37°C for 3 months retained its sensitivity and showed a slight decrease in specificity.

KEYWORDS: Sleeping Sickness; *Trypanosoma brucei gambiense*; *Trypanosoma brucei rhodesiense*; Diagnosis; Variable Antigen; Latex; Indirect Agglutination

Introduction

Parasitological diagnosis of human African trypanosomiasis is often hampered by low parasitaemia, especially in case of infection with *T. brucei gambiense*. Antibody detection tests therefore are currently used in conjunction with or prior to parasitological examination.

Sera from infected individuals contain a broad spectrum of antibodies to variable and invariable trypanosome antigens (1,3,8,9). For detection of antibodies to *T.b. gambiense* several test systems using antigens or whole parasites of selected Variable Antigen Type (VAT) have been reported, including trypanolysis, direct agglutination, indirect haemagglutination, immunofluorescence and ELISA (4-6,10,11).

For field surveys rapid slide agglutination tests are particularly well suited. The only assay of this kind presently available is the CATT, a direct agglutination card test with stained trypanosomes of VAT LiTat 1.3. Reported here is a version of an indirect agglutination card test using latex particles covalently coated with semipurified variable antigen of LiTat 1.6, another wide-spread predominant VAT of *T.b. gambiense*, earlier referred to as VAT G16/6 (11).

Materials and methods

Reagents

Latex: polystyrene carboxylated Serva Unisphere 23, 0.8 μm diameter, 100 g/l suspension, Serva.

Coupling reagent: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), Sigma.

Antigen: lyophylized semipurified variable surface glycoprotein of *Trypanosoma brucei gambiense* VAT LiTat 1.6.

Agglutination cards: Wellcome disposable card, black spots.

DEAE cellulose, Whatman DE52 pre-swollen.

Filter paper, Whatman 4.

Glycerol, 11.62 mol/l, Merck.

Ethanolamine, Merck.

Bovine serum albumin (BSA), Janssen Chimica.

Tris-buffered saline (TBS), 0.1 mol/l, 0.09 mol/l NaCl, 0.0154 mol/l NaN_3, pH 8.0.

Carbonate buffer (CB), 0.05 mol/l, 0.0154 mol/l NaN_3, pH 9.0.

Phosphate buffer (PB I), 0.01 mol/l, pH 8.0.

Phosphate buffer (PB II), 0.02 mol/l, 0.0154 mol/l NaN_3, pH 7.4.

Phosphate buffered saline (PBS), 0.01 mol/l, 0.125 mol/l NaCl, 0.0154 mol/l NaN_3, pH 7.2.

Phosphate buffered saline glucose (PSG), 0.05 mol/l, 0.036 mol/l NaCl, 0.0833 mol/l glucose, pH 8.0.

Sera

Non-sleeping sickness sera were collected from persons presenting at the Institute of Tropical Medicine, Antwerp for various reasons. A first series of 287 sera had been stored at $-70°C$ in 60 μl volumes. 84 of these sera were found positive for antibodies to *Plasmodium falciparum* in an indirect immunofluorescence assay. A second series of 420 sera, including 48 *P. falciparum* seropositives, was tested after storage at $\pm 6°C$ for less than 5 days.

Sera of patients with parasitologically confirmed *T.b. gambiense* or *T.b. rhodesiense* infection were obtained from the WHO Serum Bank for Sleeping Sickness. Samples had been frozen at $-70°C$ or lyophilized and kept at $-20°C$. *T.b. gambiense* infection sera (100) originated from Congo, Gabon, Ivory Coast, Sudan and Zaire. *T.b. rhodesiense* infection sera (26) originated from Mozambique, Uganda and Zambia (table 1).

TABLE 1

Number, storage condition and country of the trypanosomiasis sera (CG = Congo, GA = Gabon, CI = Ivory Coast, SD = Sudan, ZR = Zaire, MZ = Mozambique, UG = Uganda, ZM = Zambia)

	T.b. gambiense					*T.b. rhodesiense*		
Storage condition	CG	GA	CI	SD	ZR	MZ	UG	ZM
Lyophilized, $-20°C$		5	27		6	8		8
Frozen, $-70°C$	25			20	17		10	

Wet and dry blood samples

Heparinized blood (ORh^-) was taken from a healthy European. Packed erythrocytes were added to 9 *T.b. gambiense* and 9 non-sleeping sickness sera so as to obtain reconstituted wet blood samples with haematocrit of either 20% and 40%. Ten μl volumes of each reconstituted blood sample were spotted onto 18 "Whatman 4" filter paper discs with a diameter of 5 mm, dried for 3 hours at 37°C and overnight at room temperature.

Preparation of semipurified variable surface glycoprotein

Rats were inoculated intraperitoneally with cloned *T.b. gambiense* VAT LiTat 1.6 (7). After 3 days trypanosomes were harvested from the blood by DEAE-cellulose chromatography (2). After 3 washings with cold PSG, the final centrifugation pellet was suspended in 6 volumes of PB I. The trypanosomes were disrupted by passing the frozen suspension 6 times through an X-press (AB BIOX, Sweden). The homogenized suspension was centrifuged (90 minutes, 45000g, 4°C). The supernatant was dialyzed against PB I and fractionated on a DEAE cellulose column equilibrated with the same buffer. The first protein fraction eluted was concentrated approximately 10 times by ultrafiltration (cut-off 10.000), diluted with PB I to a protein concentration of 1.5 g/l, aliquoted into penicilline vials in 1 ml volumes, freeze-dried and stored under nitrogen at ±6°C.

Preparation of latex reagent

Freeze-dried antigen was reconstituted with 1 ml H_2O per vial. Latex was diluted with TBS up to 6.25 g/l in an erlenmeyer flask. EDC 209 mmol/l freshly dissolved in H_2O, was added to a final concentration of 19 mmol/l. Immediately thereafter, antigen was added to a final concentration of 50 mg/l. The mixture was gently shaken at room temperature on a horizontal rotator. After 3 hours the reaction was quenched by addition of freshly prepared ethanolamine 0.25 mol/l in H_2O to a final concentration of 0.01 mol/l. Shaking was continued for one hour. Next, the latex was extensively washed over a polycarbonate membrane filter (Nucleopore, pore size 0.4 μm) in an Amicon ultrafiltration cell with a least 10 volumes of CB followed by 10 volumes of PB II containing 1 g/l BSA. The final reagent consisted of 6.6 g/l sensitized latex in PB II with 10 g/l BSA and 581 mmol/l glycerol. It was stored at ±6°C.

Two batches of reagent were independently prepared from the same antigen stock.

Agglutination assay

Twofold dilutions of serum and wet blood were prepared in PBS. Eluates of dry blood analogous to the dilutions of wet blood were obtained by eluting sets of two paper discs at room temperature for one hour with doubling volumes of PBS starting with 40 μl.

On each spot of an agglutination card 20 μl latex reagent and 20 μl freshly prepared test sample were mixed with a plastic rod and spread over an area

of ±1.5 cm in diameter. The card was rocked on a horizontal rotator with a displacement of 1.5 cm at 70 rpm for 5 minutes. The reaction was scored positive when macroscopic agglutination was visible.

Results

Sensitivity and specificity of the first batch

The following serum samples were tested: 62 *T.b. gambiense* infection sera, all 26 *T.b. rhodesiense* infection sera and all 287 non-sleeping sickness sera of the first series. Sera were tested at several dilutions, starting at 1:8, with latex reagent stored at ±6°C for less than one month.

TABLE 2
Distribution of serum end-titres with latex reagent of the first batch

End-titre	T.b.g.[a]	T.b.r.[b]	non-S.S.[c]
<1:8	4 (6.5%)	8 (30.8%)	283 (98.6%)
1:8	1 (1.6%)	7 (26.9%)	2 (0.7%)
1:16	0 (0.0%)	6 (23.1%)	1 (0.3%)
1:32	23 (37.1%)	4 (15.4%)	1 (0.3%)
1:128	29 (46.8%)	1 (3.8%)	0 (0.0%)
1:512	5 (8.1%)	0 (0.0%)	0 (0.0%)

[a] *T.b. gambiense* infection sera, n=62
[b] *T.b. rhodesiense* infection sera, n=26
[c] non-sleeping sickness sera, n=287

TABLE 3
Sensitivity and specificity of the test with latex reagent of the first batch

Serum dilution	Sensitivity		Specificity	
	T.b.g.[a]	T.b.r.[b]	non-S.S.[c]	mal-S.[d]
1:8	93.5%	69.2%	98.6%	95.2%
1:16	91.9%	42.3%	99.3%	97.6%
1:32	91.9%	19.2%	99.7%	98.8%

[a] *T.b. gambiense* infection sera, n=62
[b] *T.b. rhodesiense* infection sera, n=26
[c] non-sleeping sickness sera including 84 malaria seropositives, n=287
[d] non-spleeping sickness malaria seropositives, n=84

Results are shown in table 2. Four non-sleeping sickness sera, all *P. falciparum* seropositives, showed end-titres from 1:8 to 1:32, thus yielding an overall test specificity of 98.6% at dilution 1:8 (95% confidence interval: 97.2-100%). Of the *T.b. gambiense* infection sera 93.5% were reactive with end-titres ranging from 1:8 to 1:512 (95% confidence interval: 87.4-99.6%). Of the *T.b. rhodesiense* infection sera only 69.2% were reactive with end-titres from 1:8 to 1:512 (95% confidence interval: 51.5-86.9%). Sensitivity and specificity at different serum dilutions are given in table 3.

Stability of the first batch

All 62 *T.b. gambiense* infection sera and all 287 non-sleeping sickness sera mentioned in table 2 were retested at dilution 1:16 using latex reagents

stored at ±6°C and at 37°C for 3 months. With both reagents a 1.6% increase in sensitivity was noted in conjunction with a decrease in specificity of 0.3% at ±6°C and 2.4% at 37°C (table 4). According to the confidence intervals these differences are not significant.

TABLE 4

Sensitivity and specificity of the test at serum dilution 1;16 using latex reagent of the first batch at different storage conditions; 95% confidence interval in brackets

Storage	Sensitivity T.b.g.[a]	Specificity non-S.S.[b]
<1 month at ±6°C	92.0% (85.2-98.8%)	99.3% (98.3-100%)
3 months at ±6°C	93.6% (87.5-99.7%)	99.0% (97.8-100%)
3 months at 37°C	93.6% (87.5-99.7%)	96.9% (94.9-98.9%)

[a] *T.b. gambiense* infection sera, n=62
[b] non-sleeping sickness sera, n=287

Sensitivity and specificity of the second batch

Within 3 months after preparation and storage at ±6°C the second batch was tested with a total of 100 *T.b. gambiense* infection and 707 non-sleeping sickness sera including all samples previously used with the first batch.

TABLE 5

Distribution of serum end-titres with latex reagent of the second batch

End-titre	T.b.g.[a]	non-S.S.[b]
<1:8	6 (6%)	701 (99.2%)
1:8	4 (4%)	5 (0.7%)
1:16	17 (17%)	1 (0.1%)
1:32	37 (37%)	0 (0.0%)
1:128	33 (33%)	0 (0.0%)
1:512	3 (3%)	0 (0.0%)

[a] *T.b. gambiense* infection sera, n=100
[b] non-sleeping sickness sera, n=707

TABLE 6

Sensitivity and specificity of the test with latex reagent of the second batch

Serum dilution	Sensitivity T.b.g.[a]	Specificity non-S.S.[b]	Specificity mal-S.[c]
1:8	94.0%	99.2%	96.2%
1:16	90.0%	99.9%	99.2%
1:32	73.0%	100.0%	100.0%

[a] *T.b. gambiense* infection sera, n=100
[b] non-sleeping sickness sera including 132 malaria seropositives, n=707
[c] non-sleeping sickness malaria seropositives, n=132

Six non-sleeping sickness samples of which 5 *P. falciparum* seropositives showed end-titres from 1:8 to 1:16, thus yielding an overall test specificity of 99.2% at dilution 1:8 (95% confidence interval: 98.5-99.9%). Of the *T.b. gambiense* infection sera 94.0% were reactive with end-titres ranging from 1:8 to 1:512 (95% confidence interval: 89.3-98.7%) (table 5). Sensitivity and specificity at different serum dilutions are given in table 6.

Results obtained with blood samples

The 18 serum samples and the corresponding wet and dried blood preparations were tested in parallel with reagent of the second batch that had been stored at ±6°C for 6 months. The distribution of end-titres is given in table 7. The end-titres of linked serum, wet and dry blood samples never differed more than two dilutions. As compared with serum, 20 % haematocrit wet blood showed essentially the same end-titres, whereas some of the 40 % haematocrit blood samples scored one dilution lower. Most dry blood eluates scored one or two dilutions lower than serum.

TABLE 7

End-titre distribution of serum and corresponding wet and dry blood samples obtained with reagent of the second batch. Reconstituted blood samples were prepared by adding packed ORh⁻ red blood cells to serum

	T.b.g.[a]					non-S.S.[b]				
	serum	wet blood		dry blood		serum	wet blood		dry blood	
dilution		20[c]	40[c]	20[c]	40[c]		20[c]	40[c]	20[c]	40[c]
<1:2	0	0	0	0	0	8	8	9	8	8
1:2	0	0	0	0	0	0	1		0	1
1:4	0	0	0	2	2	1			1	
1:8	0	0	2	0	2					
1:16	4	3	1	3	2					
1:32	2	2	3	1	0					
1:64	0	1	0	0	0					
1:128	0	0	0	2	3					
1:256	2	1	2	1						
1:512	1	2	1							

[a] *T.b. gambiense* infection sera, n=9
[b] non-sleeping sickness sera, n=9
[c] haematocrit (%)

Discussion

Recent progress in technology has led to increasing use of latex particles for serodiagnostic purposes. Different types of latex and coupling procedures are available for preparation of antigen coated reagents. However, the sensitivity and specificity of the resulting antibody detection test still primarily depends on the nature of the antigen. In this respect African trypanosomes with their multitude of invariable and variable antigens pose a particular problem.

Variable surface glycoprotein LiTat 1.6 has been chosen for the present reagent. It is easily obtainable in a nearly pure form and gives excellent results in ELISA with *T.b. gambiense* infection sera (11, unpublished data).

The specificity of the test version looks promising even with sera of patients with malaria, one of the most common infections that often cause cross-reactivity. Sensitivity proved satisfactory with the *T.b. gambiense* but not with the *T.b. rhodesiense* infection sera. Improved reactivity for either infection might be obtained with other parasite derived or recombinant antigens.

The test is inexpensive, easy to perform and apparently applicable to wet and dry blood samples. Providing improved stability of the reagent at ambient temperatures, developing a test kit for field evaluation would be relatively easy.

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Un test expérimental d'agglutination au latex pour la détection d'anticorps dans la trypanosomiase humaine africaine.

Résumé. — Un test d'agglutination au latex sur carte pour la détection d'anticorps dans la trypanosomiase humaine africaine est présenté. La glycoprotéine de surface semi-purifiée du Type Antigénique Variable LiTat 1.6 de *Trypanosoma brucei gambiense* a été fixée au latex de manière covalente. Les sérums de 100 patients infectés par *T.b. gambiense*, 26 patients infectés par *T.b. rhodesiense* et 707 non-trypanosomés, dont 132 séropositifs pour la malaria, ont été testés. A la dilution 1:16 la sensibilité du test est 91 % pour le groupe *T.b. gambiense* et 42,2 % pour le groupe *T.b. rhodesiense*. La spécificité est plus de 99 %. Le réactif reste stable à ±6°C pendant au moins trois mois. Après trois mois à 37°C la sensibilité n'avait pas changé tandis que la spécificité avait légèrement baissé.

Een experimentele latex agglutinatietest voor antistofopsporing bij menselijke Afrikaanse trypanosomiase.

Samenvatting. — Een latex kaart agglutinatietest voor antistofopsporing bij menselijke Afrikaanse trypanosomiase wordt voorgesteld. Halfgezuiverd oppervlakte glycoproteïne van Variabel Antigeen Type LiTat 1.6 van *Trypanosoma brucei gambiense* werd covalent gebonden aan latex. Sera van 100 patienten met *T.b. gambiense* infectie, van 26 patienten met *T.b. rhodesiense* infectie en van 707 personen zonder trypanosomiase, waaronder 132 malaria seropositieven, werden getest. Bij serumverdunning 1:16 was de gevoeligheid van de test 91 % voor de *T.b. gambiense* groep en 42,3 % voor de *T.b. rhodesiense* groep. De specificiteit was hoger dan 99 %. Het reagens bleef minstens drie maanden stabiel bij ±6°C. Na drie maanden bewaring bij 37°C was de gevoeligheid behouden en was de specificiteit licht gedaald.

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