Dot Enzyme-Linked Immunosorbent Assay for More Reliable Staging of Patients with Human African Trypanosomiasis

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Human African trypanosomiasis (HAT) or sleeping sickness is a disease characterized by a hemolymphatic stage 1 followed by a meningoencephalitic stage 2 which is fatal without specific treatment. Furthermore, due to the toxicity of drugs used to treat stage 2 (mainly melarsoprol) accurate staging is required. Actual criteria employed during field surveys are not sensitive enough for precise staging. Antineurofilament (anti-NF) and antigalactocerebrosides (anti-GalC) antibodies have been identified in cerebrospinal fluid (CSF) as potential markers of central nervous system (CNS) involvement. We describe a dot enzyme-linked immunosorbent assay (dot-ELISA) to detect anti-GalC and anti-NF antibodies and its value in staging. NF- and GalC-dotted nitrocellulose strips were first developed in our laboratory. They were then evaluated in Angola and Central African Republic on 140 CSF samples. Compared to our staging criteria (i.e., CSF cell count ≥ 20 cells/ μ l, CSF immunoglobulin M concentration ≥ 100 mg/liter, and/or the presence of trypanosomes in the CSF), combined detection of both CSF anti-NF and CSF anti-GalC by dot-ELISA showed 83.2% sensitivity and 100.0% specificity. Dot-ELISA could be a useful test to diagnose CNS involvement in HAT in the less-equipped laboratories or in the field situation and to improve patient treatment.

Human African trypanosomiasis (HAT) or sleeping sickness is caused by the hemoflagellate *Trypanosoma brucei gambiense* or *T. brucei rhodesiense*, both transmitted by tsetse fly bite. The early stage, or stage 1, corresponding to the development of the parasite in blood and lymph is followed, in the absence of treatment, by a late stage, or stage 2, which is characterized by central nervous system (CNS) involvement ranging from meningitis to meningoencephalitis and ending in death without treatment (10). Field diagnosis of HAT requires screening, diagnostic confirmation, and staging. Biological methods for diagnosis of HAT have recently been reviewed (8). The card agglutination test for trypanosomiasis (CATT) is currently used for mass screening (22). When the result is positive, the CATT test must be followed by testing for detection of parasites in blood or gland juice. If they are present, lumbar puncture is necessary for staging by cerebrospinal fluid (CSF) examination to detect trypanosomes, which is the only and obvious criteria of CNS involvement, corresponding to stage 2 of the disease. However, parasite detection depends mainly on the CSF volume obtained and the parasitological method used (18, 24, 25). Trypanosomes are not always detected, and false negatives can occur, especially in the early late stage, when

clinical signs are absent. For this reason the World Health Organization recommends complementary criteria for stage 2 diagnosis which are based on CSF cell counts (>5 cells/ μ l) or increased CSF protein levels (>450 mg/liter, depending on the techniques used) (7, 29). These biological parameters may be determined during field surveys, although their sensitivity and specificity are low, especially in early-late-stage cases. Other more sensitive CSF criteria are now available for stage 2 determination: total immunoglobulin M (IgM) levels (23) and blood-CSF barrier dysfunction and IgM intrathecal synthesis (4, 16, 19). However, they require complicated techniques that cannot be used during field surveys, with the exception of a recent latex agglutination test for detection of CSF IgM (12, 17). Biological diagnosis of CNS involvement in HAT is therefore of importance, because administration of highly toxic melarsoprol to patients with doubtful stage 2 diagnosis is difficult to accept since 5 to 10% lethality can be attributed to the drug itself. As there is no new drug for stage 2, except difluoromethylornithine (5), only the development of new specific biological tools for HAT staging may contribute to limit the use of melarsoprol to proven stage 2 cases.

During the course of the disease, dramatic B-cell activation occurs in patients and autoantibodies are commonly detected (2, 15, 21). We have previously demonstrated by an enzyme-linked immunosorbent assay (ELISA) that some antibodies recognized neurofilament (NF) proteins of neurons and galactocerebrosides (GalC) of myelin and exhibited cross-reactivities with invariant membrane antigens of *T. brucei gambiense*

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FIG. 1. Dot-ELISA for detection of CSF anti-GalC and anti-NF antibodies in patients with HAT. Each nitrocellulose strip is divided into two parts, with a dot of GalC in the upper part and a dot of NF in the lower part. After incubation with a CSF sample and then with rabbit (Fab')₂ peroxidase-labeled anti-human IgM, addition of 4-chloro-1-naphtol and hydrogen peroxide gives a more or less intense blue color when the test is positive. Absence of anti-GalC and absence of anti-NF give a colorless reaction (negative control). Presence of anti-NF gives a more intense color than presence of anti-GalC (positive control). Here, three CSF samples from patients with HAT are shown as examples: S1 is not reactive for anti-GalC and anti-NF (dots are colorless); Sint and S2* are reactive for both anti GalC and anti-NF (dots are colored in blue).

and *T. brucei rhodesiense* that might be induced by molecular mimicry (1, 3, 11, 14). Their detection in CSF was associated with stage 2 of the disease (3, 4, 11). However, ELISA is not adapted for use during field surveys.

We describe here a new, field-adapted dot-ELISA technique to detect anti-NF and anti-GalC antibodies in CSF. First, test procedures were evaluated in the laboratory. Second, the dot-ELISA method was evaluated during field surveys. We report results and discuss the value of this new test for HAT staging.

MATERIALS AND METHODS

(i) **Patients and CSF samples.** First, the dot-ELISA method was evaluated in the laboratory using stored CSF from patients identified in Angolan hospital settings. Control CSF samples were obtained from Caucasian patients hospitalized in the neurology department at the university hospital in Limoges for suspicion of meningitis.

Second, two field surveys were designed to evaluate this new dot-ELISA method, in the Central African Republic (CAR; Batangafo focus, 2001) and in Angola (Bengo focus, 2002). They were directed by "Programme National de Lutte contre la Trypanosomose Humaine Africaine (PNLTHA)" in CAR and "Instituto de Combate e Controlo das Tripanossomias (ICCT)" in Angola. All patients were included in the study after informed consent, screened by CATT, and confirmed by trypanosome detection in blood. Then, examination of CSF sampled by lumbar puncture was done for staging based on cell count and/or the presence of trypanosomes according to specific national procedures (directly using a counting chamber [PNLTHA] or double centrifugation [ICCT]). The remaining CSF samples were collected and stored at -20°C for further analysis. For ethical considerations, no CSF controls were sampled in the population of areas of endemicity, as no lumbar puncture could be justified.

(ii) **Detection of anti-NF and anti-GalC antibodies in CSF.** (a) **Dot-ELISA technique.** The dot-ELISA is based on the principle that when CSF is applied to the nitrocellulose strip spotted with the antigen, i.e., NF or GalC, the specific antibodies, when present in the CSF, bind to the antigen dot. This binding reaction is detected visually by addition of an enzyme-labeled second antibody.

NF from bovine spinal cords (Sigma, Saint Louis, MO) and a GalC mixture from bovine brain containing GalC type I and GalC type II in equal proportion (Sigma) were spotted separately onto a nitrocellulose strip (Millipore, Saint Quentin en Yvelines, France) (14). Briefly, 10 μg GalC was diluted in 10 μl of an isopropanol and water mixture (2:1 [vol/vol]) and then spotted manually on nitrocellulose strips (1 by 2 cm in size) previously conditioned in the same aqueous solution. After washing with cold phosphate-buffered saline (PBS) (pH 7.2)–0.05% (vol/vol) Tween 20 (Sigma), 3 μg of NF in 5 μl of PBS was spotted on the same nitrocellulose strips with a dot-blotter (Schleicher and Schuell, Aubervilliers, France). One strip conditioned with both GalC and NF served for the detection of anti-NF and anti-GalC antibodies in one CSF sample.

Then, all steps of the test procedure were performed at ambient temperature. After 1 h of saturation with PBS–10% (vol/vol) horse serum (Gibco, Paisley, United Kingdom), the blotted strips were incubated with CSF diluted 1:10 in

PBS for 3 h. After three washes in PBS–0.05% Tween 20, strips were further incubated for 1 h with rabbit (Fab')₂ peroxidase-labeled anti-human IgM diluted 1:100 in PBS (Dako, Glostrup, Denmark). After three washes in PBS–0.05% Tween 20, the reaction was revealed by addition of 10 mg 4-chloro-1-naphtol–5 ml ethanol–45 ml PBS–50 μl 30% H_2O_2 (Sigma) for 10 min. The reaction was stopped with distilled water, and the results were read immediately. The development of a deep-reddish-blue-colored dot was considered to show reactivity, whereas a colorless dot was considered to show nonreactivity (Fig. 1). Each CSF sample was studied in duplicate on two separate nitrocellulose strips. The entire reaction took approximately 4 h.

Mouse monoclonal anti-NF 160 (clone NN18; Sigma), mouse monoclonal anti-GalC (Sigma), and horseradish peroxidase-labeled anti-mouse Ig (Dako) were also used for positive and negative controls.

(b) **ELISA technique.** For anti-NF antibody detection, Maxisorb Nunc 96-well plates (Nunc) were coated with 250 ng of NF (Sigma) in 100 μl of PBS per well and incubated overnight at $+4^{\circ}\text{C}$. Wells were washed once with PBS and then saturated with 200 μl PBS–bovine serum albumin (BSA; Sigma) (3% [wt/vol]) at 37°C for 1 h. Wells were washed again one time with PBS, and 100 μl of diluted CSF (1:50 in PBS–3% [wt/vol] BSA) was added. After incubation overnight at $+4^{\circ}\text{C}$, wells were washed six times with PBS–0.05% Tween 20. Wells were incubated for 1 h with rabbit (Fab')₂ peroxidase-labeled anti-human IgM diluted 1:2,000 in PBS–1.5% (wt/vol) BSA (Dako). After six washes with PBS–0.05% Tween 20, the reaction was revealed with 8 ml of water–1 ml 0.4% orthophenylene diamine (Sigma)–1 ml 0.1 M citrate buffer (Sigma)–100 μl 30% H_2O_2 and stopped with 30 μl 8 N H_2SO_4 . The optical density at 492 nm (OD_{492}) was read.

For anti-GalC antibody detection, Immunosorb 96-well plates (Nunc) were coated with 1 μg GalC (Sigma) in 50 μl of a mixture of ethanol and methanol (1:1 [vol/vol]) per well. Procedures were similar to those described for anti-NF antibody detection, except PBS washes were done without Tween 20.

Each CSF sample was analyzed in duplicate for the presence of anti-NF or anti-GalC antibodies.

(iii) **CSF IgM concentration.** IgM concentrations were determined by nephelometry (Behring Diagnostic, Rueil-Malmaison, France). The threshold for the IgM concentration was 2.4 mg/liter, as recommended by the manufacturer. However, the presence of IgM in the CSF of patients was considered pathological for concentrations ≥ 100 mg/liter.

(iv) **Staging procedure.** (a) **Staging during field surveys.** In the field surveys conducted in CAR and in Angola, stage determination was based on CSF cell counts or the presence of the parasite in CSF. A patient was in stage 1 (S1) when a CSF cell count ≤ 5 cells/ μl was determined or no trypanosomes were detected. A patient was in S2 when a CSF cell count > 5 cells/ μl was determined or the presence of trypanosomes was detected.

(b) **Revised staging in the laboratory.** As already reported in the literature (16), we decided to use more-accurate staging in order to improve specificity and sensitivity of anti-NF and anti-GalC antibody detection. A patient with a CSF cell count ≤ 5 cells/ μl , no trypanosomes, and IgM < 100 mg/liter was in S1. A patient with a CSF cell count of 6 to 20 cells/ μl , no trypanosomes, and IgM < 100 mg/liter was in an undetermined stage called the intermediate stage (Sint). A patient with a CSF cell count > 20 cells/ μl , or trypanosomes, or IgM ≥ 100 mg/liter was in a new S2 (S2*).

TABLE 1. Classification of patients and controls and CSF reactivity on ELISA and dot-ELISA^a

Population	HAT stage	Total no. of samples	No. of T + sample	No. of IgM positive samples	No. of samples giving positive results for:					
					Anti-NF		Anti-GalC		Anti-NF + anti-GalC	
					Dot-ELISA	ELISA	Dot-ELISA	ELISA	Dot-ELISA	ELISA
Laboratory (<i>n</i> = 42)	S1	4	0	0	0	0	0	0	0	0
	Sint	3	0	0	1	0	1	0	1	0
	S2*	35	29	35	28	22	28	28	28	22
Survey (<i>n</i> = 140)	S1	43	0	0	1	ND	1	ND	1	ND
	Sint	65	0	0	43	ND	32	ND	32	ND
	S2*	32	7	32	32	ND	25	ND	25	ND
Control		13	0	0	0	ND	0	ND	0	ND

^a Classification is shown according to (i) the stage of HAT (S1, CSF patients with cell count ≤ 5 cells/ μ l and no trypanosomes and IgM concentration < 100 mg/liter; Sint, CSF patients with cell count between 6 to 20 cells/ μ l and IgM concentration < 100 mg/liter and no trypanosomes; S2*, CSF patients with cell count > 20 cells/ μ l or IgM concentration ≥ 100 mg/liter or presence of trypanosomes), (ii) the presence of trypanosomes in the CSF (T+), (iii) a CSF IgM concentration ≥ 100 mg/liter (IgM), and (iv) the presence of CSF anti-NF alone, CSF anti-GalC alone, and both CSF anti-NF and CSF anti-GalC antibodies detected by ELISA or dot-ELISA. A total of 42 CSF samples from Angola were studied in laboratory conditions, and 140 CSF samples were studied in the field during surveys conducted in Angola and Central African Republic. CSF controls (*n* = 13) were from Caucasian patients hospitalised for meningitis. ND, not done.

(v) **Statistical analysis.** Statistical analysis was performed using Spearman correlation to compare populations studied in the laboratory to populations studied in field surveys ($P < 0.05$ was considered significant).

Epidemiological studies and determination of sensitivity and specificity were carried out using EpiInfo 6.0 software (Centers for Disease Control and Prevention, Atlanta, Ga.).

RESULTS

(i) **Laboratory results. (a) Patients and CSF samples.** A total of 42 CSF samples from HAT patients from Angola (10 to 60 years of age [mean, 31 ± 13 years]; sex ratio, 1.6 [male/female]) were used to establish dot-ELISA procedures in laboratory conditions. They were also used for ELISA in order to compare the two techniques involved in the detection of anti-NF and anti-GalC antibodies. Four patients were classified in S1 and 38 in S2. In S2, 29 (76.3%) patients had trypanosomes in the CSF and 35 (92.1%) had IgM ≥ 100 mg/liter. According to CSF cell counts and our revised staging criteria, 35 out of the 38 S2 patients were in S2* and 3 in Sint (Table 1). In this population, CSF cell counts and IgM concentrations were statistically correlated ($r = 0.099$; $P = 0.013$) (Fig. 2).

Thirteen CSF samples from Caucasian controls (18 to 78 years of age [mean, 50 ± 18 years]) suspected for meningitis were also included. All of them were CATT negative and showed CSF cell counts < 5 cells/ μ l and IgM < 100 mg/liter.

(b) **ELISA technique.** The threshold for positivity was determined for both anti-NF and anti-GalC as the mean OD₄₉₂ plus 3 standard deviations using four CSFs from S1 patients (OD₄₉₂ = 0.077 for anti-NF; OD₄₉₂ = 0.096 for anti-GalC).

A total of 22 (57.9%) CSF S2 (Sint plus S2*) samples were anti-NF positive, and 28 (73.7%) were anti-GalC positive. A total of 22 (62.9%) CSF S2* samples were positive for anti-NF, and 28 (80.0%) were positive for anti-GalC. All CSF S1 and all CSF Sint samples were negative for both antibodies (Table 1).

Sensitivities and specificities of ELISA for different situations are reported in Table 2. For anti-NF, ELISA sensitivities were 57.9% for S2, 71.0% for S2*, 93.8% when trypanosomes were present, and 47.2% when IgM ≥ 100 mg/liter. For anti-GalC, ELISA sensitivities were 68.4%, 74.3%, 66.7%, and 69.4%, respectively. For both anti-NF and anti-GalC, ELISA

sensitivities were 67.6%, 80.1%, 93.8%, and 71.4%, respectively. For anti-NF, ELISA specificities were 100.0% both for S2 and S2*, 46.2% when trypanosomes were present, and 100.0% when IgM ≥ 100 mg/liter. For anti-GalC, ELISA specificities were 50.0%, 71.4%, 30.8%, and 50.0%, respectively. For both anti-NF and anti-GalC, ELISA specificities were 100.0%, 90.0%, 85.3%, and 93.4%, respectively.

(c) **Dot-ELISA technique.** A total of (76.3%) CSF S2 (Sint plus S2*) samples were anti-NF and anti-GalC positive. One of them was classified as Sint by use of laboratory criteria and was also positive for both anti-NF and anti-GalC (Table 1).

Reactivity of dot-ELISA compared to CSF cell count and IgM concentration is shown in Fig. 2. Sensitivities and specificities of dot-ELISA according to the different situations are in Table 2. For anti-NF, dot-ELISA sensitivities were 100.0% for S2, 94.7% for S2*, 96.6% when trypanosomes were present, and 100.0% when IgM ≥ 100 mg/liter. For anti-GalC, dot-ELISA sensitivities were 82.9%, 75.7%, 76.7%, and 83.3%, respectively. For both anti-NF and anti-GalC dot-ELISA sensitivities were 80.6%, 78.9%, 77.5% and 85.6%, respectively. For anti-NF, dot-ELISA specificities were 85.7% for S2, 100.0% for S2*, 46.2% when trypanosomes were present, and 41.7% when IgM ≥ 100 mg/liter. For anti-GalC, dot-ELISA specificities were 85.7%, 100.0%, 50.0%, and 69.2%, respectively. For both anti-NF and anti-GalC, dot-ELISA specificities were 100.0%, 95.8%, 98.8%, and 100.0%, respectively.

As shown in Fig. 3, dot-ELISA had better reactivity than ELISA for CSF from patients in Sint or S2* stages.

All CSF controls were negative for both anti-NF and anti-GalC antibodies (data not shown).

(ii) **Field surveys. (a) Patients and CSF samples.** During field surveys, the numbers of individuals screened for HAT were 1,246 in Angola and 6,734 in CAR. In Angola, 21 patients (1.68%) (4 to 53 years of age [mean, 25 ± 15 years]; sex ratio, 1.6 [male/female]) were parasitologically confirmed in the field. Four patients were in S1 and 17 in S2 (trypanosomes were found in the CSF of 5 patients), out of which 6 were in Sint and 11 in S2*. In CAR, 119 patients (1.77%) (3 to 70 years of age [mean, 22 ± 15 years]; sex ratio, 0.93 [male/female])

FIG. 2. Dot-ELISA detection of anti-GalC and anti-NF antibodies in CSF of 42 patients with HAT from Angola (laboratory study) and 140 patients from Angola and CAR (field study): comparison of dot-ELISA and CSF cell count and CSF IgM concentration (CSF anti-NF, a and b; CSF anti-GalC, c and d). ■, dot-ELISA positive; □, dot-ELISA negative.

were parasitologically confirmed in the field. Thirty-nine patients were in S1, and 80 patients were in S2 (trypanosomes were found in the CSF of 2 patients), out of which 59 were in Sint and 21 in S2* (Table 1). In Angola and in CAR, trypanosomes were found in the CSF only when the cell count was >20 cells/ μ L and IgM concentration was >100 mg/liter. In this population, CSF cell count and IgM concentration were statistically correlated ($r = 0.373$; $P = 0.0001$) (Fig. 2).

(b) Dot-ELISA technique. Anti-NF and anti-GalC antibody detection was done by dot-ELISA during field surveys (Table 1). Dot-ELISA examination of CSF showed that 42 (97.7%) of S1 patients were negative for both anti-NF and anti-GalC. However, one S1 CSF was positive for both anti-NF and anti-GalC. Forty-three (66.1%) Sint CSF were anti-NF positive, and 32 (49.2%) CSF were anti-GalC positive. In the group of 32 S2* patients, dot-ELISA showed that 32 (100.0%) were positive for anti-NF and 25 (78.1%) were positive for anti-GalC and for both anti-NF and anti-GalC. Among these 32 CSF, 32 (100.0%) showed IgM concentrations ≥ 100 mg/liter, and 7 (21.9%) showed the presence of trypanosomes.

Reactivity of dot-ELISA compared to CSF cell count and IgM concentration is shown in Fig. 2. Sensitivities and specificities of dot-ELISA for different situations are reported in Table 2. For anti-NF, dot-ELISA sensitivities were 74.4% for S2, 100.0% for S2*, 85.7% when trypanosomes were present, and 30.8% when IgM ≥ 100 mg/liter. For anti-GalC, dot-ELISA sensitivities were 58.1%, 81.0%, 81.3%, and 56.7%, respectively. For both anti-NF and anti-GalC, dot-ELISA sensitivities were 60.0%, 83.2%, 85.6%, and 70.7%, respectively.

For anti-NF, dot-ELISA specificities were 97.2% for S2, 81.5% for S2*, 50.9% when trypanosomes were present, and 69.4% when IgM ≥ 100 mg/liter. For anti-GalC, dot-ELISA specificities were 96.3%, 93.3%, 100.0%, and 73.2%, respectively. For both anti-NF and anti-GalC, dot-ELISA specificities were 76.4%, 100.0%, 92.3%, and 50.3%, respectively.

DISCUSSION

The need for precise staging to treat HAT patients with adapted drugs is well recognized (13). Staging by CSF cell count or the presence of trypanosomes in the CSF is not sensitive enough. We have developed two different methods for staging: ELISA and dot-ELISA to detect anti-NF and anti-GalC antibodies in CSF. We compared sensitivities and specificities of both tests in the laboratory for 42 CSF samples and then for 140 CSF samples in field conditions. CSF cell counts and IgM concentrations showed a similar evolution from S1 to S2* in both conditions ($P < 0.05$).

Dot-ELISA showed a better degree of sensitivity and specificity than ELISA in laboratory conditions. These data conformed to other studies that assessed that dot blot assays are most sensitive and specific for the detection of low titers of antibodies directed against bacterial lipopolysaccharide (28) or viral antigens (20). The discrepancy between these two methods is related to the high nonspecific binding of samples in ELISA plates. The replacement of the plastic binding matrix by a nitrocellulose membrane essentially solved this problem (20, 28). In addition, ELISA had the disadvantage of being a

TABLE 2. Sensitivity and specificity of ELISA and dot-ELISA detection of anti-NF and CSF anti-GalC antibodies in CSF of HAT patients

Population	Method	CSF criterion	Result for:											
			Anti-NF				Anti-GalC				Anti-NF + CSF anti-GalC			
			Sensitivity		Specificity		Sensitivity		Specificity		Sensitivity		Specificity	
			%	95% CI ^d	%	95% CI	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Laboratory (n = 42)	ELISA ^a	S2	57.9	40.9–73.3	100.0	39.6–100.0	68.4	51.2–82.0	50.0	9.2–90.8	67.6	49.4–82.0	100.0	19.8–100.0
		S2*	71.0	51.8–85.1	100.0	56.1–100.0	74.3	56.4–94.9	71.4	30.3–94.9	80.1	69.2–95.8	90.0	29.9–98.9
		T+	93.8	67.7–99.7	46.2	27.1–66.3	66.7	47.1–82.1	30.8	10.4–61.1	93.8	46.3–99.7	85.3	68.2–94.5
		IgM	47.2	30.8–64.3	100.0	51.7–100.0	69.4	51.7–83.1	50.0	13.9–86.1	71.4	61.7–82.1	93.4	64.3–98.7
	Dot-ELISA ^b	S2	100.0	87.7–100.0	85.7	42.0–99.2	82.9	65.7–92.8	85.7	22.3–77.7	80.6	63.4–91.2	100.0	39.6–100.0
		S2*	94.7	81.4–99.1	100.0	39.6–100.0	75.7	58.4–87.6	100.0	39.6–100.0	78.9	63.4–88.6	95.8	82.3–99.5
		T+	96.6	81.5–99.8	46.2	16.5–71.4	76.7	57.3–89.4	50.0	26.1–91.3	77.5	43.2–89.6	98.8	83.4–99.2
		IgM	100.0	85.9–100.0	41.7	20.4–73.9	83.3	64.5–93.7	69.2	38.9–89.6	85.6	66.5–92.7	100.0	97.9–100.0
	Survey (n = 140)	S2	74.4	63.7–82.9	97.2	89.3–99.5	58.1	47.0–68.5	96.3	79.1–99.8	60.0	46.0–70.3	76.4	45.3–85.7
		S2*	100.0	80.8–100.0	81.5	73.1–87.8	81.0	57.4–93.7	93.3	86.8–96.8	83.2	56.4–92.8	100.0	85.6–100.0
		T+	85.7	53.0–98.5	50.9	44.0–91.5	81.3	57.6–96.3	100.0	55.0–100.0	85.6	57.8–95.3	92.3	60.6–97.3
		IgM	30.8	23.6–58.4	69.4	71.6–85.3	56.7	45.3–66.8	73.2	64.2–86.4	70.7	54.8–67.8	50.3	22.6–87.6

^a Sensitivity and specificity of detection of anti-NF alone, anti-GalC alone, and both anti-NF and anti-GalC antibodies by ELISA was compared to field criteria (S2, CSF patients with cell count > 5 cells/μl or the presence of trypanosomes) and laboratory criteria (S2*, CSF patients with cell count > 20 cells/μl or IgM concentration ≥ 100 mg/liter or the presence of trypanosomes) and then compared to the presence of trypanosomes in the CSF (T+) and CSF IgM concentration ≥ 100 mg/liter.

^b Sensitivity and specificity of detection of anti-NF alone, anti-GalC alone, and both anti-NF and anti-GalC antibodies by dot-ELISA was compared to field criteria (S2) and laboratory criteria (S2*) and then compared to the presence of trypanosomes in the CSF (T+) and CSF IgM concentration ≥ 100 mg/liter in both laboratory and field conditions during two surveys (Angola and Central African Republic).

^c According to the condition studied, sensitivity and specificity were calculated as follows. True positives (TP) were S2 or S2* patients or patients with IgM concentrations ≥ 100 mg/liter or parasites in CSF and a positive ELISA or a positive dot-ELISA result. False negatives (FN) were S2 or S2* patients or patients with IgM concentrations ≥ 100 mg/liter or parasites in CSF and a negative ELISA or a negative dot-ELISA result. Sensitivity was calculated as TP/(TP + FN). True-negative (TN) patients were S1 or S1 and Sint patients or patients with IgM concentrations < 100 mg/liter or no parasites in CSF and a negative ELISA or a negative dot-ELISA result. False positives (FP) were S1 or S1 and Sint patients or patients with IgM concentrations < 100 mg/liter or no parasites in CSF and a positive ELISA or a positive dot-ELISA result. Specificity was calculated as TN/(TN + FP).

^d CI, confidence interval.

test that required expensive equipment, making it difficult to adapt in the field (8). For this reason only dot-ELISA was further used in the not-so-well-equipped laboratories and in the field situation in CAR and in Angola.

During surveys, only one CSF sample out of 43 S1 HAT patients was positive for both anti-NF and anti-GalC antibodies by dot-ELISA. Treatment failure (26) and sleep disruptions have already been described for S1 patients after polysomnography recordings (6). As no other studies were done with the patient with positive dot-ELISA results, we hypothesized that cross-reactions could also occur with other unrecognized pathologies. These findings confirm the hypothesis that usual biological criteria (CSF cell count, CSF IgM analysis) do not represent a good standard for HAT staging. Nevertheless, we think that S1 patients with a negative dot-ELISA result for both antibodies could be treated with pentamidine. Further clinical trial should be done to determine the really false-positive patients detected by dot-ELISA screening among the S1 positive patients who are followed up, especially after pentamidine treatment.

Surprisingly, 43 patients out of the 65 classified in the Sint category showed positive anti-NF results by dot-ELISA and 32 out of them were also positive for anti-GalC. As seen in laboratory conditions, all CSF samples positive for anti-GalC were also positive for anti-NF. However, CSF containing anti-NF antibodies were not systematically positive for anti-GalC. These results confirm that CSF cell counts are not sensitive enough and should not be used alone for HAT staging and that

further investigations have to be done with Sint patients. We think that dot-ELISA-positive Sint patients should be treated with melarsoprol or difluoromethylornithine but not with pentamidine, as they could be considered to be in the neurological stage of the disease. In contrast, the remaining 33 dot-ELISA-negative Sint patients might be treated with pentamidine, as we think they are in the hemolymphatic stage. These findings could be an explanation for the controversial results for treatment of Sint patients by pentamidine reported in the literature (9, 27).

Thirty-two patients out of the 32 classified in the S2* category showed positive anti-NF results by dot-ELISA, and 25 of them were also positive for anti-GalC. As in the Sint stage, all CSF samples having anti-GalC antibodies were positive for anti-NF, but the opposite was not true. The best results for sensitivity and specificity were obtained for the S2* stage when both antibodies were dot-ELISA positive: 83.2% and 100.0%, respectively.

Sensitivity and specificity of both antibodies detected by dot-ELISA during field surveys were poorly correlated with IgM ≥ 100 mg/liter.

In our study, in spite of our using double centrifugation to detect trypanosomes in the CSF during the survey in Angola, only five patients showed trypanosomes, but all of them were positive by dot-ELISA for both anti-NF and anti-GalC, as in the laboratory conditions. In the survey in CAR, trypanosomes in the CSF were identified using the counting chamber. Only two patients showed trypanosomes, but both were positive by

FIG. 3. ELISA and dot-ELISA detection of anti-GalC and anti-NF antibodies in 42 patients with HAT from Angola: comparison between ELISA and dot-ELISA results and CSF cell count (a and b) or CSF IgM concentration (c and d). ▲, positive dot-ELISA and positive ELISA; ■, positive dot-ELISA and negative ELISA; Δ, negative dot-ELISA and positive ELISA; □, negative dot-ELISA and negative ELISA.

dot-ELISA for both antibodies. The correlation between the presence of trypanosomes and dot-ELISA positivity for both antibodies was excellent, although the number of CSF samples positive for trypanosomes was low because the parasitological techniques used were often not sensitive enough (24, 25). Due to this, the specificity of dot-ELISA based upon presence of trypanosomes in CSF could not be retained. However, we think the CSF samples must be reexamined for the presence of trypanosomes in cases of dot-ELISA positivity.

To define the sensitivity of dot-ELISA assay compared to that of IgM latex (17), in the stage determination of HAT, it will be of interest to perform appropriately powered comparative trials in diverse settings and patient groups. Moreover, such studies should weight efficacy, toxicity, and ease of application of pentamidine versus melarsoprol treatment for the Sint stage and we ought to follow up Sint patients.

In conclusion, in the field situation, detection of trypanosomes in CSF remains problematical and IgM quantification somewhat difficult. When compared to CSF cell counts of >20 cells/μL, dot-ELISA for detection of both anti-NF and anti-GalC antibodies shows good sensitivity and high specificity which allow its use for mass screening, thus reducing the workload of parasite detection, which will remain imperative for unequivocal diagnosis. In addition, dot-ELISA seems to be very useful for classifying patients in the Sint stage when the CSF cell count is 6 to 20 cells/μL and the treatment to be administered difficult to choose accurately. However, some CSF of Sint patients have an elevated IgM concentration in CSF in contrast to the negativity of the dot-ELISA test. This

could be related to the aim of the dot-ELISA assay, which was established to detect specific IgM raised against glycolipidic and proteic autoantigens during HAT as previously described (1, 3, 11, 14) and not irrelevant IgM that could be increased during the course of infectious diseases. Therefore, dot-ELISA could be proposed for HAT staging in conjunction with conventional biological CSF tests and new tests such as the recently developed IgM latex (17). Dot-ELISA may be of interest for countries and health organizations concerned but must be applied in large-scale study before approval for use during a mass screening.

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