

# *Trypanosoma brucei gambiense* Spliced Leader RNA Is a More Specific Marker for Cure of Human African Trypanosomiasis Than *T. b. gambiense* DNA

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**To assess the efficacy of treatment for human African trypanosomiasis, accurate tests that can discriminate relapse from cure are needed. We report the first data that the spliced leader (SL) RNA is a more specific marker for cure of human African trypanosomiasis than parasite DNA. In blood samples obtained from 61 patients in whom human African trypanosomiasis was cured, SL RNA detection had specificities of 98.4%–100%, while DNA detection had a specificity of only 77%. Data from our proof-of-concept study show that SL RNA detection has high potential as a test of cure.**

**Keywords.** human African trypanosomiasis; diagnosis; test of cure; RNA; specificity.

*Trypanosoma brucei gambiense* is responsible for the majority of human African trypanosomiasis cases. Patients with human African trypanosomiasis due to peripheral *T. b. gambiense* infection (stage 1) are treated with pentamidine, whereas those with disease due to central nervous infection (stage 2) are usually treated with nifurtimox-eflornithine combination therapy or

melarsoprol. In routine practice, systematic posttreatment follow-up by blood and cerebrospinal fluid (CSF) testing is no longer recommended for pentamidine and nifurtimox-eflornithine combination therapy because of their high efficacies [1]. In the rare situations in which melarsoprol is still used or in clinical trials of new drug compounds or combinations, patients should be examined 6, 12, and 18 months after treatment. This 18-month follow-up period, which is required to determine treatment efficacy, slows down the drug-development process. In addition, accurate diagnosis of patients with relapse is difficult because additional lumbar punctures are required, there is low compliance with the follow-up appointments, and diagnosis is often based on proxy indicators, such as white blood cell (WBC) count in CSF, with arbitrary cutoffs. New tools for accurate, early, and noninvasive assessment of treatment outcome would be helpful in future drug efficacy studies.

Molecular tests for detecting the parasite's DNA have been suggested to shorten follow-up after treatment [2]. However, a study of 360 patients with *T. b. gambiense* human African trypanosomiasis in the Democratic Republic of the Congo reported trypanosomal DNA in blood and CSF specimens from 8%–20% and 16%–44% of cured patients, respectively, up to 2 years after treatment [3]. The mechanism behind this persistence of trypanosome DNA after cure remains unknown. Messenger RNA (mRNA) detection has been suggested for a faster assessment of treatment efficacy [4]. The spliced leader (SL) RNA is an interesting candidate target because it has a conserved sequence in the *Trypanozoon* subgenus, is highly abundant (>9000 molecules) [4] and is part of the mRNA, which is considered one of the best surrogate markers for viable organisms [5]. This 39-bp SL RNA sequence is attached to the 5' of all mRNA in the parasite during the mRNA maturation process [6]. Recently, González-Andrade et al developed a sensitive and specific reverse transcription real-time PCR test for *Trypanozoon* SL RNA detection in clinical specimens [4]. In the present study, we tested whether the SL RNA is a more specific marker for cure assessment than DNA in *T. b. gambiense* human African trypanosomiasis.

## METHODS

### Patients and Controls

This study is part of a larger project aiming to improve human African trypanosomiasis diagnosis; approval for the project was obtained from the ethical committee (Comité Consultatif de Déontologie et d'Éthique) of the Institut de Recherche pour le Développement. Sixty-one patients with human African trypanosomiasis and 32 healthy controls from areas of

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endemicity were recruited in the main human African trypanosomiasis foci along the Guinean coast (Forecariah, Dubreka, and Boffa) during 2007–2010. Patients with human African trypanosomiasis had a positive result of the card agglutination test for trypanosomiasis and trypanolysis assays for antibody detection in blood [7, 8], and trypanosomes were detected in their lymph node aspirate by microscopy and/or in blood by the mini anion exchange centrifugation technique [9]. Eleven patients had stage 1 infection (WBC count,  $\leq 5$  cells/ $\mu\text{L}$  of CSF), 23 had early stage 2 infection (6–20 cells/ $\mu\text{L}$  of CSF), and 27 had late stage 2 infection ( $>20$  cells/ $\mu\text{L}$  of CSF). Among controls, the card agglutination test for trypanosomiasis and the immune trypanolysis assay did not detect antibodies against *T. b. gambiense* [8]. For RNA isolation, 2.5 mL blood samples were collected in PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland), transported at  $-20^{\circ}\text{C}$ , and immediately stored at  $-80^{\circ}\text{C}$  until the RNA was isolated. For DNA isolation, 500- $\mu\text{L}$  aliquots of blood were stored at  $-80^{\circ}\text{C}$  until further extraction. Blood samples obtained before treatment were collected at the time of diagnosis. Patients with stage 1 and early stage 2 infection were treated with pentamidine, and patients with late stage 2 infection were treated with melarsoprol. Patients were followed up for at least 6 months after treatment, with a minimum of 2 visits per patient. Blood samples for DNA and RNA testing were collected during the first visit, which varied between 16 and 96 weeks after treatment, and were stored in the same way as pretreatment samples. A patient was considered cured if no trypanosomes were observed in blood or CSF specimens at all follow-up visits, the WBC count was  $<20$  cells/ $\mu\text{L}$  of CSF, and there were no clinical signs of relapsing human African trypanosomiasis at the last visit. All 32 controls were a subset of the control cohort used in the previous study of SL RNA detection [4], as were samples from 7 of 61 patients with human African trypanosomiasis.

### Nucleic Acid Isolation

Total RNA from 2.5 mL of blood stored in PAXgene tubes was extracted with the PAXgene Blood RNA kit (PreAnalytiX) according to the manufacturer's instructions, and RNA was eluted in 80  $\mu\text{L}$  of diethylpyrocarbonate-treated water. Total DNA from 500  $\mu\text{L}$  of blood was extracted using the DNeasy Tissue kit (Qiagen) as described previously [10].

### SL RNA Detection

The trypanosome's SL RNA was copied into complementary DNA by reverse transcription and amplified by real-time PCR in a Mx3005P thermocycler (Agilent, USA) using the SensiFAST SYBR No-ROX One-Step Kit (Bioline, United Kingdom) and primers 5'-CAATATAGTACAGAACTG-3' (cSL) and 5'-AACTAACGCTATTATTAGAA-3' (SL-F). Reaction mixtures and cycling conditions were as described in the kit's manual but with primer annealing and elongation temperatures of  $50^{\circ}\text{C}$  and  $60^{\circ}\text{C}$ , respectively. Postamplification melting curves

were recorded from  $45^{\circ}\text{C}$  to  $95^{\circ}\text{C}$  with increments of  $0.1^{\circ}\text{C}/\text{second}$ . The fluorescence threshold value was set at 2000 fluorescence intensity values in each run. Threshold cycle (Ct) values of  $\geq 34$  cycles were scored as invalid. This cutoff Ct value was based on an optimal combination of sensitivity and specificity. Each run contained 1 positive and 2 negative PCR controls, and all tests were done in duplicate to assess the repeatability of the SL RNA assay.

### Satellite DNA Detection

Samples were tested in PCR once. We used *Trypanozoon*-specific TBR1/2 primers targeting satellite DNA and reaction conditions as described earlier [10].

### Statistical Analysis

Sensitivities and specificities were calculated from data entered into contingency tables. Sensitivity was defined as the proportion of patients with confirmed sleeping sickness who had positive results of the index test, and specificity was defined as the proportion of controls who had negative results of the index test. Proportions with exact binomial confidence intervals (CIs) were calculated, and agreement between results of duplicate SL RNA runs was measured by the Cohen  $\kappa$ . Interplate variation was measured by the standard deviation of the mean Ct value of the positive control sample in each plate.

## RESULTS

Results of the SL RNA assay and TBR PCR performed on blood samples obtained from the study participants are summarized in Table 1. All healthy controls had negative test results for both runs of the SL RNA assay and the TBR PCR, indicating 100% specificity (1-sided 97.5% CI, 89.1%–100%) for both tests. At the time of diagnosis, of the 61 patients with human African trypanosomiasis, 59 and 58, respectively, had positive SL RNA test results for the 2 runs, indicating sensitivities of 96.7% (95% CI, 88.7%–99.6%) and 95.1% (95% CI, 86.3%–99.0%). The TBR PCR yielded

**Table 1. Accuracies of the *Trypanozoon* Spliced Leader (SL) RNA Assay and Polymerase Chain Reaction (TBR PCR) for Diagnosis and Cure Assessment of Human African Trypanosomiasis**

Group <sup>a</sup>	Samples, No.	Positive Test Result Before Treatment, No.		Positive Test Result After Treatment, No.			
		SL RNA		TBR PCR			
		Run 1	Run 2	Run 1	Run 2		
Patients	61	59	58	57	0	1	14
Controls	32	0	0	0	NA	NA	NA

Abbreviation: NA, not applicable.

<sup>a</sup> Groups denote patients with *T. b. gambiense* human African trypanosomiasis and healthy controls from an area of human African trypanosomiasis endemicity.

positive results for 57 patients with human African trypanosomiasis (93.4%; 95% CI, 84.1%–98.2%). As a test of cure, the SL RNA assay scored a specificity of 100% (1-sided 97.55% CI, 94.1%–100.0%) in run 1 and 98.4% (95% CI, 91.2%–100.0%) in run 2. The TBR PCR showed a significantly lower specificity for cure of 77.0% (95% CI, 64.5%–86.8%;  $P \leq .0003$ ). Interplate variation of the SL RNA assay showed an SD of 0.31 Ct. Agreement between all 154 duplicate SL RNA runs was 98.7%, with a  $\kappa$  of 97.3%.

## DISCUSSION

We reported the first data on the accuracy of SL RNA detection as a test of cure of human African trypanosomiasis, compared with DNA detection by the TBR PCR. Of the patients in whom *T. b. gambiense* trypanosomiasis was cured, 23% had positive TBR PCR test results for blood specimens collected 16–96 weeks after treatment. This confirms previous findings from a study conducted in the Democratic Republic of the Congo, in which 8%–20% of the cured patients showed *Trypanozoon* DNA in their blood, even 2 years after successful treatment [11]. In contrast, SL RNA was detected in the blood of only 0%–1.6% at the same time point after treatment, indicating the exceptionally high specificity of the SL RNA assay for cure, compared with DNA detection.

Some limitations of our study should be taken into account. Only blood specimens from cured patients was tested, because no follow-up CSF RNA samples were available. Nonetheless, the WBC count and parasite detection in CSF are usually more sensitive for detection of relapses than parasitological findings in blood [12]. Another limitation of our sample set was that no blood specimens from patients with relapse were available, and thus no estimation of the sensitivity of SL RNA detection for relapse could be provided.

Candidate drugs for safe and oral treatment of both stages of *T. b. gambiense* human African trypanosomiasis are finally in the pipeline [13]. Clinical trials that assess the efficacies of such new compounds require accurate tests for early detection of treatment failure. The SL RNA assay is ideally suited for standardized and high-throughput testing in clinical trials. Blood specimens are collected directly from patients into PAXgene RNA tubes, compatible with the BD Vacutainer blood collection system, which also limits potential contamination. RNA can be extracted in reference centers with a commercial kit and analyzed in 96-well plates with a high throughput. Neopterin as a host inflammation biomarker in CSF has been previously suggested as a marker for treatment efficacy assessment [14]. Both tests could therefore provide complementary information. Furthermore, the SL RNA assay can be performed on both blood and CSF specimens.

Further evaluation studies are required, but the proof-of-concept data reported here indicate that, in blood, SL RNA is a better marker than parasite DNA for the assessment of

human African trypanosomiasis cure. If its accuracy for detecting patients with relapse after minimal follow-up time can be demonstrated, SL RNA detection has the potential to revolutionize future drug efficacy studies.

## Notes

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