

RESEARCH ARTICLE

Trypanosome SL-RNA detection in blood and cerebrospinal fluid to demonstrate active gambiense human African trypanosomiasis infection

Ipos Ngay Lukusa¹, Nick Van Reet², Dieudonné Mumba Ngoyi¹, Erick Mwamba Miaka³, Justin Masumu¹, Pati Patient Pyana¹, Wilfried Mutombo³, Digas Ngolo³, Vincent Kobo³, Felix Akwaso³, Médard Ilunga³, Lewis Kaninda³, Sylvain Mutanda³, Dieudonné Mpoyi Muamba³, Olaf Valverde Mordt⁴, Antoine Tarral⁴, Sandra Rembry⁴, Philippe Büscher², Veerle Lejon^{5*}

1 Department of Parasitology, Institut National de Recherche Biomédicale, Kinshasa, Democratic Republic of the Congo, **2** Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium, **3** Programme National de Lutte contre la Trypanosomiase Humaine Africaine (PNLTHA), Ministry of Health, Kinshasa, Democratic Republic of the Congo, **4** Drugs for Neglected Diseases initiative, Geneva, Switzerland, **5** Mixed Research Unit 177 Intertryp, Institut de Recherche pour le Développement, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, University of Montpellier, Montpellier, France

* veerle.lejon@ird.fr



OPEN ACCESS

Citation: Ngay Lukusa I, Van Reet N, Mumba Ngoyi D, Miaka EM, Masumu J, Patient Pyana P, et al. (2021) Trypanosome SL-RNA detection in blood and cerebrospinal fluid to demonstrate active gambiense human African trypanosomiasis infection. *PLoS Negl Trop Dis* 15(9): e0009739. <https://doi.org/10.1371/journal.pntd.0009739>

Editor: Epco Hasker, ITM, BELGIUM

Received: February 3, 2021

Accepted: August 17, 2021

Published: September 17, 2021

Copyright: © 2021 Ngay Lukusa et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript files.

Funding: The DiTECT-HAT project, coordinated by VL, is part of the European & Developing Countries Clinical Trials Partnership (EDCTP2, <https://www.edctp.org/>) programme supported by the European Union (grant number DRIA-2014-306-DIRECT-HAT). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Spliced Leader (SL) trypanosome RNA is detectable only in the presence of live trypanosomes, is abundant and the *Trypanozoon* subgenus has a unique sequence. As previously shown in blood from Guinean human African trypanosomiasis (HAT) patients, SL-RNA is an accurate target for diagnosis. Detection of SL-RNA in the cerebrospinal fluid (CSF) has never been attempted. In a large group of Congolese gambiense HAT patients, the present study aims i) to confirm the sensitivity of SL-RNA detection in the blood and; ii) to assess the diagnostic performance of SL-RNA compared to trypanosome detection in CSF.

Methodology/Principal findings

Blood and CSF from 97 confirmed gambiense HAT patients from the Democratic Republic of Congo were collected using PAXgene blood RNA Tubes. Before RNA extraction, specimens were supplemented with internal extraction control RNA to monitor the extraction, which was performed with a PAXgene Blood RNA Kit. SL-RNA qPCR was carried out with and without reverse transcriptase to monitor DNA contamination. In blood, 92/97 (94.8%) HAT patients tested SL-RNA positive, which was significantly more than combined trypanosome detection in lymph and blood (78/97 positive, 80.4%, $p = 0.001$). Of 96 CSF RNA specimens, 65 (67.7%) were SL-RNA positive, but there was no significant difference between sensitivity of SL-RNA and trypanosome detection in CSF. The contribution of DNA

Competing interests: The authors have declared that no competing interests exist.

to the Cq values was negligible. In CSF with normal cell counts, a fraction of SL-RNA might have been lost during extraction as indicated by higher internal extraction control Cq values.

Conclusions/Significance

Detection of SL-RNA in blood and CSF allows sensitive demonstration of active gambiense HAT infection, even if trypanosomes remain undetectable in blood or lymph. As this condition often occurs in treatment failures, SL-RNA detection in blood and CSF for early detection of relapses after treatment deserves further investigation.

Trial registration

This study was an integral part of the diagnostic trial "New Diagnostic Tools for Elimination of Sleeping Sickness and Clinical Trials: Early tests of Cure" (DiTECT-HAT-WP4, Clinical-Trials.gov Identifier: [NCT03112655](https://clinicaltrials.gov/ct2/show/study/NCT03112655)).

Author summary

Human African trypanosomiasis is a parasitic infection occurring in sub-Saharan Africa, which is fatal if left untreated. Diagnosis relies on demonstration of trypanosomes, which may occur at such low concentrations that they remain microscopically undetectable. Nucleic acid detection offers an alternative, in particular RNA, which is unstable and a better marker for live organisms than DNA. Trypanosomal SL-RNA detection in blood by reverse transcriptase quantitative PCR has hitherto only been tested twice. Although in cerebrospinal fluid, trypanosome presence indicates brain infection, SL-RNA detection has never been attempted. We evaluated sensitivity of SL-RNA detection in blood and cerebrospinal fluid. For each specimen, 2 controls were included: presence of genomic DNA contamination and efficacy of RNA extraction. Sensitivity of SL-RNA detection in blood was higher than of combined blood and lymph microscopy. In cerebrospinal fluid, SL-RNA and trypanosome detection had similar sensitivity. In a few specimens, traces of DNA were amplified. In some cerebrospinal fluids, some RNA was lost during extraction. Performing both internal controls is crucial, to ensure that negative SL-RNA cerebrospinal fluid findings are not due to a failed extraction and, in particular when testing treated patients, to differentiate live parasite RNA from reminiscent DNA.

Introduction

Human African trypanosomiasis (HAT) or sleeping sickness is a neglected tropical disease caused by *Trypanosoma brucei* (*T.b.*) *gambiense* in Central and West Africa and by *T.b. rhodesiense* in East Africa. In humans, presence of the trypanosome in the blood and lymph only, corresponds to the first stage of the disease, whereas the second stage is characterized by trypanosome invasion of the central nervous system.

The diagnosis of HAT is primarily based on the detection of the parasite in the blood, lymph or cerebrospinal fluid (CSF) [1]. Performance of diagnostic tests is influenced, for *T.b. gambiense* HAT, by the low and fluctuating trypanosomal load in these body fluids, intrinsic to the parasite and its interaction with the host's immune response. The resulting low

sensitivity of microscopic diagnosis, implies a risk of failure to detect the parasite although a patient is actually infected [2].

PCR targeting parasite DNA presents an alternative for the diagnosis of HAT [3]. However, DNA detection does not fully differentiate active infection, when the parasite is present, from a cleared infection. In some patients, long-term persistence of DNA has been observed, even after successful treatment [4,5].

The detection of trypanosomal RNA, which is unstable and produced only by live parasites, demonstrates an active infection in the host and thus enables to establish a definite diagnosis. Spliced Leader (SL) trypanosome RNA can be used for diagnostic purposes, as it is abundant in trypanosomes and the *Trypanozoon* subgenus has a unique sequence. SL-RNA is involved in the maturation of messenger RNA to which it is attached [6]. The diagnostic sensitivity of SL-RNA detection in blood of sleeping sickness patients from Guinea with trypanosomes detected in blood and/or lymph reached 91.7–96.7%, while in the same studies specificities were 95.5 and 100% [5,6]. Also for post-treatment follow-up of patients with *T.b. gambiense*, the specificity of SL-RNA detection in blood was higher than that of DNA detection by PCR [5]. To date, SL-RNA detection has never been applied on the blood of gambiense HAT patients from other countries. Also, the diagnostic performance of SL-RNA demonstration in CSF of HAT patients remains unknown.

Although presence of trypanosomes in CSF has traditionally been a marker for 2nd stage HAT, stage determination loses importance as new stage-independent treatments for sleeping sickness emerge [7–9]. Nevertheless, parasitological examination of CSF remains relevant. A fraction of gambiense HAT patients can only be confirmed by trypanosome detection in CSF, despite using the most sensitive techniques on blood [10]. Also for detection of relapses, CSF examinations are usually more sensitive than parasitology on blood [11,12]. The present study therefore aims i) to confirm the sensitivity of SL-RNA detection in the blood of a large group of gambiense HAT patients from the Democratic Republic of Congo (DRC) and; ii) to assess the diagnostic performance of SL-RNA detection in CSF of those patients.

Methods

Ethics statement

This study was an integral part of the diagnostic trial "New Diagnostic Tools for Elimination of Sleeping Sickness and Clinical Trials: Early tests of Cure" (DiTECT-HAT-WP4, ClinicalTrials.gov Identifier: NCT03112655). The DiTECT-HAT-WP4 study has received approval from the "Comité Consultatif de Déontologie et d'Ethique" (CCDE) of the French National Institute for Sustainable Development Research (IRD, June 2016 session), from the institutional Review Board of the Institute of Tropical Medicine (reference 1096/16), and from the Ethics Committees of the Ministry of Health, DRC through the Ngaliema Clinic of Kinshasa (September 2016 session) and the School of Public Health of the University of Kinshasa (reference ESP/CE/136/2020). It was carried out in conjunction with a prospective therapeutic trial conducted by DNDi (DNDi-OXA-02-HAT, registered as NCT03087955) on the new drug Acoziborole for HAT that was approved by the CERSAC, Ethics Committees of the Ministry of Health, DRC through the Ngaliema Clinic of Kinshasa and the CNES (Comité National d'Ethique de la Santé), DRC.

Participants first gave their informed consent to participate in the therapeutic trial. A separate written informed consent was obtained next before their inclusion in the DiTECT-HAT-WP4 study. For minors participating in the DiTECT-HAT-WP4 study, an additional written consent was obtained from their legal representative.

Study sites

The DiTECT-HAT-WP4 study was conducted in Democratic Republic of the Congo from February 2017 until March 2019. Patients were recruited in five reference district hospitals that were conducting the DNDi-OXA-02-HAT therapeutic trial under the supervision of the PNLTHA, located in Dipumba and Katanda in the province of Kasai Oriental, Ngandajika in the province of Lomami, Masi Manimba in the province of Kwilu and in Kinshasa.

Patients

Patients included in the DiTECT-HAT-WP4 study were enrolled first in the DNDi-OXA-02-HAT study. The inclusion criteria for DiTECT-HAT-WP4 corresponded to those applied in the DNDi-OXA-02-HAT study: confirmed HAT with *T.b. gambiense* parasites in blood, lymph and/or CSF; age ≥ 15 years; give and sign the study consent form; Karnofsky index > 50 ; able to take tablets orally; have a permanent address or being retrievable by other people; able to comply to the follow-up visits schedule and to other constraints of the study and accept to be hospitalized for acoziborole treatment and study procedures. As all HAT patients participating in DiTECT-HAT-WP4 were first enrolled in the DNDi-OXA-02 study, they were treated with acoziborole, independent of their disease stage.

Diagnosis

Patients included in this study were diagnosed either in the village by mobile teams (active screening), or at the reference district hospital (passive screening). Screening consisted of identifying characteristic clinical signs, in particular swollen cervical lymph nodes, and serological tests (CAT1/*T.b. gambiense* test or a rapid diagnostic test for gambiense HAT). Individuals who were positive in a serological test underwent parasitological examination. For persons with swollen cervical lymph nodes, a lymph aspirate was examined for trypanosomes by microscopy. For persons positive in a serological test but negative in lymph or without swollen lymph nodes, blood was examined for trypanosome presence by mini anion-exchange centrifugation technique on buffy coat (mAECT-BC)[13]. If mAECT-BC was unfeasible, blood was examined by mAECT or the haematocrit centrifugation technique. All HAT patients detected by active screening were reconfirmed at the reference district hospital before inclusion. After parasitological confirmation, or if there was a strong clinical and serological suspicion, a lumbar puncture was performed. The CSF was examined for white blood cell (WBC) number. Trypanosomes were searched for during the cell counting procedure, and, if no trypanosomes had been observed, by the modified single centrifugation technique of the CSF [14]. The patient was classified as early or intermediate stage with absence of trypanosomes in the CSF and a WBC count in the CSF of respectively ≤ 5 or 6–20 WBC/ μl . The late stage of the disease was defined by the presence of trypanosomes in the CSF or > 20 WBC/ μl . As a quality control, video recordings were made to certify presence of viable trypanosomes in lymph, blood and/or CSF.

From each HAT patient, 2.5 ml of blood were collected in a PAXgene Blood RNA Tube (PreAnalytiX, www.PreAnalytiX.com) according to the instructions of the manufacturer. After the collection of CSF for direct examination, the PAXgene Blood Tube was opened and 2.5 ml of CSF were collected by holding the tube under the lumbar puncture needle, after which the tube was closed. Both blood and CSF samples collected in PAXgene Blood RNA Tubes were inverted at least 10 times, and decanted immediately into 10 ml cryotubes. They were stored at -20°C for maximum 2 months before transport in liquid nitrogen to the HAT National Reference Laboratory at the Institut National de Recherche Biomédicale (INRB) in Kinshasa, where they were stored at -80°C until RNA extraction.

RNA extraction

RNA extraction on blood and CSF samples collected in PAXgene Blood RNA Tubes was performed using a PAXgene Blood RNA Kit IVD following the guidelines of the manufacturer (www.PreAnalytiX.com, PAXgene Blood RNA Kit Handbook 04/2008). The samples were thawed by placing them at ambient temperature for 4 hours to ensure full lysis before centrifugation and start of the extraction process. The pellet was lysed and supplemented after centrifugation with 4 μ l RNA internal extraction control (IEC) according to manufacturer's recommendations (Primer design Ltd, York House, School Lane, Chandlers Ford, UK SO53 4DG). Briefly, the resuspended pellet was proteinase K treated, and residual cell debris removed through PAXgene shredder spin column centrifugation. Ethanol was added to the flow-through fraction and the lysate applied to a PAXgene RNA spin column centrifugation. The bound RNA was treated with DNase I and after several wash steps, RNA was eluted in 80 μ l and stored at -80°C . RNA extracted from human blood spiked with *T.b. gambiense* LiTat 1.3 served as positive control.

SL-RNA quantitative PCR

SL-RNA was amplified using SensiFAST SYBR Lo-ROX One-Step Mix (Bioline, United Kingdom) with 400 nM of primers 5'-CAATATAGTACAGAAACTG-3' (cSL) and 5'-AAC-TAACGCTATTATTAGAA-3' (SL-F) (IDT Integrated DNA Technologies) and 4 μ l RNA eluate in a final volume of 20 μ l. All RNA extracts were tested in duplicate, and with (RT) or without (noRT) addition of reverse transcriptase (RT) (Bioline, United Kingdom). Cycling conditions using AriaMX Real-Time PCR System Machine (Agilent Technologies, USA) were 45°C for 10 minutes, 95°C for 2 minutes, and 40 cycles of 95°C for 2 seconds, 50°C for 10 seconds and 60°C for 5 seconds. The melting curve was recorded between 45°C and 95°C , using increments of 0.5°C for 5 seconds. Each qPCR run included a positive control from the spiked *T.b. gambiense* RNA extract and a negative control.

An RNA extract was considered positive for SL-RNA if both RT replicates were associated with a melting temperature (T_m) between 65 and 68°C . When detected, the C_q of the noRT reaction was used to calculate the percentage contribution of genomic DNA to the C_q -value of the RT reaction according to the following equation [15]:

$$\%gDNA = \left(\frac{2^{-C_q \text{ noRT}}}{2^{-C_q \text{ RT}}} \right) \times 100$$

Internal extraction control qPCR

The IEC was amplified using qScript XLT One-Step RT-qPCR ToughMix, Low ROX, using 1 μ l of IEC primer/probe (Primer design Ltd, UK) and 5 μ l of RNA extract in a 20 μ l reaction. Cycling conditions using AriaMX Real-Time PCR System Machine (Agilent Technologies, USA) were 50°C for 10 minutes, 95°C for 2 minutes and 40 cycles of 95°C for 2 seconds, 50°C for 10 seconds and 60°C for 5 seconds.

Data analysis

For proportions, binomial confidence intervals with a 95% confidence level (CI) were calculated. The categorical results of trypanosome and SL-RNA detection in the same patients were compared by the Mc Nemar Chi square test. The C_q values of the IEC of two groups of patients were compared by an unpaired t-test, determining the two-tailed p value.

Results

Patient characteristics

In total, 97 parasitologically confirmed HAT patients were included in DiTECT-HAT-WP4, 53 in Masi Manimba, 23 in Dipumba, 9 in Katanda, 10 in Ngandajika and 2 in Kinshasa. The median age of the participants was 31 years (interquartile range 24–44). The sex ratio was 50 females for 47 males. The CATT test was positive in 100% of patients on whom it was performed (95/95), the remaining two patients, on whom CATT was not performed, were positive in SD Bioline HAT. The parasitological results of these HAT patients are summarized in [Table 1](#).

Swollen lymph nodes could be punctured in 53 patients, and lymph was trypanosome positive for 35/53 HAT patients included in the study (66.0%, CI 52.6–77.4%). Next, blood was examined for 68 patients, and trypanosomes were detected in blood for 45 of 68 included HAT patients (66.2%, CI 54.3–76.3%). Lymph or blood were trypanosome positive in 78/97 HAT patients (80.4%, CI 71.1–87.8%). In total 69/97 (71.1%, CI 61.0–79.9%) HAT patients had trypanosomes in CSF, including all 19 patients in whom trypanosome infection could not be demonstrated previously in lymph and blood.

Fourteen HAT patients were classified in early stage (14.4%, CI 8.1–23.0%), 7/97 in intermediate stage (7.2%, CI 3.0–14.3%) and 76/97 in late stage (78.4%, CI 68.8–86.1%). All of them were SL-RNA positive, either in CSF and/or blood. Detailed results are shown in [Fig 1](#).

RT-qPCR results on blood specimens

The internal extraction control added to each blood specimen during the RNA extraction procedure was detected by RT-qPCR in all 97 extracted blood specimens with a mean Cq value of 20.9 (standard deviation 1.0).

Of 97 confirmed HAT patients, 92 (94.8%, CI 88.2–98.1%) tested SL-RNA positive in blood ([Table 1](#)). The contribution of genomic DNA to the RT detection of SL-RNA could not be calculated for 30 reactions since no amplification in noRT replicates was observed (grey symbols in [Fig 1](#)), was below 4% in 61 reactions and was 39% in one reaction (black to red gradient in [Fig 1](#)).

Table 1. Results of RT-qPCR on blood and CSF from 97 gambiense HAT patients by disease stage, according to demonstration of trypanosomes in blood, lymph and CSF, and to CSF WBC count.

	N	SL-RNA	
		Blood positive	CSF positive
Early stage (N = 14)			
Tryp. in lymph and/or blood, no Tryp. in CSF, WBC <5	14	14	0
Intermediate stage (N = 7)			
Tryp. in lymph and/or blood, no Tryp. in CSF, WBC 5–20	7	7	1
Late stage (N = 76)		71	64/75*
Tryp. in lymph and/or blood, Tryp. in CSF, WBC <20	3	3	0
Tryp. in lymph and/or blood, Tryp. in CSF, WBC >20	46	45	44/45*
Tryp. in lymph and/or blood, Tryp. in CSF, WBC not done	1	1	1
Tryp. in lymph and/or blood, no Tryp. in CSF, WBC >20	7	7	2
No tryp. in lymph and/or blood, Tryp. in CSF, WBC <20	1	1	0
No tryp. in lymph and/or blood, Tryp. in CSF, WBC >20	18	14	17

N = number of patients; CSF = cerebrospinal fluid; Tryp. = trypanosome

* CSF specimen missing for 1 patient; WBC = CSF white blood cell count/μl

<https://doi.org/10.1371/journal.pntd.0009739.t001>

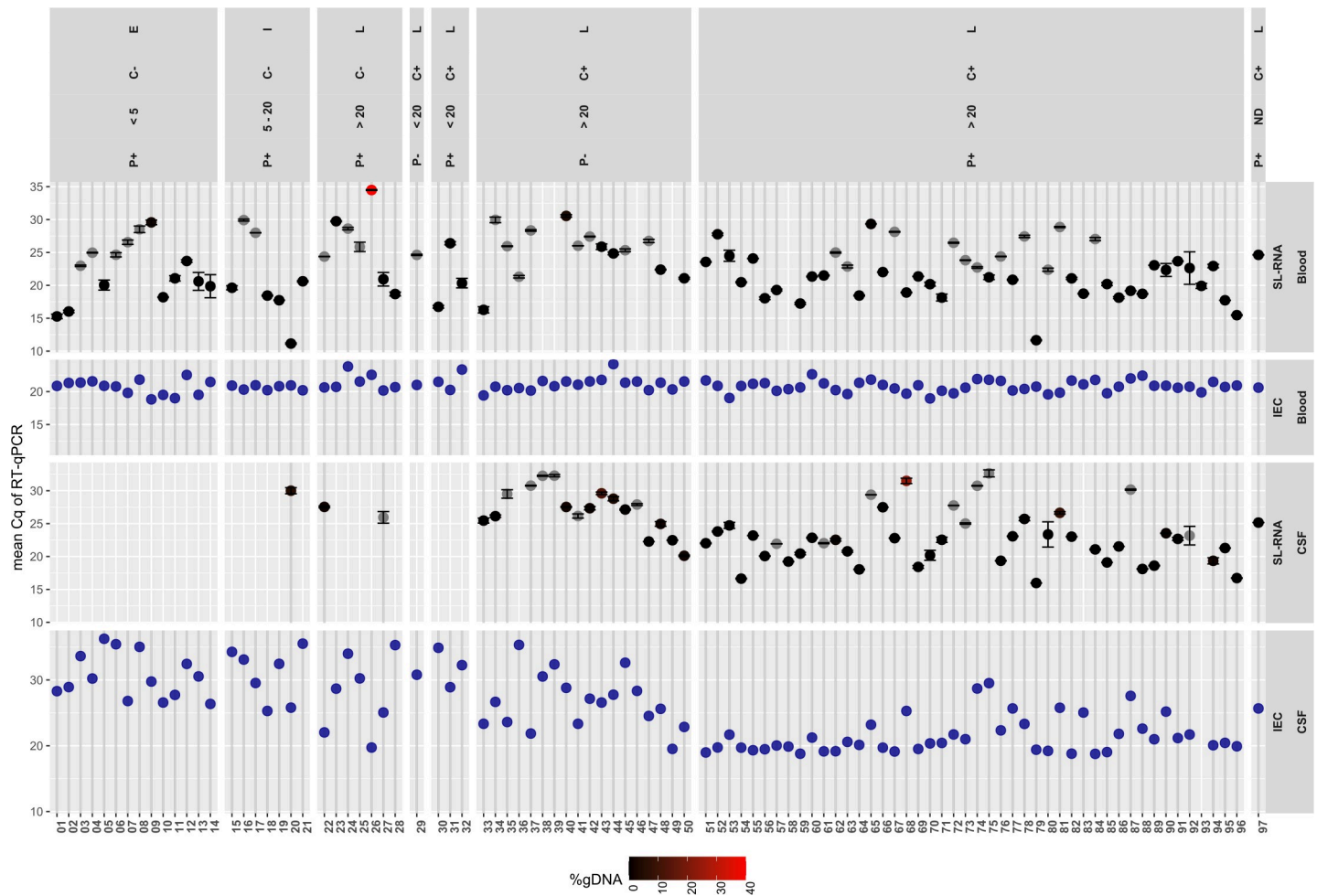


Fig 1. Cq values of RT-qPCR SL-RNA detection in blood and CSF of gambiense HAT patients. The results are combined with RT-qPCR for internal extraction control (IEC), according the disease stage (E early, I Intermediate and L late stage), parasitological test result in blood and lymph (P), parasitological test result in CSF (C), and CSF WBC count/ μ l. + trypanosome presence;—absence of trypanosomes. ND not determined.

<https://doi.org/10.1371/journal.pntd.0009739.g001>

Four out of 5 SL-RNA negative specimens (patients 38, 39, 46, 58) did not show amplification (no Cq value) and/or had a RT Cq value <40 but a melting temperature far outside the 65–68°C range. One negative specimen (patient 49) showed one positive reaction (Cq 33.6 and melting temperature 66°C) and one negative reaction (no Cq value). In only one of these blood SL-RNA negative patients, trypanosomes had been detected in blood and CSF (patient 58), while in the other 4 (patients 38, 39, 46, 49), trypanosomes had been detected in CSF only. Video recordings reconfirmed microscopic parasite finding for these 5 SL-RNA negatives. In the 68 patients in whom trypanosome presence in blood had been examined, SL-RNA was significantly more positive than trypanosome detection ($p = 0.0001$, 63 SL-RNA versus 45 parasite positives). SL-RNA detection in blood was also significantly more positive than combined lymph and blood parasite detection ($p = 0.001$, 92 SL-RNA versus 78 blood and/or lymph parasite positives in 97 HAT patients).

Of 14 patients in early stage and 7 in intermediate stage, respectively 14 (100%, CI 76.8–100%) and 7 (100%, CI 59.0–100%) were SL-RNA positive. Of 76 patients in late stage, 71 (94.7%, CI 85.2–97.5%) had SL-RNA positive results (Table 1).

RT-qPCR results on CSF specimens

Of 97 patients included, 96 CSF RNA specimens were available (CSF RNA specimen of patient 93 missing). The internal RNA extraction control added to each CSF specimen during the RNA extraction procedure was detected by RT-qPCR in all 96 specimens with a mean Cq corresponding to 25.4 (standard deviation 5.3, Fig 1). As shown in Fig 2, extraction of RNA appeared more efficient (low IEC Cq values) in CSF specimens with high WBC counts, and decreased with the WBC count, as witnessed by increasing IEC Cq values. There was a significant difference ($p < 0.0001$) between the IEC Cq of 30.8 (standard deviation 3.4, $n = 25$) of CSF with WBC count $\leq 20/\mu\text{l}$, and IEC Cq of 23.4 (standard deviation 4.4, $n = 70$) of CSF with WBC counts of $>20/\mu\text{l}$, indicating more efficient RNA extraction in the latter (WBC count not available for 1 specimen).

In CSF, 65/96 patients (67.7%, CI 57.8–76.35%) were SL-RNA positive (Table 1). Again, the gDNA contribution to RT SL-RNA detection was absent or small (Fig 1): 1 RNA extract was detected with 24% gDNA and 1 with 12%, 10 RNA extracts contained between 10–1% gDNA, 37 extracts had $< 1\%$ gDNA contribution, while in 16 RNA extracts no noRT reaction was observed. For eight SL-RNA negative patients, one RNA replicate tested positive while the other tested negative (patients 6, 12, 17, 23, 26, 31, 32, 83).

Of 14 early stage patients, zero (0.0%, CI 0.0–25.2%) had SL-RNA in CSF, while of 7 patients in intermediate stage, one (14.3%, CI 0.53–53.4%) reacted SL-RNA positive (patient 20). Of 75 CSF specimens from late stage patients, 64 (85.3%, CI 75.4–91.8%) contained SL-RNA. From 7 patients in late stage without trypanosomes in CSF, 2 (28.6%, CI 7.6–64.8%) were CSF SL-RNA positive (patients 22 and 27). From 68 late stage CSF samples containing

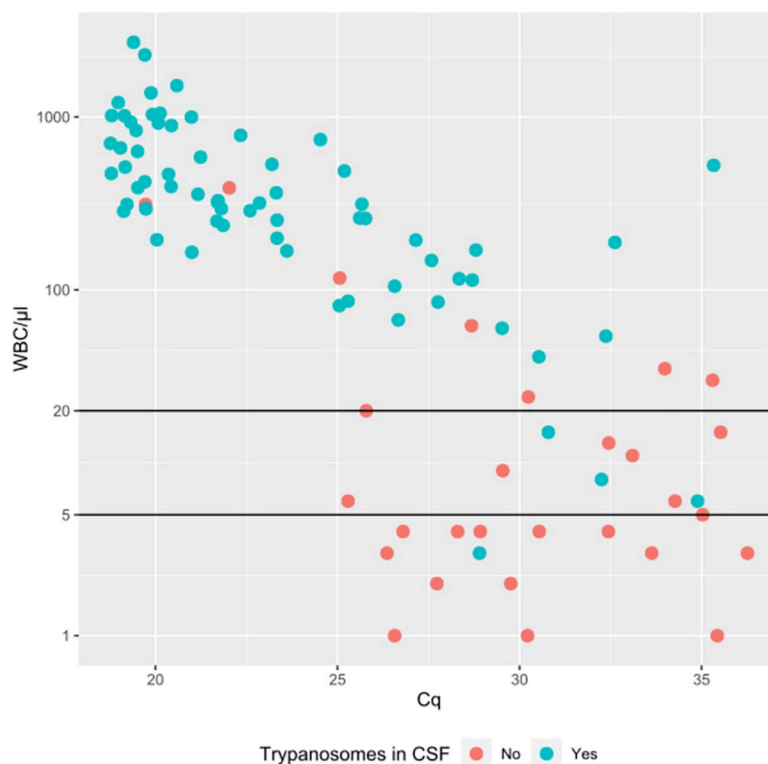


Fig 2. Cq values of the internal extraction control RT-qPCR in CSF in function of the CSF white blood cell count/ μl . Blue dots: trypanosomes in CSF. Red dots: no trypanosomes in CSF.

<https://doi.org/10.1371/journal.pntd.0009739.g002>

trypanosomes, 62 (91.2%, CI 81.7–96.2%) were SL-RNA positive. In 6 patients who were SL-RNA negative but reported as modified single centrifugation positive (patients 29–32, 36 and 83), retrospective analysis of the video recordings reconfirmed presence of live trypanosomes in the CSF. There was no significant difference in the number of SL-RNA or trypanosome positives ($p = 0.5$, 65 SL-RNA versus 68 modified single centrifugation positives).

Discussion

The obtained results confirm the high sensitivity of SL-RNA detection in blood for diagnosis of HAT, which exceeds the sensitivity of parasitological diagnosis in blood and lymph. For the first time, trypanosomal RNA was detected in the CSF of HAT patients, and the technique has similar sensitivity as the single modified centrifugation of CSF for trypanosome detection.

A number of strengths from the present study merit to be highlighted. An important improvement was the integration of an internal extraction control, through the addition of exogenous RNA to the test specimens, in order to verify the efficiency of the RNA extraction process. In particular for CSF, which was processed for the first time, inclusion of this extraction control seems a must when specimens with relatively normal WBC counts ($\leq 20/\mu\text{l}$) are processed. A second control step introduced in the protocol was parallel qPCR analysis of the specimen without addition of reverse transcriptase. This enables the calculation of the contribution of genomic DNA to the obtained RNA result, thus guaranteeing that RNA was effectively detected instead of remnant DNA. Moreover, as it has been estimated that half-life of most trypanosomal mRNA is less than 100 minutes [16], the detection of SL-RNA in a specimen thus implies ongoing active *Trypanozoon* infection. This is of particular importance once specimens of treated patients will be analysed, as trypanosomal DNA may circulate long after cure, especially in CSF. Indeed, previous observations suggest presence of trypanosomal DNA in respectively 24.6% and 35.4% of blood and CSF of successfully cured patients, even between 12 and 24 months after HAT treatment [4]. Another strength is the collaboration with a therapeutic clinical trial, which led to the collection of well-characterized and high quality specimens, with a quality control on positive parasitological tests through video recordings. This allowed re-confirmation of parasite positive results *a posteriori*. Similar therapeutic trials will probably not be conducted anymore in the near future as different new oral drugs have emerged to treat all stages of HAT, implying that the systematic lumbar punctures for disease staging will no longer be needed. The treatment regimen with fexinidazole requires a lumbar puncture only if clinical signs suggest severe second stage HAT [9], which is usually accompanied by a cytorachia of more than 100 WBC/ μl [17]. In the future, lumbar puncture will probably be limited to clinically suspected HAT cases in whom no trypanosomes were detectable in lymph or blood and that need parasitological confirmation in CSF, including relapses after treatment. The present trial was therefore probably the last opportunity to test the feasibility of RNA extraction and detection in a larger number of gambiense HAT patient CSF specimens.

The present study also has some limitations. First, although we were able to demonstrate the high sensitivity of RNA detection in blood of HAT patients, we could not determine the specificity of the test, as no specimens from non-HAT control individuals had been collected. However, in 2 previous studies using a similar protocol in Guinea, specificities of respectively 96 and 100% were observed in 49 and 32 endemic healthy controls [5,6]. The strong disparity of the C_q values observed for the internal extraction control in CSF, might indicate that the PAXgene collection and extraction protocol, which is efficient for blood, is suboptimal for the purification of RNA from normal non-inflammatory CSF, which, due to the low WBC number, probably has a low total RNA content. Finally, none of the participating HAT patients has been tested for presence of trypanosomes in the skin [18,19]. Although it cannot be excluded

that blood parasitemia in some patients was below the detection limit of mAECT-BC, lymph or skin trypanosomes could be at the origin of RNA detected in their blood.

In this study, the sensitivity of SL-RNA detection in the blood for diagnosis of HAT was 95.8% (CI 89.8–98.8%). In the past, the value of this parameter was determined at 92% (CI 78–97%) [6] and also at 96.7% (CI 88.7–99.6%), and 95.1% (CI 86.3–99.0%) [5]. However, in those previous studies, trypanosomes had always been detected in lymph and/or blood of the tested individuals, which was not the case here. In the present study, for 19% of the participants, confirmatory diagnosis of HAT was not obtained in lymph or blood and could only be obtained through the examination of the CSF. A previous study [10], which was also conducted in Kwilu, one of the actual study areas in DR Congo, also observed that 4% of gambiense HAT patients are only confirmed by the detection of trypanosomes in the CSF, despite the use of the most sensitive techniques on the blood. This not only underlines the importance of CSF examination for diagnosis of HAT in cases of high suspicion, but also the ability of SL-RNA detection in blood to pick up such patients. This observation also suggests that SL-RNA detection in blood could be an appropriate candidate for blood diagnosis of relapses after treatment, which often present with trypanosomes in the CSF only [11,12].

Detection of trypanosomes in CSF is a marker for the diagnosis of second stage HAT [1]. However, it is not always easy to detect the parasite in this fluid because it is known to be an unsuitable environment for survival of the trypanosome [20]. SL-RNA detection in CSF also stood out for its performance, with a sensitivity assessed at 95.6% (87.6–99.0%) compared to parasitological tests in CSF. There was no significant difference between SL-RNA detection in CSF and modified single centrifugation. The study found that 3 patients with negative CSF parasitology, including one patient in intermediate stage, had a positive SL-RNA test in CSF. In fact, also PCR has sometimes been positive in CSF where CSF parasitology remained negative, or in early or intermediate stage patients [21,22]. In addition, it is known that stage 1 and intermediate stage patients may be PCR positive in the CSF but still successfully treated with first stage drugs [4,23,24]. Although in such cases, PCR positivity could be explained by presence of trypanosome DNA in the CSF without actual presence of the parasite [4,23], presence of SL-RNA in CSF as demonstrated in the actual study, seems to confirm presence of live trypanosomes and active infection. In experimental models, trypanosomes indeed pass the blood brain barrier and are able to invade the central nervous system shortly after entering the bloodstream, and before meningoencephalitis is established [25]. Caution has therefore already been expressed about the interpretation of positive PCR results in otherwise normal CSF [4,26], and the same might apply to RNA detection.

The high sensitivity of SL-RNA RT-qPCR opens the door for future diagnostic applications. However, as for PCR in the past, SL-RNA is, for the moment, not really suitable for routine diagnosis of HAT under field conditions in rural areas where HAT occurs. It requires demanding and critical logistics, including correct specimen collection on RNA stabilization buffer, and transport of the stabilized RNA soon after collection or frozen from the HAT treatment center to a reference lab for proper RNA extraction, which cannot be expected in routine [3]. In addition, RNA collection materials and RNA extraction remain relatively expensive. Complexity of specimen collection and processing, affordability and need of specific equipment therefore represent the main barriers for implementation of RT-qPCR for diagnosis of HAT. In particular circumstances, RNA detection could however correct the shortcomings of DNA detection, especially when high sensitivity is required such as in latent infections, which do not progress to clinical disease and may remain undiagnosed [27], or when demonstration of active infection is critical and DNA detection is not reliable, such as for diagnosis of relapse after treatment [4,5]. Another potential application could be the *a posteriori* confirmation of

HAT in serological suspects who undergo treatment without parasitological examination, as could be the case in future “test and treat” algorithms [28].

Future research should confirm specificity of RNA detection and confirm its sensitivity in CSF. The performance of detection of SL-RNA as a marker for the diagnosis of relapses during post-treatment follow-up of patients with *T.b. gambiense* should be explored further. In particular the high sensitivity of RNA detection in blood could open the door for less invasive diagnosis of relapses, avoiding lumbar puncture. Furthermore, as recently new RNA detection tests have emerged which have only been tested experimentally, including 7SL-derived small RNA [29], future research should also include evaluation of the diagnostic performance of detection of new potentially more sensitive RNA targets on blood and CSF specimens from HAT patients.

In conclusion, with the present study, we confirmed the elevated sensitivity of SL-RNA detection in the blood of HAT patients and demonstrated the diagnostic performance of SL-RNA detection in CSF for the first time, which is a first step towards new applications of RNA detection.

Acknowledgments

We acknowledge all the technicians and health personnel of the General Reference Hospitals of Dipumba, Katanda, Ngandajika, Masi Manimba and Roi Baudouin who have contributed to the study and taken care of the patients. We also acknowledge the personnel of the PNLTHA, INRB and ITM who participated in the study, as well as the assistance of Geneva and Kinshasa DNDi teams, including Christelle Perdrieu, Pere Simarro and Adeline Prêtre.

Author Contributions

Conceptualization: Nick Van Reet, Dieudonné Mumba Ngoyi, Olaf Valverde Mordt, Philippe Büscher, Veerle Lejon.

Data curation: Ipos Ngay Lukusa, Philippe Büscher.

Formal analysis: Ipos Ngay Lukusa, Nick Van Reet, Veerle Lejon.

Funding acquisition: Veerle Lejon.

Investigation: Ipos Ngay Lukusa, Wilfried Mutombo, Digas Ngolo, Sandra Rembry.

Methodology: Nick Van Reet, Sandra Rembry.

Project administration: Dieudonné Mumba Ngoyi, Erick Mwamba Miaka, Wilfried Mutombo, Digas Ngolo, Olaf Valverde Mordt, Antoine Tarral, Sandra Rembry, Philippe Büscher, Veerle Lejon.

Resources: Justin Masumu, Pati Patient Pyana, Vincent Kobo, Felix Akwaso, Médard Ilunga, Lewis Kaninda, Sylvain Mutanda, Dieudonné Mpoyi Muamba.

Supervision: Dieudonné Mumba Ngoyi, Erick Mwamba Miaka, Antoine Tarral, Philippe Büscher, Veerle Lejon.

Validation: Nick Van Reet, Philippe Büscher.

Visualization: Nick Van Reet.

Writing – original draft: Ipos Ngay Lukusa, Veerle Lejon.

Writing – review & editing: Nick Van Reet, Dieudonné Mumba Ngoyi, Erick Mwamba Miaka, Justin Masumu, Pati Patient Pyana, Wilfried Mutombo, Digas Ngolo, Vincent

Kobo, Felix Akwaso, Médard Ilunga, Lewis Kaninda, Sylvain Mutanda, Dieudonné Mpoyi Muamba, Olaf Valverde Mordt, Antoine Tarral, Sandra Rembry, Philippe Büscher.

References

1. WHO, 2013. Control and surveillance of human African trypanosomiasis: report of a WHO Expert Committee. Geneva: World Health Organization; 2013. 237 p. (WHO technical report series).
2. Chappuis F, Loutan L, Simarro P, Lejon V, Buscher P. Options for Field Diagnosis of Human African Trypanosomiasis. *Clin Microbiol Rev.* 2005; 18(1):133–46. <https://doi.org/10.1128/CMR.18.1.133-146.2005> PMID: 15653823
3. Büscher P, Deborggraeve S. How can molecular diagnostics contribute to the elimination of human African trypanosomiasis? *Expert Rev Mol Diagn.* 2015; 15(5):607–15. <https://doi.org/10.1586/14737159.2015.1027195> PMID: 25786994
4. Deborggraeve S, Lejon V, Ekangu RA, Mumba Ngoyi D, Pati Pyana P, Ilunga M, et al. Diagnostic Accuracy of PCR in gambiense Sleeping Sickness Diagnosis, Staging and Post-Treatment Follow-Up: A 2-year Longitudinal Study. *PLoS Negl Trop Dis.* 2011; 5(2):e972. <https://doi.org/10.1371/journal.pntd.0000972> PMID: 21364966
5. Ilboudo H, Camara O, Ravel S, Bucheton B, Lejon V, Camara M, et al. *Trypanosoma brucei gambiense* Spliced Leader RNA Is a More Specific Marker for Cure of Human African Trypanosomiasis Than *T. b. gambiense* DNA. *J Infect Dis.* 2015; 212(12):1996–8. <https://doi.org/10.1093/infdis/jiv337> PMID: 26080371
6. González-Andrade P, Camara M, Ilboudo H, Bucheton B, Jamonneau V, Deborggraeve S. Diagnosis of Trypanosomatid Infections. *J Mol Diagn.* 2014; 16(4):400–4. <https://doi.org/10.1016/j.jmoldx.2014.02.006> PMID: 24814957
7. Nare B, Wring S, Bacchi C, Beaudet B, Bowling T, Brun R, et al. Discovery of Novel Orally Bioavailable Oxaborole 6-Carboxamides That Demonstrate Cure in a Murine Model of Late-Stage Central Nervous System African Trypanosomiasis. *Antimicrob Agents Chemother.* 2010; 54(10):4379–88. <https://doi.org/10.1128/AAC.00498-10> PMID: 20660666
8. Mesu VKBK, Kalonji WM, Bardonneau C, Mordt OV, Blesson S, Simon F, et al. Oral fexinidazole for late-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a pivotal multicentre, randomised, non-inferiority trial. *The Lancet.* 2018; 391(10116):144–54. [https://doi.org/10.1016/S0140-6736\(17\)32758-7](https://doi.org/10.1016/S0140-6736(17)32758-7) PMID: 29113731
9. Lindner AK, Lejon V, Chappuis F, Seixas J, Kazumba L, Barrett MP, et al. New WHO guidelines for treatment of gambiense human African trypanosomiasis including fexinidazole: substantial changes for clinical practice. *Lancet Infect Dis.* 2020; 20(2):e38–46. [https://doi.org/10.1016/S1473-3099\(19\)30612-7](https://doi.org/10.1016/S1473-3099(19)30612-7) PMID: 31879061
10. Mumba Ngoyi D, Ali Ekangu R, Mumvumba Kodi MF, Pyana PP, Balharbi F, Decq M, et al. Performance of Parasitological and Molecular Techniques for the Diagnosis and Surveillance of Gambiense Sleeping Sickness. Ndung'u JM, editor. *PLoS Negl Trop Dis.* 2014; 8(6):e2954. <https://doi.org/10.1371/journal.pntd.0002954> PMID: 24921941
11. Miezan TW, Dje NN, Doua F, Boa F. Trypanosomose humaine africaine en Côte d'ivoire: caractéristiques biologiques après traitement. *Bull Soc Pathol Exot.* 2002; 95(5):362–5 PMID: 12696377
12. Bisser S, N'Siesi F, Lejon V, Preux P, Van Nieuwenhove S, Miaka Mia Bilenge C, et al. Equivalence Trial of Melarsoprol and Nifurtimox Monotherapy and Combination Therapy for the Treatment of Second-Stage *Trypanosoma brucei gambiense* Sleeping Sickness. *J Infect Dis.* 2007; 195(3):322–9. <https://doi.org/10.1086/510534> PMID: 17205469
13. Camara M, Camara O, Ilboudo H, et al., "Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. *Trop Med Int Health* 2010; 15(7): 796–9. <https://doi.org/10.1111/j.1365-3156.2010.02546.x> PMID: 20497407
14. Büscher P, Mumba Ngoyi D, Kaboré J et al. Improved Models of Mini Anion Exchange Centrifugation Technique (mAECT) and Modified Single Centrifugation (MSC) for Sleeping Sickness Diagnosis and Staging. *PLoS Negl Trop Dis.* 2009; 3(11): e471. <https://doi.org/10.1371/journal.pntd.0000471> PMID: 19936296
15. Laurell H, Iacovoni JS, Abot A, Svec D, Maoret J-J. Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. *Nucleic Acids Res.* 2012; 40(7):10. <https://doi.org/10.1093/nar/gkr1259> PMID: 22228834
16. Fadda A, Ryten M, Droll D et al. Transcriptome-wide analysis of trypanosome mRNA decay reveals complex degradation kinetics and suggests a role for co-transcriptional degradation in determining mRNA levels: Transcriptome-wide mRNA decay in trypanosomes. *Mol Microbiol.* 2014; 94(2): 307–26. <https://doi.org/10.1111/mmi.12764> PMID: 25145465

17. Blum J, Schmid C, Burri C. Clinical aspects of 2541 patients with second stage human African trypanosomiasis. *Acta Trop*. 2006; 97(1):55–64. <https://doi.org/10.1016/j.actatropica.2005.08.001> PMID: [16157286](https://pubmed.ncbi.nlm.nih.gov/16157286/)
18. Capewell P, Cren-Travaillé C, Marchesi F, Johnston P, Clucas C, Benson RA, et al. The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes. *eLife*. 2016; 5: e17716. <https://doi.org/10.7554/eLife.17716> PMID: [27653219](https://pubmed.ncbi.nlm.nih.gov/27653219/)
19. Camara M, Soumah AM, Ilboudo H, Travaillé C, Clucas C, Cooper A, et al. Extravascular Dermal Trypanosomes in Suspected and Confirmed Cases of gambiense Human African Trypanosomiasis. *Clin Infect Dis*. 2020; <https://doi.org/10.1093/cid/ciaa897> PMID: [32638003](https://pubmed.ncbi.nlm.nih.gov/32638003/)
20. Pentreath VW, Owolabi AO, Doua F. Survival of *Trypanosoma brucei brucei* in cerebrospinal fluid. *Ann Trop Med Parasitol*. 1992; 86(1):29–34. <https://doi.org/10.1080/00034983.1992.11812627> PMID: [1616393](https://pubmed.ncbi.nlm.nih.gov/1616393/)
21. Truc P, Jamonneau V, Cuny G. Use of polymerase chain reaction in human African trypanosomiasis stage determination and follow-up. *Bull World Health Organ*. 1999; 77(9):745–8. PMID: [10534898](https://pubmed.ncbi.nlm.nih.gov/10534898/)
22. Kyambadde JW, Enyaru JCK, Matovu E, Odiit M, Carasco JF. Detection of trypanosomes in suspected sleeping sickness patients in Uganda using the polymerase chain reaction. *Bull World Health Organ*. 2000; 78(1):119–24. PMID: [10686746](https://pubmed.ncbi.nlm.nih.gov/10686746/)
23. Jamonneau V, Solano P, Garcia A, Lejon V, Dje N, Miezan TW, et al. Stage determination and therapeutic decision in human African trypanosomiasis: value of polymerase chain reaction and immunoglobulin M quantification on the cerebrospinal fluid of sleeping sickness patients in Cote d'Ivoire. *Trop Med Int Health*. 2003; 8(7):589–94. <https://doi.org/10.1046/j.1365-3156.2003.01079.x> PMID: [12828540](https://pubmed.ncbi.nlm.nih.gov/12828540/)
24. Truc P, Lando A, Penchenier L, Vatunga G, Josenando T. Human African trypanosomiasis in Angola: clinical observations, treatment, and use of PCR for stage determination of early stage of the disease. *Trans R Soc Trop Med Hyg*. 2012; 106(1):10–4. <https://doi.org/10.1016/j.trstmh.2011.10.002> PMID: [22093811](https://pubmed.ncbi.nlm.nih.gov/22093811/)
25. Frevert U, Movila A, Nikolskaia OV, Raper J, Mackey ZB, Abdulla M, et al. Early Invasion of Brain Parenchyma by African Trypanosomes. *PLoS ONE*. 2012; 7(8):e43913. <https://doi.org/10.1371/journal.pone.0043913> PMID: [22952808](https://pubmed.ncbi.nlm.nih.gov/22952808/)
26. Jamonneau V, Solano P, Koffi M, Denizot M, Cuny G. Apports et limites du diagnostic de la trypanosomiase humaine africaine. *Med Sci*. 2004; 20(10):871–5.
27. Büscher P, Bart J-M, Boelaert M, Bucheton B, Cecchi G, Chitnis N, et al. Do Cryptic Reservoirs Threaten Gambiense-Sleeping Sickness Elimination? *Trends Parasitol*. 2018; 34(3):197–207. <https://doi.org/10.1016/j.pt.2017.11.008> PMID: [29396200](https://pubmed.ncbi.nlm.nih.gov/29396200/)
28. Eperon G, Balasegaram M, Potet J, Mowbray C, Valverde O, Chappuis F. Treatment options for second-stage gambiense human African trypanosomiasis. *Expert Rev Anti Infect Ther*. 2014; 12(11): 1407–17. <https://doi.org/10.1586/14787210.2014.959496> PMID: [25204360](https://pubmed.ncbi.nlm.nih.gov/25204360/)
29. Chiweshe SM, Steketee PC, Jayaraman S, Paxton E, Neophytou K, Erasmus H, et al. Parasite specific 7SL-derived small RNA is an effective target for diagnosis of active trypanosomiasis infection. *Caljon G*, editor. *PLoS Negl Trop Dis*. 2019; 13(2):e0007189. <https://doi.org/10.1371/journal.pntd.0007189> PMID: [30779758](https://pubmed.ncbi.nlm.nih.gov/30779758/)