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Diagnosis of Trypanosomatid Infections

Targeting the Spliced Leader RNA

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Trypanosomatids transcribe their genes in large polycistronic clusters that are further processed into mature mRNA molecules by trans-splicing. During this maturation process, a conserved spliced leader RNA (SL-RNA) sequence of 39 bp is physically linked to the 5′ end of the pre-mRNA molecules. Trypanosomatid infections cause a series of devastating diseases in man (sleeping sickness, leishmaniasis, Chagas disease) and animals (nagana, surra, dourine). Here, we investigated the SL-RNA molecule for its diagnostic potential using reverse transcription followed by real-time PCR. As a model, we used *Trypanosoma brucei gambiense*, which causes sleeping sickness in west and central Africa. We showed that the copy number of the SL-RNA molecule in one single parasitic cell is at least 8600. We observed a lower detection limit of the SL-RNA assay in spiked blood samples of 100 trypanosomes per milliliter of blood. We also proved that we can detect the trypanosome's SL-RNA in the blood of sleeping sickness patients with a sensitivity of 92% (95% CI, 78%—97%) and a specificity of 96% (95% CI, 86%—99%). The SL-RNA is thus an attractive new molecular target for next-generation diagnostics in diseases caused by trypanosomatids. *(J Mol Diagn 2014, 16: 400—404; http://dx.doi.org/10.1016/j.jmoldx.2014.02.006)*

Trypanosomatids are early eukaryotic protozoans that possess unique molecular features. Genes typically do not harbor introns but are arranged in large polygenic clusters that are transcribed polycistronically.[1] Polycistronic pre-mRNA is further processed into functional monocistronic mRNA molecules by trans-splicing and polyadenylation. During this RNA maturation process, a conserved spliced leader RNA (SL-RNA) molecule is donated to the 5′ end of each individual mRNA.[2] The SL-RNA sequence of the trypanosomatids can be 39 to 41 nucleotides long and carries methyl groups on the first four ribose moieties and on the first and fourth bases following the conserved 7-methylguanosine (m7G).[2,3]

Among the trypanosomatids are three major human pathogens: *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* spp. Their trans-splicing mechanisms have been extensively investigated for understanding eukaryotic cell biology, but the SL-RNA has never been explored as a diagnostic target, although this molecule has several attractive diagnostic features. It is a short, noncoding RNA sequence that is conserved, but unique, for each species and is present in each mRNA molecule in the cell. Importantly, mRNA is considered to be

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the best surrogate marker for viable organisms,[4,5] because it is rapidly degraded after death of the organism with a typical half-life of 3 minutes.[6] Here, we describe the first study that explores the trypanosomatid SL-RNA molecule for its diagnostic potential.

As a model, we used sleeping sickness, a devastating disease that is endemic in sub-Saharan Africa and caused by the *T. brucei* subspecies *gambiense* and *rhodesiense*. The trypanosomes are transmitted to humans by tsetse flies and invade the brain, which in most cases is fatal if not treated in time. *T. brucei gambiense* is associated with the chronic form of sleeping sickness in west and central Africa, whereas *T. brucei rhodesiense* causes acute sleeping sickness in east Africa. Here, we show that the *T. brucei* SL-RNA is an excellent target molecule for the detection of the trypanosomes in clinical specimens.

Materials and Methods

Oligonucleotides

Oligonucleotides were synthesized and purified using high-performance liquid chromatography by Integrated DNA Technologies (IDT, Leuven, Belgium). Nucleic acid sequences were as follows: SL-RNA molecule, 5′-AACUAACGCUAUUAUUAGAACAGUUUCUGUACUAUAUUG-3′; SL-RNA–specific primer for reverse transcription and real-time PCR (cSL), 5′-CAATATAGTACAGAAACTG-3′; forward primer for real-time PCR (SL-F), 5′-AACTAACGCTATTATTAGAA-3′.

Parasites

T. brucei gambiense (LiTat 1.3), *T. brucei rhodesiense* (AnTat 25.1), *T. brucei brucei* (Lister 427), *T. evansi* (Kazakstan), *T. equiperdum* (OVI), and *T. vivax* (ILRAD700) were propagated in OF1 mice or HsdCpb:WU rats (Harlan Laboratories, Horst, The Netherlands). Trypanosomes were separated from the blood using diethylaminoethyl cellulose chromatography[7] followed by repeated centrifugation and sediment washes with PBS glucose [38 mmol/L $Na_2HPO_4 \cdot H_2O$, 2 mmol/L $NaHPO_4$, 80 mmol/L glucose, 29 mmol/L NaCl (pH 8.0)]. *T. congolense* (Savannah type, KTT/MSORO/M7), *T. rangeli* (ScTb), and *Leishmania donovani* (BPK282/0cl4) were propagated *in vitro* as described previously.[8–10] *In vitro*–cultured parasites were harvested by repeated centrifugation and washing in PBS glucose. Parasite concentrations in PBS glucose were measured using Uriglass counting chambers (Menarini, Vienna, Austria).

Patients and Controls

Thirty-six blood samples from sleeping sickness patients and 49 blood samples from endemic controls were analyzed with the SL-RNA assay. Patients and controls were recruited in the main sleeping sickness foci along the Guinean coast (Forecariah, Dubreka, and Boffa) between 2007 and 2009 as reported by Ilboudo et al[11] in 2012. This study is part of a larger project aiming to improve sleeping sickness diagnosis for which approval was obtained from the World Health Organization (Research Ethics Review Committee) and from the ethical committee of the Institut de Recherche pour le Développement (Comité Consultatif de Déontologie et d'Ethique). All participants were informed of the objectives of the project in their own language and signed a written informed consent form. For participants <18 years of age, informed consent was obtained from their parents. Sleeping sickness patients had a positive result in the card agglutination test for trypanosomiasis (CATT) for antibody detection in blood,[12] and trypanosomes were detected in their blood by the mini anion exchange centrifugation technique.[13] Endemic controls did not show antibodies against *T.b. gambiense* in the CATT and in the immune trypanolysis assay with LiTat 1.3, LiTat 1.5, and LiTat 1.6 *T.b. gambiense* parasites.[14] For RNA isolation, 2 mL of blood was taken in PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) from patients and controls, and transported at −20°C to Centre International de Recherche-Développement sur l'Elevage en Zone Subhumide (Bobo-Dioulasso, Burkina-Faso). On arrival, samples were immediately stored at −80°C until the RNA was isolated.

RNA Isolation and cDNA Preparation

Total RNA from laboratory-cultured parasites and spiked human blood samples were extracted using TRIzol (Ambion, Austin, TX). Briefly, 10 volumes of TRIzol reagent and two volumes of chloroform were added to the sample, followed by centrifugation at 12,000 × *g* for 15 minutes. The RNA in the aqueous phase was precipitated in isopropanol, washed in 75% ethanol, and then dissolved in 50 μL of diethylpyrocarbonate-treated water. Total RNA from the blood of patients and controls was extracted with the PAXgene Blood RNA kit (PreAnalytiX), and RNA was eluted in 80 μL of diethylpyrocarbonate-treated water. Copy DNA (cDNA) of the SL-RNA was prepared using the SL-RNA–specific cSL primer and SuperScript II Reverse Transcriptase (Invitrogen/Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Real-Time PCR

SL-RNA cDNA levels were measured by real-time PCR in the LightCycler 480 PCR system (Roche Applied Science, Penzberg, Germany). Two and a half microliters of cDNA were mixed with 0.25 μmol/L cSL and SL-F primers in a 25-μL reaction containing 1× SensiMix SYBR green No-Rox (Bioline, London, UK). Thermal cycling conditions were 10 minutes incubation at 95°C, followed by 40 cycles of 94°C for 15 seconds, 50°C for 15 seconds, and 60°C for 15 seconds with data acquisition. Post-amplification melting curves were recorded from 45°C to 95°C, with increments

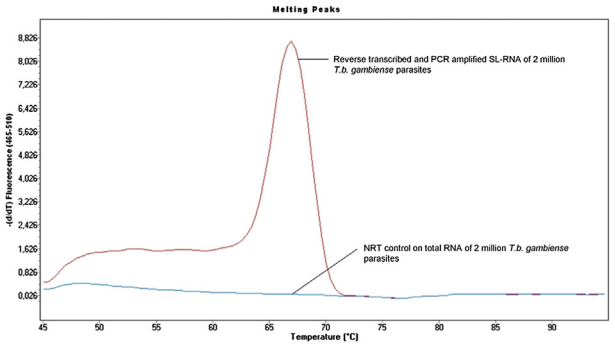

Figure 1 Melting peak of reverse-transcribed and PCR-amplified SL-RNA in total RNA from 2 million *T.b. gambiense* parasites. Non-reverse-transcribed (NRT) total RNA was used as a negative control.

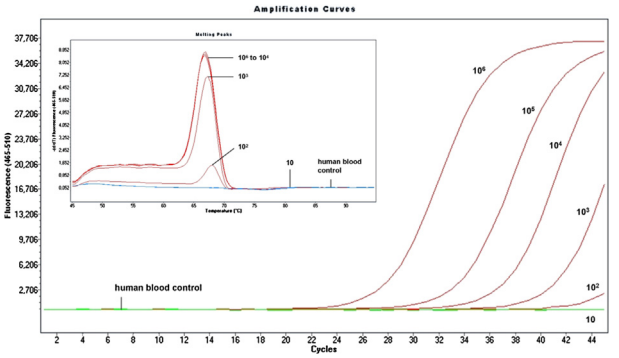

Figure 2 Amplification curves and melting peaks (**inset**) of the SL-RNA in 10-fold serial dilutions of *T.b. gambiense* parasites in human blood. Expressed as parasites per milliliter of blood.

of 0.1°C per second. Genomic DNA contamination in the RNA samples was verified by subjecting the RNA to real-time PCR without cDNA preparation.

Statistics

Mean threshold cycle (C_t) values of duplicate samples in real-time PCR were calculated with one-sigma SD (σ). Sensitivities and specificities of the SL-RNA assay in sleeping sickness patients and endemic controls were calculated at a 95% CI using Wilson's score. Sensitivity is defined as the proportion of confirmed sleeping sickness patients who are positive by the index test, and specificity as the proportion of endemic controls who are negative by the index test.

Results

Proof-of-Concept

The SL-RNA sequences of *T.b. gambiense* and *T.b. rhodesiense* were aligned *in silico*, together with those from the closely related *T.b. brucei*, *T. equiperdum*, *T. evansi*, *T. congolense*, *T. vivax*, *T. rangeli*, *T. cruzi*, and *L. donovani* using Clustal Omega version 1.2.1 (Supplemental Figure S1). The alignment shows a *Trypanozoon*-specific central region from bp 11 to 20 and a hyperconserved region from bp 21 to 39. The *Trypanozoon* subgenus consists of the human infective *T. brucei gambiense* and *T. brucei rhodesiense*, as well as the animal pathogens *T. brucei brucei*, *T. evansi*, and *T. equiperdum*. The cSL primer used during reverse transcription of the SL-RNA is complementary to the 19-bp hyperconserved sequence. The additional primer that is used during PCR is situated adjacent and has its 3′ region in the *Trypanozoon*-specific region. To deliver the proof of concept, we extracted total RNA from 2 million *T.b. gambiense* parasites and detected the SL-RNA molecules by reverse transcription and real-time PCR. The melting peak of the amplified cDNA showed a melting temperature of 67°C (Figure 1), which corresponds to the *in silico*—predicted melting temperature of the 39-bp *T.b. gambiense* SL-RNA sequence (66.5°C). Non-reverse-transcribed total RNA did not show any amplified DNA during the real-time PCR. Using standards of chemically synthesized SL-RNA molecules and the equation

$$\left[\frac{\text{gRNA}}{(\text{length} \times 340)}\right] \times 6.023 \times 10^{23} = \text{number of RNA molecules}, \quad (1)$$

we have estimated the number of SL-RNA molecules per parasite. We found that there are at least 8600 (σ = 1080) SL-RNA molecules in one single trypanosome.

Analytical Sensitivity

We determined the analytical sensitivity by applying the SL-RNA assay to RNA extracts from naive human blood samples spiked with known numbers of *T.b. gambiense* parasites, ranging from 1,000,000 to 10 parasites per milliliter (p/mL) of blood in a 10-fold dilution series. All samples were prepared in duplicate, and the analytical sensitivity of the assay was defined as the lower dilution that showed a positive test result in both duplicates. The analytical sensitivity of the SL-RNA assay is 100 p/mL of blood (Figure 2). Mean Ct values of the duplicate samples were 29.0 (σ = 0.80) for 1,000,000 p/mL, 33.35 (σ = 0.98) for 100,000 p/mL, 36.35 (σ = 0.80)

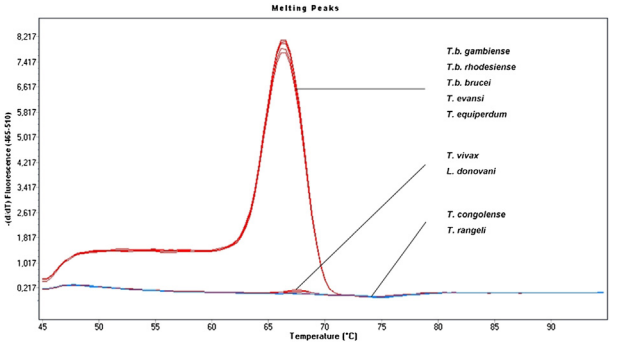

Figure 3 Melting peaks of reverse-transcribed and PCR-amplified total RNA (10 pg) from different trypanosomatids.

for 10,000 p/mL, and >40 for 1000 and 100 p/mL. Non-spiked blood remained negative in the assay.

Analytical Specificity

To determine the analytical specificity of the SL-RNA assay, we tested 10 pg of total RNA from different *Trypanosoma* species and from *Leishmania* (Figure 3). Our assay only detects the parasites of the *Trypanozoon* subgenus *T. vivax* and *Leishmania* showed a minor amplification signal, and the SL-RNA of *T. rangeli* and *T. congolense* were not amplified at all. These observations are fully consistent with the *in silico* alignments of the SL-RNA sequences from these organisms (Supplemental Figure S1).

Patients and Controls

Out of the 36 sleeping sickness patients, 33 were positive in the SL-RNA assay, whereas 47 of 49 endemic controls remained negative. This corresponds with a test sensitivity of 92% (95% CI, 78%−97%) and a test specificity of 96% (86%−99%). No DNA contamination was observed in the analyzed RNA samples.

Discussion

Technological advancements are continuously expanding our portfolio of diagnostic tests for infectious diseases. In parallel with the search for more accurate technologies to detect infectious agents, there is a need to provide the best target molecule for such a diagnostic test. Here, we show that the spliced leader RNA is an excellent target for accurate detection of trypanosomatids in blood samples. The proof of concept was delivered for sleeping sickness, but the idea can be exploited for Chagas disease and leishmaniasis, as well as for important animal diseases such as nagana and surra. Furthermore, nematodes share a similar trans-splicing mechanism to process their mRNA,[15] and a similar approach can thus be used to detect the SL-RNA of parasitic nematodes in man and animals.

We reverse transcribed the 39-bp SL-RNA RNA of *T.b. gambiense* and analyzed the cDNA by real-time PCR in experimentally prepared samples and in blood samples from sleeping sickness patients. Despite the very short length, the assay was highly efficient in detecting trypanosomes and did not show any amplification artifacts such as primer dimers. Crucial steps during the optimization of the assay were efficient RNA extractions without genomic DNA contamination, the annealing temperature of the oligonucleotides during reverse transcription, and the annealing and elongation temperatures during real-time PCR. In blood samples spiked with known numbers of *T.b. gambiense,* we were able to detect the parasite's SL-RNA in as few as 100 parasites per milliliter of blood. This is in the same range as DNA detection methods such as PCR and LAMP, which target multicopy DNA sequences of *T. brucei*,[16−18] and a nucleic acid sequence−based amplification assay, which targets the ribosomal RNA.[19] However, the major advantage of our SL-RNA assay is that we target the parasite's mRNA, which is considered to be a better marker for viable organisms than DNA or rRNA.[4,5] Analytical specificity experiments showed that the assay did not detect RNA from genetically closely related trypanosomatids. *In silico* analysis of our primers did not indicate any potential cross-reaction with other microorganisms that are endemic in sleeping sickness foci and cause diseases with similar clinical symptoms, such as malaria parasites for fever and meningococcal bacteria for neurological symptoms (data not shown). We also verified whether we could detect the *T.b. gambiense* SL-RNA in the blood of patients with sleeping sickness. Our SL-RNA assay showed a sensitivity of 92% and specificity of 96% in 36 patients and 49 endemic controls from Guinea. The two endemic controls that showed positive SL-RNA test results may be true sleeping sickness cases that were missed by the reference tests, although this is unlikely because the serological reference tests make use of three different immunodominant antigens, the LiTat 1.3, LiTat 1.5, and LiTat 1.6 variant surface glycoproteins. Because the SL-RNA assay detects the *Trypanozoon* subgenus, we cannot exclude a transient infection with *T.b. brucei*, which is not detected by the serological reference tests used in this study. *T.b. brucei* is generally not infectious to man but has been reported in atypical human infections.[20] Although not observed during the optimization and the analytical sensitivity and specificity experiments, we cannot exclude amplification artifacts in the SL-RNA assay. By including standards of chemically synthesized SL-RNA in the assay, we showed that the number of SL-RNA molecules in one single *T.b. gambiense* cell is at least 8600. This is consistent with what we theoretically expect based on previous studies. The *T. brucei* genome project reported 9000 protein coding genes,[1] and a subsequent transcriptome study showed that 75% of these genes generate 1 to 10 mRNAs per cell.[21]

Trypanosomatids are responsible for several diseases in man, animals, and even plants.[22] Sleeping sickness, leishmaniasis, and Chagas disease have the worst reputation because they cause >100,000 deaths each year. Accurate diagnosis of these diseases is challenging because of the extremely low parasite load in the blood of patients. Nucleic acid−based tests are often considered to be the future reference methods because they generally show high sensitivity and specificity.[16] In this study, we have shown for the first time the great potential of the SL-RNA as a marker for trypanosomatids in the blood of patients. This is an important new step in expanding our biomarker portfolio for diagnosing diseases caused by trypanosomatids, as well as by other pathogens sharing a similar trans-splicing mechanism such as nematodes.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2014.02.006*.

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