EXPERT REVIEWS

# Recent progress in molecular diagnosis of sleeping sickness

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This article will review the most recent progress in the molecular diagnosis of sleeping sickness and its potential role in patient management and disease control. While PCR remains restricted to research and reference laboratories, promising alternative molecular platforms have emerged over the last few years. Several loop-mediated isothermal amplification assays have been developed for detection and identification of the parasite with reported high analytical sensitivity and specificity. Simplified loop-mediated isothermal amplification formats have been designed and are undergoing evaluation studies in the field. Accurate diagnosis based on specific detection of the parasite's ribosomal RNA has been made possible by the isothermal nucleic acid sequence-based amplification and by direct hybridization with fluorescent detection probes. In addition to the technological progress, the authors also discuss the diagnostic performance of molecular tests in the most recent clinical evaluation studies and briefly present some viewpoints for the near future.

**KEYWORDS:** diagnosis • diagnostic accuracy • DNA • hybridization • LAMP • NASBA • PCR • RNA • sleeping sickness • *Trypanosoma brucei gambiense* • *Trypanosoma brucei rhodesiense*

Sleeping sickness (human African trypanosomiasis) is a life-threatening infectious disease endemic in sub-Saharan Africa. The disease is caused by infection with either *Trypanosoma brucei* (*T.b.*) *gambiense* (Western and Central Africa) or *T.b. rhodesiense* (Eastern Africa) through the bite of an infected *Glossina* tsetse fly. *T.b. gambiense* accounts for >90% of all reported cases and is associated with chronic sleeping sickness (months to years) while *T.b. rhodesiense* causes an acute form of the disease (weeks to months). In contrast to *T.b. gambiense*, the reservoir hosts of *T.b. rhodesiense* are cattle and wild animals, while humans are rather an accidental host. The disease starts with a hemolymphatic (first) stage in which the trypanosomes dwell in blood, lymph and other peripheral tissues of the patient, generating nonspecific clinical symptoms such as fever, general malaise, headaches and joint pains. The neurological (second) stage is initiated by parasites crossing the blood–brain barrier and settling in the central nervous system. Patients in the second stage show severe behavioral changes, sensory disturbances, abnormal sleep–wake rhythm and poor coordination, albeit these typical symptoms are generally observed in *gambiense* patients while many *rhodesiense* patients succumb from heart failure. Thanks to sustained control activities in the last two decades, combined with changing land-use, the number of reported sleeping sickness cases dropped below 10,000 in 2009 (FIGURE 1) [SIMARRO P, UNPUBLISHED DATA] [1]. However, the actual number of infected cases is probably three to four times higher [2], as the disease is endemic in sociopolitically unstable or poorly accessible areas, hindering accurate mapping of the disease. Elimination of sleeping sickness is in sight [3,4], but will rely on long-term control activities including tsetse control, diagnosis and treatment of patients, followed by surveillance of cleared disease foci. In travel medicine, sleeping sickness is rare with only a couple of dozen cases reported in the last 20 years [5,6].

## Standard diagnostic tools & their limitations

Despite the technological progress in recent decades, diagnosis of sleeping sickness in Africa is still based on two techniques: antibody detection in an agglutination test and/or parasite detection by microscopy. *Gambiense* sleeping sickness is often diagnosed via systematic screening by mobile teams of the populations at risk in endemic areas. Persons are tested for antibodies against *T.b. gambiense* in their blood using the card agglutination test for trypanosomiasis (CATT) [7]. CATT-positive individuals then undergo microscopic examination of their lymph and/or blood to confirm the presence of trypanosomes. Given the low parasite load

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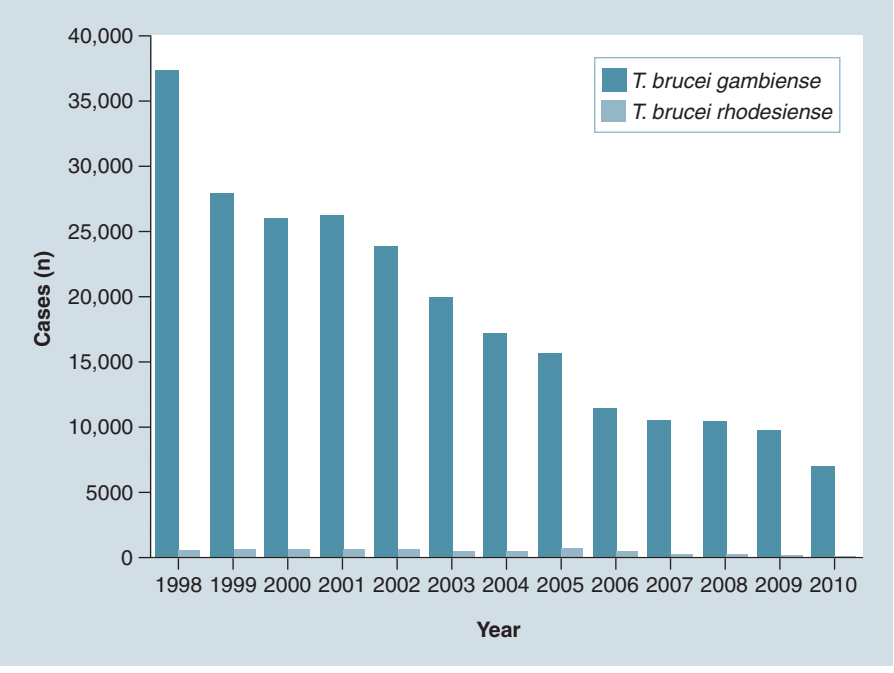


**Figure 1. Number of reported human cases of *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* infections between 1998 and 2010.**
Data provided by P Simarro (WHO).

in the blood of *gambiense* sleeping sickness patients, a parasite concentration technique is often implemented before microscopic examination. Field-applicable concentration methods include the microhematocrit centrifugation technique [8], the quantitative buffy coat (QBC) technique [9] and the mini-anion exchange centrifugation technique (mAECT) [10]. The CATT test has formed the basis for diagnosis of *gambiense* sleeping sickness for more than 30 years owing to its ease of use, low cost and high sensitivity. However, its limited specificity results in a substantial number of nonconfirmed seropositive individuals and in some disease foci, the sensitivity of the CATT is rather low [11]. The mAECT technique is the most sensitive trypanosome detection format with a detection limit of <50 parasites per ml blood [12,13]. Despite its high sensitivity and specificity, large-scale use of mAECT is hampered by its high cost and irregular production. *Rhodesiense* sleeping sickness patients are generally diagnosed when they seek medical care and, owing to the presentation of nonspecific symptoms, many cases are first misdiagnosed as malaria [14]. In the absence of a serological test, infection with *T. b. rhodesiense* is determined by parasite detection in the blood. In addition to parasitological confirmation of the infection, accurate disease staging is paramount since first and second disease stage patients require different drugs and treatment regimens [15,16]. Thus, patients with parasites detected in the lymph or blood as well as nonconfirmed CATT seropositives (end titer >1/8) with neurological symptoms undergo lumbar puncture to examine the cerebrospinal fluid (CSF) for markers of neuro-invasion. These markers are the presence of parasites, best detected by the modified single centrifugation of the CSF [17], or increased number of white blood cells in the CSF (>5 cells per µl). After treatment, sleeping sickness patients should be followed-up for 2 years before definite cure can be confirmed [18]. The 6-monthly hospital visits and lumbar punctures during this follow-up are highly demanding for the patient, with poor adherence as a result. By defining new follow-up criteria, Mumba *et al.* showed that it is possible to shorten the follow-up period to a maximum of 12 months [19].

## Recent progress in molecular diagnosis of sleeping sickness

### *Sample collection & nucleic acid extraction*

Molecular diagnostics for sleeping sickness detect the parasite's DNA or RNA in a clinical specimen taken from the patient (Table 1). Most techniques are based on enzymatic amplification of parasite-specific nucleic acid sequences and require prior DNA or RNA purification to minimize enzyme inhibition. As well as factors affecting enzyme activity, biological specimens also contain nucleases that rapidly degrade nucleic acids. Therefore, nucleic acid degradation should be kept minimal during storage and transport of the specimen to the reference laboratories that are usually far away from the sleeping sickness foci. Basiye *et al.* recently compared seven different protocols for DNA and RNA stabilization in whole blood at -20°C, 4°C and 26°C for up to 10 weeks [20]. No RNA degradation was observed when blood was stored at -20°C or 4°C in a homemade guanidium-based L3 buffer and the DNA quality remained unaffected even at 26°C. Long-term storage of DNA in whole blood is also possible using Flinders Technology Associates (FTA) cards (Whatman, UK). These filter paper cards contain a chemically treated fiber matrix that lyses cells and protects DNA from degradation. Ahmed *et al.* compared five different techniques for blood sample processing from FTA cards for *T.b.* diagnosis in 300 cattle from Uganda [21]. The authors recommended DNA elution from FTA cards using Chelex® 100 (Bio-Rad, CA, USA). Using the same FTA/Chelex approach, *T.b. rhodesiense* DNA was successfully detected in blood of 128 sleeping sickness patients from Uganda and Tanzania [22]. A low-cost alternative for FTA cards are plain cellulose filters such as Whatman 4, which work well for DNA storage [23]. In conclusion, for RNA preservation in clinical specimens, commercial buffers or quality-controlled homemade guanidium-based buffers are recommended. From personal experience, for DNA preservation, the authors also prefer guanidium buffers, such as the AS1 buffer (Qiagen, Germany) over FTA cards for several reasons: low cost, compatibility with automated or semi-automated extraction protocols, closed tube format and the larger blood volume that can be stored. In particular, using larger volumes of specimen for DNA extraction increases the repeatability and sensitivity of molecular diagnosis of sleeping sickness [Büscher P, Unpublished data].

**Table 1. Reported detection limits of different molecular diagnostics for sleeping sickness.**

| Study (year) | Target group | Assay | Target NA | Primer and probe sequences (5′–3′ direction) | | LOD† | Ref. |
|---|---|---|---|---|---|---|---|
| Moser *et al.* (1989) | *Trypanozoon* | Single PCR | Satellite DNA | TBR-1 | 5′-CGAATGAATATTAAACAATGCGCAG-3′ | 0.1 | [24] |
| | | | | TBR-2 | 5′-AGAACCATTTATTAGCTTTGTTGC-3′ | | |
| Holland *et al.* (2001) | *Trypanozoon* | | *ESAG 6/7* gene | ESAG6/7-F | 5′-ACATTCCAGCAGGAGTTGGAG-3′ | 5 | [25] |
| | | | | ESAG6/7-R | 5′-CACGTGAATCCTCAATTTTGT-3′ | | |
| Desquesnes *et al.* (2001) | *Trypanozoon* | | ITS1 DNA | Kin1 | 5′-GCGTTCAAAGATTGGGCAAT-3′ | 100–10,000 | [26] |
| | | | | Kin2 | 5′-CGCCCGAAAGTTCACC-3′ | | |
| Njiru *et al.* (2005) | *Trypanozoon* | | ITS1 DNA | ITS1 CF | 5′-CCGGAAGTTCACCGATATTG-3′ | 100–1000 | [27] |
| | | | | ITS1 BR | 5′-TTGCTGCGTTCTTCAACGAA-3′ | | |
| Kabiri *et al.* (1999) | *Trypanozoon* | Nested PCR | *ESAG 6/7* gene | Museq1 | 5′-GCGTTAGCAGCAGCTGCAGCTGGG-3′ | 0.1 | [28] |
| | | | | Museq2 | 5′-CCTCCTCGGATATTTTCCGCACCC-3′ | | |
| Cox *et al.* (2005) | *Trypanozoon* | | ITS1 DNA | ITS1 | 5′-GATTACGTCCCTGCCATTTG-3′ | 1 | [29] |
| | | | | ITS2 | 5′-TTGTTCGCTATCGGTCTTCC-3′ | | |
| | | | | ITS3 | 5′-GGAAGCAAAAGTCGTAACAAGG-3′ | | |
| | | | | ITS4 | 5′-TGTTTTCTTTTCCTCCGCTG-3′ | | |
| Adams *et al.* (2006) | *Trypanozoon* | | ITS1 DNA | TRYP1 | 5′-AAGCCAAGTCATCCATCG-3′ | 1 | [30] |
| | | | | TRYP2 | 5′-TAGAGGAAGCAAAAG-3′ | | |
| | | | | TRYP3 | 5′-TGCAATTATTGGTCGCGC-3′ | | |
| | | | | TRYP4 | 5′-CTTTGCTGCGTTCTT-3′ | | |
| Becker *et al.* (2004) | *Trypanozoon* | Real-time PCR | Satellite DNA | Tb177F | 5′-AACAATGCGCAGTTAACGCTAT-3′ | 0.1 | [31] |
| | | | | Tb177R | 5′-ACATTAAACACTAAAGAACAGCGTTG-3′ | | |
| Deborggraeve *et al.* (2006) | *Trypanozoon* | PCR-LF | 18S rRNA gene | 18S-F | 5′-CGCCAAGCTAATACATGAACCAA-3′ | 0.05 | [32] |
| | | | | 18S-R | 5′-TAATTTCATTCATTCGCTGGACG-3′ | | |
| | | | | Probe | 5′-TTGTGTTTACGCACTTG-3′ | | |
| Kuboki *et al.* (2003) | *Trypanozoon* | LAMP | *PFRA* gene | A1-F3 | 5′-TCACAACAAGACTCGCACG-3′ | 10 | [33] |
| | | | | A1-B3 | 5′-GGGCTTTGATCTGCTCCTC-3′ | | |
| | | | | A1-FIP | 5′-TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC-3′ | | |
| | | | | A1-BIP | 5′-CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT-3′ | | |

†LOD = number of parasites detected per reaction; one parasite contains approximately 0.1 pg genomic DNA.
ESAG6/7: Expression site-associated genes 6/7; ITS1: First internal transcribed spacer; LOD: Limit of detection; LAMP: Loop-mediated isothermal amplification; LAMP-LF: LAMP lateral flow; MDA: Multiple displacement amplification; NA: Nucleic acid; NASBA: Nucleic acid sequence-based amplification; NASBA-LF: NASBA lateral flow; PCR-LF: PCR lateral flow; PFRA: Paraflagellar rod protein A gene; PNA-FISH: Peptide nucleic acid FISH; RIME: Repetitive insertion mobile element; rRNA: Ribosomal RNA; SRA: Serum resistance-associated gene; TgsGP: *Trypanosoma brucei gambiense*-specific glycoprotein.

**Table 1. Reported detection limits of different molecular diagnostics for sleeping sickness (cont.).**

| Study | Target group | Assay | Target NA | Primer and probe sequences (5′–3′ direction) | | LOD† | Ref. |
|---|---|---|---|---|---|---|---|
| Njiru *et al.* (2008) | *Trypanozoon* | LAMP | RIME DNA | RIME-F3 | 5′-CTGTCCGGTGATGTGGAAC-3′ | 0.001 | [34] |
| | | | | RIME-B3 | 5′-CGTGCCTTCGTGAGAGTTTC-3′ | | |
| | | | | RIME-FIP | 5′-GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA-3′ | | |
| | | | | RIME-BIP | 5′-AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC-3′ | | |
| | | | | RIME-LF | 5′-GCCTCCCACCCTGGACTC-3′ | | |
| | | | | RIME-LB | 5′-AGACCGATAGCATCTCAG-3′ | | |
| Njiru *et al.* (2011) | *Trypanozoon* | LAMP-LF | 18S rRNA gene | RIME-F3 | 5′-CTGTCCGGTGATGTGGAAC-3′ | 0.001 | [35] |
| | | | | RIME-B3 | 5′-CGTGCCTTCGTGAGAGTTTC-3′ | | |
| | | | | RIME-FIP | 5′-GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA-3′ | | |
| | | | | RIME-BIP | 5′-AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC-3′ | | |
| | | | | RIME-LF | 5′-GCCTCCCACCCTGGACTC-3′ | | |
| | | | | RIME-LB | 5′-AGACCGATAGCATCTCAG-3′ | | |
| | | | | FITC-Probe | 5′-GTTCATATGCGGAAATACAACAA-3′ | | |
| Mugasa *et al.* (2008) | *Trypanozoon* | NASBA | 18S rRNA | TrypNasF7 | 5′-GGATTCCTTGCTTTTCGC-3′ | 0.005 | [36] |
| | | | | TrypNas6T7rev | 5′-AATTCTAATACGACTCACTATAGGGAGAAGGCTCGGACTCTTGTTCTC-3′ | | |
| | | | | TrypNasMB3 | 5′-CGCGATCCAGGTCTGTGATGCTCCTCAATGTGATCGCG-3′ | | |
| Mugasa *et al.* (2009) | *Trypanozoon* | NASBA-LF | 18S rRNA | TrypNasF7b | 5′-GATGCAAGGTCGCATATGAG GGATTCCTTGCTTTTCGC-3′ | 0.005 | [37] |
| | | | | TrypNas6T7rev | 5′-AATTCTAATACGACTCACTATAGGGAGAAGGCTCGGACTCTTGTTCTC-3′ | | |
| | | | | Capture probe | 5′-GCAAGGTGAGATTTTGGGCA-3′ | | |
| | | | | Detection probe | 5′-CAGGTCTGTGATGCTCCTCAATG-3′ | | |
| Radwanska *et al.* (2002) | *Trypanozoon* | PNA-FISH | 18S rRNA | Tbr7 PNA probe | 5′-CGGAACCCAGCCA-3′ | 1 | [38] |
| | | | | Tbr16 PNA probe | 5′-GCCCTAACAGGTGTG-3′ | | |
| | | | | Tbr18 PNA probe | 5′-GTTGCCACCAGCAGT-3′ | | |
| Shiraishi *et al.* (2011) | *Trypanozoon* | PNA probe colocalization | 18S rRNA | PNA3533 probe | 5′-CCGCTCCCGTGTTTCTTG-Lys-NH2-3′ | 0.08 | [39] |
| | | | | PNA3534 probe | 5′-GAAACACCGACCCAAGGC-Lys-NH2-3′ | | |

†LOD = number of parasites detected per reaction; one parasite contains approximately 0.1 pg genomic DNA.
ESAG6/7: Expression site-associated genes 6/7; ITS1: First internal transcribed spacer; LOD: Limit of detection; LAMP: Loop-mediated isothermal amplification; LAMP-LF: LAMP lateral flow; MDA: Multiple displacement amplification; NA: Nucleic acid; NASBA: Nucleic acid sequence-based amplification; NASBA-LF: NASBA lateral flow; PCR-LF: PCR lateral flow; PFRA: Paraflagellar rod protein A gene; PNA-FISH: Peptide nucleic acid FISH; RIME: Repetitive insertion mobile element; rRNA: Ribosomal RNA; SRA: Serum resistance-associated gene; TgsGP: *Trypanosoma brucei gambiense*-specific glycoprotein.

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| Study | Target group | Assay | Target NA | Primer and probe sequences (5′–3′ direction) | | LOD† | Ref. |
|---|---|---|---|---|---|---|---|
| Radwanska *et al.* (2002) | *Trypanosoma brucei gambiense* | Single PCR | TgsGP gene | Forward | 5′-GCTGCTGTGTTCGGAGAGC-3′ | 100 | [40] |
| | | | | Reverse | 5′-GCCATCGTGCTTGCCGCTC-3′ | | |
| Njiru *et al.* (2011) | *T. b. gambiense* | LAMP | TgsGP gene | *TgsGP-F3* | 5′-GTTCGGAGAGCTCAGACAG-3′ | 1–10 | [41] |
| | | | | *TgsGP-B3* | 5′-CCAACCGTTCCCAGTGTTG-3′ | | |
| | | | | *TgsGP-FIP* | 5′-TTGCTCCTTATCGCCGCCAGGCAAGAGCACAAAACCACAG-3′ | | |
| | | | | *TgsGP-BIP* | 5′-TGACGGGGACAACGGCTATCTATTTAACGCAGACACCGCC-3′ | | |
| | | | | *TgsGP-LF* | 5′-CCGCCCTGATCCCGCCTG-3′ | | |
| | | | | *TgsGP-LB* | 5′-GCAACTGCACAGGAACGGCG-3′ | | |
| Radwanska *et al.* (2002) | *Trypanosoma brucei rhodesiense* | Single PCR | SRA gene | Forward | 5′-ATAGTGACAAGATGCGTACTCAACGC-3′ | Not reported | [42] |
| | | | | Reverse | 5′-AATGTGTTCGAGTACTTCGGTCACGCT-3′ | | |
| Welburn *et al.* (2001) | *T. b. rhodesiense* | Single PCR | SRA gene | B537 | 5′-CCATGGCCTTTGACGAAGAGCCCG-3′ | Not reported | [43] |
| | | | | B538 | 5′-CTCGAGTTTGCTTTTCTGTATTTTTCCC-3′ | | |
| Picozzi *et al.* (2008) | *T. b. rhodesiense* | Single PCR | SRA gene | 651 | 5′-GAAGAGCCCGTCAAGAAGGTTTG-3′ | 1–10 | [44] |
| | | | | 652 | 5′-TTTTGAGCCTTCCACAAGCTTGGG-3′ | | |
| Njiru *et al.* (2008) | *T. b. rhodesiense* | LAMP | SRA gene | *SRA-F3* | 5′-GCGGAAGCAAGAATGACC-3′ | 1–10 | [45] |
| | | | | *SRA-B3* | 5′-TCTTACCTTGTGACGCCTG-3′ | | |
| | | | | *SRA-FIP* | 5′-GGACTGCGTTGAGTACGCATCCGCAAGCACAGACCACAGC-3′ | | |
| | | | | *SRA-BIP* | 5′-CGCTCTTACAAGTCTTGCGCCCTTCTGAGATGTGCCCACTG-3′ | | |
| | | | | *SRA-LF* | 5′-CGCGGCATAAAGCGCTGAG-3′ | | |
| | | | | SRA-LB | 5′-GCAGCGACCAACGGAGCC-3′ | | |

†LOD = number of parasites detected per reaction; one parasite contains approximately 0.1 pg genomic DNA.
ESAG6/7: Expression site-associated genes 6/7; ITS1: First internal transcribed spacer; LOD: Limit of detection; LAMP: Loop-mediated isothermal amplification; LAMP-LF: LAMP lateral flow; MDA: Multiple displacement amplification; NA: Nucleic acid; NASBA: Nucleic acid sequence-based amplification; NASBA-LF: NASBA lateral flow; PCR-LF: PCR lateral flow; PFRA: Paraflagellar rod protein A gene; PNA-FISH: Peptide nucleic acid FISH; RIME: Repetitive insertion mobile element; rRNA: Ribosomal RNA; SRA: Serum resistance-associated gene; TgsGP: *Trypanosoma brucei gambiense*-specific glycoprotein.

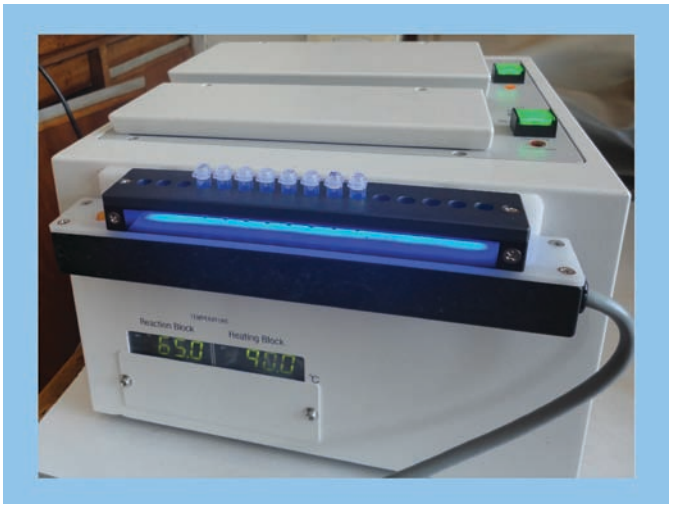

**Figure 2. The Loopamp™ (MAST group) LF-160 incubator for DNA amplification by loop-mediated isothermal amplification and product visualization by light from light-emitting diodes.**
Courtesy of E Matovu (Makerere University, Kampala, Uganda).

### *DNA detection platforms*

PCR is still the most widely used method for the detection of *Trypanosoma* DNA in clinical specimens. In our most recent review in 2010, the authors identified more than ten different PCR assays and five different target DNA sequences (satellite DNA, ribosomal DNA, ITS1, ESAG 6/7 and kinetoplast DNA) for the detection of the *Trypanozoon* group (*T. brucei*, *T. evansi* and *T. equiperdum*) [46]. Highest sensitivities were reported for PCRs targeting the satellite and ribosomal DNA with detection limits below one parasite per reaction [24,31,32]. Current formats for the detection of amplified *Trypanozoon* DNA are conventional electrophoresis in agarose gels [24], SYBR green staining in real-time [31] and oligochromatography [32]. Differentiation between *T.b. gambiense* and *T.b. rhodesiense* by PCR is based on two subspecies-specific genes: the *TgsGP* [47] and *SRA* [48] genes. Sensitivities of *TgsGP* [40] and *SRA* PCRs [42,43] tend to be relatively low (~100 parasites per reaction) as both genes are present only as single copies in the trypanosome genome. A way to overcome this limitation is whole-genome amplification prior to parasite-specific DNA amplification. Morrison *et al*. applied multiple displacement amplification as a simple isothermal whole-genome amplification step prior to PCR [49]. The authors observed a 20-fold increase in analytical sensitivity of a single-copy microsatellite targeting PCR after multiple displacement amplification relative to nested PCR.

Over the last few years, a promising alternative DNA amplification technique has been successfully applied for the detection of trypanosomes in biological specimens (Figure 2). In contrast to PCR, loop-mediated isothermal amplification (LAMP) allows isothermal amplification of target DNA, which significantly simplifies the procedure. In the last decade, more than ten studies on the development and optimization of LAMP assays for diagnosis of human and animal African trypanosomiasis have been published, illustrating the strong interest in this technique. LAMP assays have been developed for the isothermal amplification of *Trypanozoon*-specific DNA sequences in the *PFRA* gene [33] and the repetitive insertion mobile element (RIME) [34]. The RIME LAMP shows a detection limit of 0.001 parasites per reaction, which is approximately 10,000-fold lower than the *PFRA* gene LAMP. This study also demonstrated that the LAMP assay is able to detect trypanosome DNA in the supernatant of boiled blood and CSF, thus significantly reducing sample preparation steps. However, this procedure should be carefully evaluated to guarantee that there is no loss in sensitivity and that the procedure is robust. *T.b. gambiense*- and *T.b. rhodesiense*-specific LAMP assays have been developed by Njiru *et al*. [41,45]. LAMP targeting the *SRA* gene for diagnosis of *rhodesiense* sleeping sickness showed a detection limit of one trypanosome per ml blood using heat-treated buffy coat, which is 1000-fold more sensitive than conventional SRA PCR [45]. A similar low detection level was reached for the *T.b. gambiense* LAMP using the *TgsGP* gene [41]. LAMP product visualization with hydroxynaphtol blue was scored favorable over turbidimetry and colorimetry with Quant-iT™ PicoGreen® and calcein-$MnCl_2$ in a comparative study performed in 2010 [50]. In 2011, Njiru successfully coupled the RIME LAMP to a lateral-flow dipstick device for simple and rapid read-out [35]. However, this approach requires that the sample tube is opened after the reaction, posing a major risk for cross-contamination with amplified products, especially in low-resource laboratories. Progress on sample preparation prior to LAMP has also been made. Matovu and coworkers reported high sensitivities (>90%) of the RIME LAMP as well as the *SRA* gene LAMP on blood from 128 *T.b. rhodesiense* sleeping sickness patients collected on FTA cards [22]. Recently, it was shown that the sensitivity of LAMP on CSF samples and on blood spotted on 903 Protein Saver cards (Whatman, UK) significantly increases when Triton X-100 is used during sample preparation [51].

### *RNA detection platforms*

Whereas LAMP detects DNA, a similar isothermal technique for RNA amplification has been applied to diagnosis of sleeping sickness. The nucleic acid sequence-based amplification (NASBA) technology is based on the simultaneous activity of reverse transcriptase, T7 RNA polymerase and RNase to amplify target RNA without the need for thermocycling conditions. Mugasa *et al*. developed two different NASBA formats that target the 18S ribosomal RNA of the *Trypanozoon* group. The real-time NASBA assay [36] is a single-tube format that uses a molecular beacon approach for the detection of amplified product. The same group also developed a lateral flow format for the detection of NASBA products based on oligochromatography dipstick technology [37]. The analytical detection limits of the NASBA assays are in the same range as the most sensitive LAMP test (RIME). A drawback of NASBA is that RNA is more prone to degradation by nucleases and hydrolysis compared with DNA. On the other hand, RNA-targeted diagnostics might have an advantage over DNA-detection tests for cure assessment after treatment [52]. FISH uses fluorescent oligonucleotide probes to detect and localize specific target nucleic acid sequences and can thus be categorized as a molecular test. The technique was

developed in the early 1980s and has been frequently used in the field of cytogenetics [53] and infectious diseases [54]. In 2002, Radwanska *et al.* applied FISH with AlexaFluo-labeled (Molecular Probes®, OR, USA) peptide nucleic acid (PNA) probes hybridizing with the *Trypanozoon* rRNA for the diagnosis of sleeping sickness [38]. This PNA-FISH test was able to detect one single trypanosome in a 2-µl blood slide but never reached the field owing to the lack of robust, low-cost fluorescence microscopes. Recently, Shiraishi *et al.* delivered the proof-of-principle for specific detection of *T. brucei* rRNA by PNA hybridization-directed colocalization of fluorescent beads (Figure 3) [39]. A pair of PNA probes are coupled to two fluorescent beads differing in size and color and show easily detectable colocalization when simultaneously hybridizing to *T. brucei* rRNA. The assay was able to detect 1.6 ng of total *T. brucei* RNA, corresponding to approximately 300 parasites.

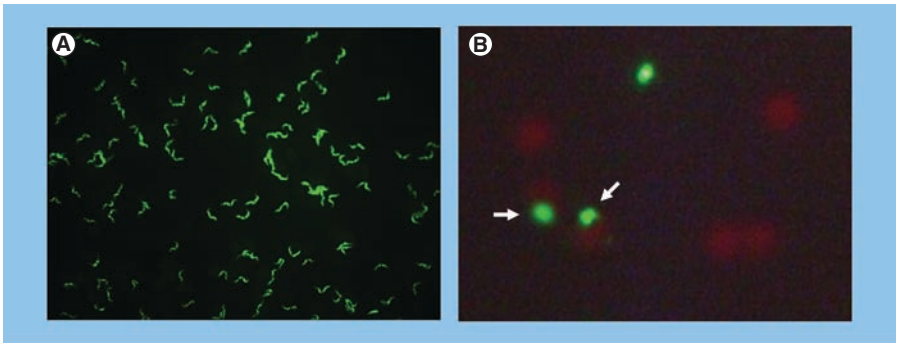


**Figure 3. Microscopic detection of parasite RNA by hybridization with fluorescent probes. (A)** FISH in *Trypanosoma brucei* parasites with Alexa488-labeled rRNA-targeting peptide nucleic acid probes and **(B)** peptide nucleic acid-directed colocalization of fluorescent beads in solution for the specific detection of *Trypanosoma brucei* ribosomal RNA.
Image **(B)** courtesy of T Shiraishi (University of Copenhagen, Copenhagen, Denmark).

### *Fluorescence microscopy in the field*

With the recent introduction of field-applicable fluorescence microscopes [55–57], fluorescence-based diagnostics gained attention. These microscopes contain light-emitting diodes (LEDs) and are portable, battery-powered and low-cost, opening new ways for fluorescence-based molecular diagnosis in the field (Figure 4). The quantitive buffy coat (QBC), introduced in sleeping sickness diagnosis more than 30 years ago [9,58], is based on trypanosome concentration by centrifugation in capillary tubes, as well as nucleus and kinetoplast staining with acridine orange. Although the test is highly sensitive, QBC has never been widely adopted by field workers for reasons including costs, need for a nonstandard microcentrifuge and the lack of a field-adapted UV source. The latter disadvantage has been overcome by the availability of a LED directly attached to the QBC objective (ParaLens™, QBC Diagnostic Inc., PA, USA) [59], and this might revive the use of QBC in sleeping sickness diagnosis. In a recent study, Biéler *et al.* showed that the sensitivity of an acridine orange-based parasite-detection test could be significantly increased by lysis of 3-ml blood followed by centrifugation [60]. The authors reported a sensitivity of approximately 50% using acridine orange and 20% using giemsa staining of specimens with 50 parasites per ml blood. In this context, a comparative evaluation study of the diagnostic accuracy and field applicability of the modified QBC, PNA-FISH and PNA-directed colocalization tests would be highly informative given the recent parallel progress in low-tech molecular diagnostics and LED fluorescence microscopy.

## Diagnostic performance of molecular tests in clinical studies

Before new diagnostic tests are adopted in clinical practice, they should have been subjected to multiple evaluation studies

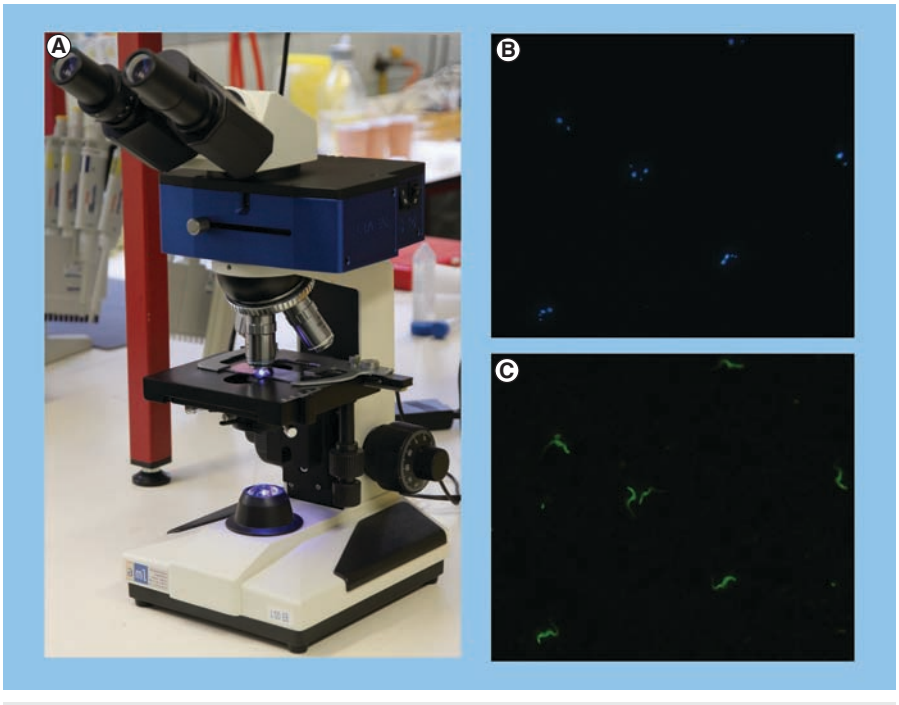


**Figure 4. Field-applicable fluorescence microscopy with light-emitting diode illumination. (A)** FLUOLED™ (Fraen corporation, MA, USA) 2CSL reflected light fluorescence illuminator on a L-120 field microscope (Advanced Microscopy Laboratory, TN, USA), allowing simultaneous visualization of **(B)** the parasite's DNA stained with 4',6-diamidino-2-phenylindole and **(C)** the parasite's surface coat with anti-ISG75 IgY and anti-IgY-Alexa488 secondary antibodies.

to prove their accuracy and efficacy in diagnosis of the disease [61,62]. To improve the quality of reports on diagnostic accuracy studies, the Standards for Reporting of Diagnostic Accuracy (STARD) initiative was launched in 2003 [63]. STARD provides a checklist of issues that should be addressed in reports on diagnostic evaluation studies, as well as a study flow diagram. It is highly important that reports on sensitivities and specificities of molecular diagnostics on sleeping sickness patients implement these guidelines to provide the reader with uniform and complete data presentation. For an overview of the diagnostic accuracies of the molecular tests that have been evaluated on clinical specimens from sleeping sickness patients, the authors refer to the recent systematic review performed by Mugasa *et al*. [64]. The review includes 12 evaluation studies on PCR, two on NASBA, one on LAMP and one study comparing both PCR and NASBA on the same specimens. Diagnostic sensitivities of the tests for trypanosome detection in patient blood ranged between 82 and 100%, while specificities assessed on non-human African trypanosomiasis controls were much more heterogeneous, ranging between 59 and 100%. Low specificities are mostly observed in studies with consecutive patient enrolment and are probably due to the fact that the index test is more sensitive than the standard test. To tackle this limitation, a latent class statistical model can be used to estimate sensitivities and specificities in the absence of gold standard test. De Clare Bronsvoort recently applied this approach for the estimation of the sensitivity and specificity of two PCR assays for the detection of *T. brucei*, in the wider sense, in livestock in western Kenya [65]. Besides sensitivity and specificity, it is also important that results of diagnostic tests are repeatable and reproducible. In 2010, Mugasa *et al*.[64] estimated the repeatability and reproducibility of a PCR and NASBA assay coupled to oligochromatographic detection in a multicenter ring trial with seven participating laboratories [66]. Next to their use in primary diagnosis of sleeping sickness, molecular diagnostics have been proposed for disease staging and for cure assessment after treatment. Studies that evaluated the diagnostic accuracies of molecular diagnostics for staging on CSF specimens were included in the systematic review reported by Mugasa *et al*. Sensitivities were 89% or higher but specificities were low (14–75%). A recent study on the evaluation of PCR in diagnosis, staging and cure assessment in the Democratic Republic of the Congo comprising 360 treated *T.b. gambiense* sleeping sickness patients showed 88.4% sensitivity and a 82.9% specificity of PCR for disease staging. In the same study, patients were followed during 2 years post-treatment and a poor predictive value of PCR for cure was observed [67]. In 20% of the cured patients, trypanosomal DNA was detected in the CSF up to 2 years post-treatment. While the biological mechanism of this phenomenon still has to be elucidated, it would be interesting to investigate whether RNA-targeting diagnostics are more accurate as a test of cure.

## Expert commentary

Molecular diagnostics for infectious diseases is a rapidly evolving field. Despite the high number of articles reporting the development of new molecular diagnostics for sleeping sickness, a field-applicable and low-cost DNA or RNA test remains elusive. The reasons for this slow progress are multiple. First, the parasite load in the blood of sleeping sickness patients is generally extremely low and thus requires highly sensitive tests. Most of the currently available molecular diagnostics are indeed very sensitive but depend on highly sophisticated equipment and expensive reagents. Sleeping sickness is endemic in sub-Saharan Africa and most often in remote areas without access to electricity and any laboratory infrastructure. Hence, tests for diagnosis of patients in the field should be very robust with minimal manipulations and not dependent on continuous supply of electricity and fragile equipment. In this context, conventional PCR and real-time PCR are far from application to the field diagnosis of sleeping sickness. The need for a continuous supply of electricity, expensive equipment and consumables, and purified DNA as starting material limits their use to research laboratories. PCR has been successfully applied in disease surveillance [23,68], travel medicine [5,69] and identification of atypical human infections with animal-infecting trypanosomes [70–72]. In disease surveillance, specimens are transported to a central laboratory for nucleic acid extraction and analysis. Comparative studies on blood collection and storage (e.g., FTA cards vs guanidium buffer), extraction methods and *T. brucei* nucleic acid amplification assays (PCR, LAMP, NASBA) on the same patient cohort would be highly informative to establish a standard protocol. In contrast to PCR, LAMP and NASBA do not need thermocycling conditions for nucleic acid amplification and can thus be carried out in a simple incubator or water bath. The major advantage of NASBA is that it targets RNA and thus might have greater potential as a test of cure compared with DNA-targeting molecular tests. However, NASBA is probably not yet ready to be applied to field conditions due to the need for purified RNA as starting material and the lack of a field-applicable single-tube NASBA format. LAMP is probably the most close to molecular diagnosis of sleeping sickness in the field. It can be performed on crude lysates of blood and CSF, amplification is carried out at one single temperature, it has similar or even greater sensitivity compared to PCR, a single tube format is available and lyophilization of the reaction mixture is possible [73]. Now it is important that LAMP undergoes evaluations in various field settings to assess its diagnostic accuracy for sleeping sickness patients. Further specific studies will have to indicate whether LAMP has greater impact and is more cost-efficient than the most sensitive parasite detection techniques, such as mAECT and modified single centrifugation. Molecular diagnostics that can discriminate between *T.b. gambiense* and *T.b. rhodesiense* suffer from low sensitivity because the subspecies-specific genes are present in single copy in the parasite's genome. LAMP seems to have greater sensitivity compared with PCR but the TgsGP and the SRA LAMP assays are still to be evaluated on patient samples. PNA-FISH and fluorescent bead colocalization for trypanosomal rRNA detection have the potential for diagnosis of sleeping sickness in the field because they are simple and enzyme-free assays. However, the PNA-FISH should be further

simplified by shorter incubation times and having the complete assay performed at an ambient temperature. The fluorescent bead colocalization assay is still in an exploratory phase and has to be transformed in a user-friendly assay. The major limitation of the current rRNA hybridization formats is their limited sensitivity. In order to be able to compete with mAECT, elegant approaches for parasite concentration from a larger blood volume prior to rRNA detection will definitively be needed. Finally, we would like to draw the reader's attention to some recent observations that do not match with the classical dogmas in sleeping sickness and may place sensitive molecular diagnostics in a different light. In collaboration with our colleagues in the Democratic Republic of the Congo, our group observed continued PCR positivity after treatment in a significant fraction of treated *gambiense* sleeping sickness patients [67]. In parallel, other studies showed the existence of silent *T.b. gambiense* infections by which individuals remain in a latent stage and do not develop disease [68,74]. In such cases, PCR results often do not correspond with the patient's serology status, which is in line with similar observations in *Leishmania donovani*-infected asymptomatic individuals [75]. Clearly, more research is needed to understand the biological mechanism of silent infections and we believe that sensitive molecular diagnostics will play an important role in this process.

## Five-year view

The major challenge in the development of molecular diagnostics for sleeping sickness is finding the equilibrium between high sensitivity and field applicability. In this context, the LAMP assays have made most progress toward the use of molecular diagnostics in patient management. While we definitely see the potential of LAMP in sleeping sickness diagnosis, the tests have not been evaluated outside the laboratory environment. We expect that in the coming years, the diagnostic accuracies of the LAMP assays are fully evaluated in field studies, as well as the assessment of its impact and cost-efficacy relative to the standard tests. Next generation biosensors based on the lab-on-chip concept are expected to enter the area of neglected tropical diseases. What we need are rapid, robust and simple molecular diagnostics with high sensitivity and specificity. Test developers should also keep in mind that the price of a test is an important parameter for successful implementation in sleeping sickness control programs. Accurate but expensive diagnostics can be donated by international health organizations but this is not sustainable in the long term. Multiplexed formats with other infectious diseases might be attractive but researchers should carefully evaluate whether there is a need for this. For instance, *T.b. gambiense* sleeping sickness patients are, for a large proportion of them, diagnosed by specialized mobile teams actively screening populations in endemic foci and the impact of multidisease diagnostics might be limited. By contrast, *T.b. rhodesiense* sleeping sickness is diagnosed by passive case detection at health centers and the disease is often misdiagnosed as malaria [76]. In this context, a diagnostic format multiplexing *T.b. rhodesiense* sleeping sickness and other infections leading to similar syndromes in one single test would be welcome. Furthermore, relative to the best parasite detection techniques currently available, novel molecular diagnostics should be more sensitive or show a similar sensitivity but be more simple, rapid and cost-effective. Finally, a large-scale comparative study on the different molecular diagnostics available and on methods for sample collection, storage and nucleic acid isolation would provide researchers and clinicians with standardized protocols for use in epidemiological studies and disease surveillance. The value of such a study was recently demonstrated for *Trypanosoma cruzi* detection by PCR in the diagnosis of Chagas disease [77]. In this multicenter evaluation study with 26 participating laboratories, a Chagas specimen panel was tested by more than 40 different *Trypanosoma cruzi*-specific PCRs, yielding a transparent overview of their diagnostic performances. We also believe that there can be a leading role for the WHO in providing information and guidance on evaluation and implementation of molecular tests for sleeping sickness diagnosis and control.

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## Key issues

- Sleeping sickness is an infectious disease endemic in remote areas in sub-Saharan Africa. Diagnostics should fulfill the specific requirements for use in low-resource settings: accurate, simple, robust and affordable.
- Major advancements in the development of molecular diagnostics for sleeping sickness have been made. Tests are based on specific detection of the parasite's DNA or RNA by amplification methods or by direct hybridization. While very useful for research purposes and disease surveillance, very few of the molecular diagnostics meet the requirements for use in patient management in the field. We need accurate diagnostics that are simple, robust and low cost.
- Several loop-mediated isothermal amplification assays for isothermal DNA amplification have been developed for *Trypanosoma* detection and identification in low-resource settings. The tests showed high analytical sensitivity and specificity in laboratory studies and are ready to enter Phase I, II and III studies in the field.
- The potential role of sensitive nucleic acid-based tests as test-of-cure after sleeping sickness treatment is still under debate. The only large-scale evaluation study performed to date indicated poor predictive value of PCR for treatment outcome. More evaluations are definitely needed to fully assess the accuracy of molecular diagnostics for treatment outcome.

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•• of considerable interest

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