ISOTHERMAL AMPLIFICATION IN MOLECULAR DIAGNOSIS OF SLEEPING SICKNESS

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The Institute of Tropical Medicine Antwerp

Innovative research, social impact

Capacity-building, equal partnership

Medical services, healthy travelling

Study at ITM, broaden your horizon
The Parasite Diagnostics Unit

We study human African trypanosomiasis [sleeping sickness] to

1. develop and evaluate novel molecular and serological diagnostics

2. understand pathogenesis, tissue tropism and drug resistance

We are the WHO collaborating center for diagnosis of sleeping sickness
Sleeping sickness

- caused by *Trypanosoma brucei*
- extracellular parasite, 20 µm length
- long flagellum attached by undulating membrane
- transmitted by tsetse flies
- two disease stages: (1) haemolymphatic $\rightarrow$ (2) neurological
  - *T. brucei gambiense* = chronic disease (months – years)
  - *T. brucei rhodesiense* = acute disease (weeks – months)
- low parasite load in the blood (especially in *T.b. gambiense*)
Sleeping sickness

Populations at risk for sleeping sickness

Risk of *T. b. gambiense* infection
[No. cases/inhabitants/year]

- Very high ≥ 1/10^3
- High < 1/10^3 to ≥ 1/10^9
- Moderate < 1/10^9 to ≥ 1/10^10
- Low < 1/10^10 to ≥ 1/10^11
- Very low < 1/10^11 to ≥ 1/10^12

Risk of *T. b. rhodesiense* infection
[No. cases/inhabitants/year]

- Very high ≥ 1/10^3
- High < 1/10^3 to ≥ 1/10^9
- Moderate < 1/10^9 to ≥ 1/10^10
- Low < 1/10^10 to ≥ 1/10^11
- Very low < 1/10^11 to ≥ 1/10^12

Sleeping sickness

Number of reported sleeping sickness cases between 1998 and 2010

Adapted from Simarro et al. (2011) PLoS Negl Trop Dis 5: e1007
Diagnosis of sleeping sickness

Active case finding for *T.b. gambiense* sleeping sickness
**Diagnosis of sleeping sickness**

**T. brucei gambiense**

**ACTIVE CASE FINDING**

- screening: serology
  - confirmation: parasitology
  - staging
  - treatment stage 1 / stage 2
  - follow-up: 2 years

**T. brucei rhodesiense**

**PASSIVE CASE FINDING**

- confirmation: parasitology
  - staging
  - treatment stage 1 / stage 2
  - follow-up: 2 years
Diagnosis of sleeping sickness

Tools to diagnose sleeping sickness in the field:

- **serological tests**
  - FIELD

- **parasitological tests**
  - FIELD

- **staging tests**
  - FIELD
Molecular diagnostics for sleeping sickness

Molecular tools to diagnose sleeping sickness in reference labs / district hospitals:

ISOTHERMAL AMPLIFICATION TOOLS
Isothermal amplification tools

Polymerase chain reaction = DNA / RNA amplification using thermal cycling

Isothermal amplification = DNA / RNA amplification without thermal cycling

- Nucleic Acid Sequence-Based Amplification (NASBA)
- Loop-Mediated Isothermal Amplification (LAMP)
- Transcription Mediated Amplification (TMA)
- Signal Mediated Amplification of RNA Technology (SMART)
- Strand Displacement Amplification (SDA)
- Rolling Circle Amplification (RCA)
- Isothermal Multiple Displacement Amplification (IMDA)
- Helicase-dependent amplification (HDA)
- Circular Helicase-Dependent Amplification (cHDA)
- Single Primer Isothermal Amplification (SPIA)
- Strand Invasion Based Amplification (SIBA)

“simple and fast”
NASBA for diagnosis of sleeping sickness

EC funded Euro-African Network for the development of simplified and rapid molecular assays for diagnosis of leishmaniasis and human African trypanosomiasis
NASBA = Nucleic Acid Sequence Based Amplification

Concerted action of three enzymes:
1. reverse transcriptase
2. RNase H
3. T7 RNA polymerase
NASBA for diagnosis of sleeping sickness

NASBA-Oligochromatography (NASBA-OligoC)

<table>
<thead>
<tr>
<th>Sample</th>
<th>18S rRNA NASBA</th>
<th>Dipstick – detection</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heater</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 minutes</td>
<td></td>
</tr>
</tbody>
</table>

NASBA for diagnosis of sleeping sickness

*T. brucei* NASBA OligoC-TesT: standardization and quality control

- Shipped at RT, stored at RT
  - Test tubes and stoppers
  - 20 OligoC dipsticks
  - Migration buffer

- Shipped at RT, stored at -20°C
  - Positive control
  - PCR mix

QC

Coris BioConcept
NASBA for diagnosis of sleeping sickness

Diagnostic evaluation studies

- **Test development**
  - Phase I evaluation
    - Case / control design
    - > 20 patients
    - > 20 controls
    - Proof-of-principle
  - Multicentre ring trial
    - Ring design
    - Repeatability
    - Reproducibility
    - Experimental samples
  - Phase II evaluation
    - Case / control design
    - > 100 patients
    - > 100 controls
    - Diverse control group
  - Phase III evaluation
    - Prospective design
    - > 300 patients
    - > 300 controls
    - Consecutive enrolment
NASBA for diagnosis of sleeping sickness

Ring trial to assess the repeatability and reproducibility of the test

→ **Repeatability:** % chance of obtaining the same result in identical specimens in the same lab by the same person using the same equipment *

→ **Reproducibility:** % chance of obtaining the same result in identical specimens in different labs by different persons using different equipment *

* Accordance and concordance % estimates using the formulae described by van der Voet et al. 2004

**Material**

- *T. brucei* OligoC-TeST kits
- Blinded specimen panel:
  1. Dilution series of *T. brucei* RNA in buffer
  2. Dilution series of parasites in human blood containing a stabilisation buffer

**Participants**

- Belgium lab 1
- Belgium lab 2
- The Netherlands
- D.R. Congo
- Uganda

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Table 3: Overview of the accordance and concordance of the *Trypanozoon* and *Leishmania* OligoC-Test on DNA and blood specimens analysed during the ring trial

<table>
<thead>
<tr>
<th>Test</th>
<th>Accordance % (95% CI)</th>
<th>Concordance % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dilution series of DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tryps</em>-PCR-OC test</td>
<td>95.5 (92.6–98.5)</td>
<td>91.7 (87.0–97.9)</td>
</tr>
<tr>
<td><em>Leish</em>-PCR-OC test</td>
<td>98.1 (94.4–100)</td>
<td>95.9 (91.7–100)</td>
</tr>
<tr>
<td><strong>Dilution series of RNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tryps</em>-NASBA-OC test</td>
<td>100</td>
<td>95.8 (92.0–100)</td>
</tr>
<tr>
<td><em>Leish</em>-NASBA-OC test</td>
<td>98.2 (94.4–100)</td>
<td>92.3 (87.0–100)</td>
</tr>
<tr>
<td><strong>Dilution series of parasites in blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Tryps</em>-PCR-OC test</td>
<td>86.6 (75.6–97.8)</td>
<td>78.4 (68.5–91.1)</td>
</tr>
<tr>
<td><em>Leish</em>-PCR-OC test</td>
<td>91.7 (88.9%–97.2)</td>
<td>87.1% (80.9–96.2)</td>
</tr>
<tr>
<td><em>Tryps</em>-NASBA-OC test</td>
<td>89.0 (82.2–95.6)</td>
<td>81.5 (72.4–90.7)</td>
</tr>
<tr>
<td><em>Leish</em>-NASBA-OC test</td>
<td>86.2 (77.8–94.4)</td>
<td>74.8 (67.0–89.2)</td>
</tr>
</tbody>
</table>

Accordance, intralaboratory repeatability; Concordance, interlaboratory reproducibility; 95% CI, 95% confidence interval by bootstrapping.
NASBA for diagnosis of sleeping sickness

Phase II evaluation of *T. brucei* NASBA-OC and PCR-OC in 143 patients and 187 controls

- **Serere:** 50 *T. b. rhodesiense* patients
  36 healthy endemic controls
- **Namungalwe:** 25 *T. b. rhodesiense* patients
  29 healthy endemic controls
- **Mbuji-Mayi:** 68 *T. b. gambiense* patients
  25 healthy endemic controls
- **Kinshasa:** 97 healthy endemic controls

*Matovu et al. (2010) PLoS NTD 4: e737*
NASBA for diagnosis of sleeping sickness

Phase II evaluation of *T. brucei* NASBA-OC and PCR-OC in 143 patients and 187 controls

Table 2. Sensitivities and specificities of the PCR-OC and NASBA-OC on the blood of HAT patients and healthy endemic controls from D.R. Congo (DRC) and Uganda.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Total N°</th>
<th>N° positive</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
<th>N° positive</th>
<th>Sensitivity % (95% CI)</th>
<th>Specificity % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HAT patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>143</td>
<td>117</td>
<td>81.8 (74.7–87.3)</td>
<td></td>
<td>129</td>
<td>90.2 (84.2–94.1)</td>
<td></td>
</tr>
<tr>
<td>DRC</td>
<td>68</td>
<td>56</td>
<td>82.4 (71.6–89.6)</td>
<td></td>
<td>66</td>
<td>97.1 (90.0–99.2)</td>
<td></td>
</tr>
<tr>
<td>Uganda</td>
<td>75</td>
<td>61</td>
<td>81.3 (71.1–88.5)</td>
<td></td>
<td>63</td>
<td>84.0 (74.1–90.6)</td>
<td></td>
</tr>
<tr>
<td><strong>Endemic controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>187</td>
<td>6</td>
<td>96.8 (93.2–98.5)</td>
<td></td>
<td>2</td>
<td>98.9 (96.2–99.0)</td>
<td></td>
</tr>
<tr>
<td>DRC</td>
<td>122</td>
<td>1</td>
<td>99.2 (95.5–99.9)</td>
<td></td>
<td>1</td>
<td>99.2 (95.6–99.9)</td>
<td></td>
</tr>
<tr>
<td>Uganda</td>
<td>65</td>
<td>5</td>
<td>92.3 (83.2–96.7)</td>
<td></td>
<td>1</td>
<td>98.5 (91.8–99.7)</td>
<td></td>
</tr>
</tbody>
</table>

Note. N° = number; CI = confidence interval.
doi:10.1371/journal.pntd.0000737.t002
Collaborative project between Find Diagnostics (Geneva) and the Institute of Tropical Medicine (Antwerp) on the laboratory evaluation of the LAMP test for diagnosis of sleeping sickness
LAMP for diagnosis of sleeping sickness

Benefits of LAMP

• No temperature cycling - single temperature
• Simple DNA/RNA extraction procedure
• High tolerance to inhibitors that would normally inhibit PCR
• Similar/higher sensitivity as PCR
• High specificity: 6 primer sites
• Very fast ~ results within 30 minutes!
• Integrated detection formats

Key components in the LAMP reaction

• 6 primer sites
• Bst DNA polymerase (65°C + strand displacement activity)
LAMP for diagnosis of sleeping sickness

Primer design

LAMP for diagnosis of sleeping sickness

Initiation reaction

LAMP for diagnosis of sleeping sickness

Amplification reaction

LAMP for diagnosis of sleeping sickness

The Loopamp™ *Trypanosoma brucei* detection assay

Loopamp™ *Trypanosoma brucei* detection kit

The Loopamp™ LF-160 incubator
LAMP for diagnosis of sleeping sickness

The Loopamp™ *Trypanosoma brucei* detection assay
Phase II diagnostic accuracy study of the Loopamp™ *Trypanosoma brucei* kit

Table 1. Sensitivities and specificities of replicate RIME LAMP and 18S PCR on the blood of HAT patients and healthy endemic controls.

<table>
<thead>
<tr>
<th>Test</th>
<th>HAT patients (n = 142)</th>
<th>Healthy endemic controls (n = 111)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive results</td>
<td>Sensitivity% (95% CI)</td>
</tr>
<tr>
<td>LAMP</td>
<td>Run 1</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>Run 2</td>
<td>124</td>
</tr>
<tr>
<td>PCR</td>
<td>Run 1</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>Run 2</td>
<td>128</td>
</tr>
</tbody>
</table>

Note. n: number of specimens, CI: confidence interval.
doi:10.1371/journal.pntd.0002504.t001

*Mitashi et al. (2013) PLoS NTD 7: e2504*
Recently launched: large scale implementation study in the D.R. Congo

LAMP for diagnosis of sleeping sickness

Study PI: Epco Hasker, ITM Antwerp
The spliced leader RNA: the next molecular target?

The *Trypanosoma* spliced leader RNA (SL-RNA): the next molecular target for isothermal amplification assays?
The spliced leader RNA: a novel molecular target

- Trypanozoon RNA sequence of 39 bp
- Chimera with all mRNA in the cell
- Added to each mRNA during trans-splicing
- mRNA is considered as the best marker for viable parasites

Would the SL RNA be a good target for molecular diagnostics?

The spliced leader RNA: a novel molecular target

REVERSE TRANSCRIPTION

SL-RNA cDNA (39 bp)

DETECTION BY QUANTITATIVE REAL-TIME PCR

González-Andrade et al. J Mol Diagn, in press
The spliced leader RNA: a novel molecular target

Proof-of-concept

González-Andrade et al. J Mol Diagn, in press
The spliced leader RNA: a novel molecular target

Analytical sensitivity in blood samples spiked with *T. brucei* parasites

González-Andrade et al. J Mol Diagn, in press
The spliced leader RNA: a novel molecular target

Analytical specificity in blood samples spiked with *T. brucei* parasites

<table>
<thead>
<tr>
<th>Organism</th>
<th>Spliced Leader RNA Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. b. gambiense</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. b. rhodesiense</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. b. brucei</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. evansi</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. equiperdum</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. congoles</em></td>
<td>AACUAACGCUAUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. vivax</em></td>
<td>AACUAACGCUUUUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. cruzi</em></td>
<td>AACUAACGCUAUUUAUGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>T. rangeli</em></td>
<td>AACUAACGCUAUUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>AACUAACGCUAUUUAUAGAGACAGUUCUGUACUAAUUG</td>
<td>39</td>
</tr>
</tbody>
</table>

**Melting Peaks**

- *T. b. gambiense*
- *T. b. rhodesiense*
- *T. b. brucei*
- *T. evansi*
- *T. equiperdum*
- *T. congoles*
- *T. vivax*
- *L. donovani*
- *T. cruzi*
- *T. rangeli*
The spliced leader RNA: a novel molecular target

How many SL-RNA molecules has one single trypanosome?

\[
\text{g RNA} \div (\text{length} \times 340) \times 6.023 \times 10^{23} \quad \rightarrow \quad > 9000 \text{ SL-RNA molecules/cell}
\]
Conclusions

- NASBA has been developed for isothermal amplification of *T. brucei* RNA
- LAMP has been developed for isothermal amplification of *T. brucei* DNA
- NASBA has been coupled to a chromatographic read-out device (dipstick)
- LAMP has been translated into a single-tube format for use in low-resource settings
- Both tests have been successfully validated in phase I, II and ring studies (NASBA)
- Sensitivities are similar to PCR
- Simple and fast
- The spliced-leader RNA (SL-RNA) showed great potential as a new molecular target
ACKNOWLEDGEMENTS