

# **Development of simplified molecular tools for the diagnosis of kinetoplast diseases**

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## SUMMARY

Kinetoplastid parasites cause disease in plants, animals and in humans and severely affect health and impede development of agriculture in developing countries where these parasites are endemic. Human African Trypanosomiasis (HAT) is inevitably fatal if not treated, and leishmaniasis may not be fatal in some forms (cutaneous and mucocutaneous), while the visceral form is highly fatal if left untreated or treated inadequately. In order to improve treatment outcome, early and proper diagnosis is important. This can not be over emphasized in HAT where treatment is dependant on the stage of the disease. The laboratory diagnosis of leishmaniasis and HAT is routinely done by microscopy, but due to technical and operational limitations of this technique, these diseases can be missed and patients denied treatment. To improve case finding, more sensitive diagnostic tests are required and one such option is the application of molecular tests. Molecular diagnostics, in particular PCR, have taken centre stage in diagnosis of various infectious diseases. However, the application of PCR in the diagnosis of leishmaniasis and HAT still lags behind due to resource poor laboratories in the deprived rural areas where these diseases are endemic. Therefore, the possible application of molecular tests in resource poor laboratories will require at least simplification of these tests to circumvent the need for expensive sophisticated equipment and possibly electricity. This thesis describes the development and simplification of molecular amplification tests for the diagnosis of kinetoplastid diseases, and their evaluation for use as confirmatory tools in disease endemic regions.

The first step in the development of a sensitive molecular test that can enhance HAT case finding was the development of a real-time nucleic acid sequence-based amplification assay (NASBA) based on amplification and concurrent detection of small subunit rRNA (18S rRNA) of *Trypanosoma brucei*, which is described in chapter 2 of this thesis. The sensitivity of the assay was evaluated on nucleic acids from *in vitro* cultured parasites and blood spiked with various parasite quantities. The assay detected 10 parasites/ml using cultured parasites as well as spiked blood.

A sensitive assay such as the real-time NASBA has the potential to become an alternative tool to confirm diagnosis of human African trypanosomiasis. However, despite good analytical sensitivity and specificity, this technique may not be implemented in HAT control programs due to high cost of necessary equipment. Therefore in chapter 3, a simplified technique, oligochromatography (OC), was applied for the detection of amplification products of *T. brucei* 18S rRNA by NASBA. The *T. brucei* NASBA-OC test had analytical sensitivity of 1 - 10 parasites/ml on nucleic acids extracted from parasite culture and 10 parasites/ml on spiked blood. The test showed no reaction with non-target pathogens or with blood from healthy controls. When NASBA-OC was compared to the composite standard applied in the study, i.e., parasitological confirmation of a HAT case by direct microscopy or by microscopy after concentration of parasites using either a microhematocrit centrifugation technique or a mini-anion-exchange centrifugation technique, the developed test showed a sensitivity of 73.0% (95% confidence interval [CI:] 60 to 83%) on blood samples, while standard expert microscopy had a sensitivity of 57.1% (95% CI: 44 to 69%). On cerebrospinal fluid samples, NASBA-OC had a sensitivity of 88.2% (95% CI: 75 to 95%) and standard microscopy had a sensitivity of 86.2% (95% CI: 64 to 88%). The simplified NASBA-OC test can be employed in field laboratories, because it does not require a thermocycler; a simple heat block or a water bath maintained at two different temperatures is sufficient for amplification. Furthermore, the employed read-out system is very simple in use, extremely rapid (less than 10 minutes) and does not produce toxic waste products. This readout system is comparable to CATT which takes 5 minutes to read the results.

Chapter 4 describes the development and evaluation of NASBA-OC to detect *Leishmania* parasite RNA in clinical specimens of visceral leishmaniasis patients from East Africa (n = 30) and of cutaneous leishmaniasis cases from South America (n = 70) and appropriate control samples. The test yielded analytical sensitivities of 10 parasites/ml of spiked blood, and 1 parasite/ml of culture material. Diagnostic sensitivity of NASBA-OC for leishmaniasis was 93.3% (95% CI: 76.5-98.8%) and specificity was 100% (95% CI: 91.1-100%) on blood samples, while sensitivity and specificity on skin biopsy samples was 98.6% (95% CI: 91.2-99.9%) and 100% (95% CI: 46.3-100%), respectively.

The NASBA-OC showed promising accuracy results for diagnosis of HAT and leishmaniasis as reported in chapters 3 and 4. However before a test can be implemented or even be evaluated on a large sample size, it has to be evaluated for repeatability and reproducibility. In chapter 5, four simplified molecular assays for the diagnosis of *Trypanosoma brucei* spp. or *Leishmania* spp. were evaluated in a multicentre ring trial with seven participating laboratories. The tests are based on PCR or NASBA amplification of the nucleic acids of parasites followed by rapid read-out by oligochromatographic dipstick (PCR-OC and NASBA-OC). Two tests are thus available for HAT: Tryp-PCR-OC and Tryp-NASBA-OC. Two other tests are available for leishmaniasis: Leish-PCR-OC and Leish-NASBA-OC. On purified nucleic acid specimens, the repeatability and reproducibility of the tests were as follows: Tryp-PCR-OC, 91.7% and 95.5%; Tryp-NASBA-OC, 95.8% and 100%; Leish-PCR-OC, 95.9% and 98.1%; Leish-NASBA-OC, 92.3% and 98.2%. On blood specimens spiked with parasites, the repeatability and reproducibility of the tests were as follows: Tryp-PCR-OC, 78.4% and 86.6%; Tryp-NASBA-OC, 81.5% and 89.0%; Leish-PCR-OC, 87.1% and 91.7%; Leish-NASBA-OC, 74.8% and 86.2%. As repeatability and reproducibility of the tests were satisfactory, further phase II and III evaluations in clinical and population specimens from disease endemic countries are recommended.

A phase II evaluation of PCR-OC and NASBA-OC for the diagnosis of HAT is presented in Chapter 6. Both tests were evaluated in a case-control design on 143 HAT patients and 187 endemic controls from the Democratic Republic of Congo (DRC) and from Uganda. The overall sensitivity of PCR-OC was 81.8% (95% confidence interval [CI]: 74.7-87.3%) and the specificity was 96.8% (93.2-98.5%). The PCR-OC showed a sensitivity and specificity of 82.4% (95% CI: 71.6-89.6%) and 99.2% (95% CI: 95.5-99.9%) on the specimens from DRC and 81.3% (95% CI: 71.1-88.5%) and 92.3% (95% CI: 83.2-96.7%) on the specimens from Uganda. NASBA-OC yielded an overall sensitivity of 90.2% (95% CI: 84.2-94.1%), and a specificity of 98.9% (95% CI: 96.2% - 99.7%). The sensitivity and specificity of NASBA-OC on the specimens from DRC was 97.1 (95% CI: 90.0- 99.2%) and 99.2% (95% CI: 95.6-99.9%) respectively. For the specimens from

Uganda a sensitivity of 84.0% (95% CI: 74.1-90.6%) and a specificity of 98.5% (95% CI: 91.8-99.7%) was observed. The tests showed satisfactory sensitivity and specificity for the *T. b. gambiense* HAT in DRC but a rather low sensitivity for *T. b. rhodesiense* HAT in Uganda. The latter could have resulted from loss of DNA quality during preparation or the long storage. The sensitivity and specificity were generally higher for NASBA-OC than PCR-OC. These tests need to be evaluated in a larger scale study with freshly prepared nucleic acids to fully elucidate their potential as HAT diagnostics.

A number of molecular amplification tests for HAT diagnosis have been developed in various studies and varying accuracy results are reported. In Chapter 7, the diagnostic accuracy of molecular amplification tests for HAT was reviewed as well as reasons for variation in accuracy amongst tests. Relevant articles were identified in Medline from January 1984 to June 2010, by reference tracking and from the “grey” literature. The studies assessed the diagnostic accuracy of molecular amplification tests of HAT in case-control, cross sectional and cohort studies compared to microscopy as the reference standard. Fifteen studies were included in the systematic review and 10 studies in the meta-analysis. Ten PCR (including PCR and PCR-OC) studies were pooled with a sensitivity of 99.6 (95% CI 88.5 to 99.9%) and specificity of 97.7 (95% CI 90.3 to 99.5%). The accuracy of NASBA tests could not be pooled because the studies were too few, but the sensitivity ranged between 89.0% (95% CI: 75 to 96%) and 97% (95% CI: 95% CI 85 to 100%), and specificity between 14% (95% CI 0% to 58%) and 99% (95% CI: 96 to 1.00). The PCR tests showed a high sensitivity compared to microscopy. This systematic review was also able to determine that this high sensitivity was coupled with an equally acceptable specificity. The PCR accuracy results on blood were generally homogeneous among the studies and therefore allow for the results to be generalized. Based on this review, PCR may be adopted for routine HAT diagnosis as an alternative to microscopy without need for further assessment of accuracy. No conclusion could be made on the accuracy of NASBA tests because there were too few studies, therefore more studies may be needed to precisely assess the diagnostic accuracy of these particular molecular amplification tests and determine their added value to both PCR and microscopy.