

Sensitivity and specificity of the *Leishmania* OligoC-TesT and NASBA-oligochromatography for diagnosis of visceral leishmaniasis in Kenya

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Summary

OBJECTIVE To estimate the sensitivity and specificity of the OligoC-TesT and nucleic acid sequence-based amplification coupled to oligochromatography (NASBA-OC) for molecular detection of *Leishmania* in blood from patients with confirmed visceral leishmaniasis (VL) and healthy endemic controls from Kenya.

METHODS Blood specimens of 84 patients with confirmed VL and 98 endemic healthy controls from Baringo district in Kenya were submitted to both assays.

RESULTS The *Leishmania* OligoC-TesT showed a sensitivity of 96.4% (95% confidence interval [CI]: 90–98.8%) and a specificity of 88.8% (95% CI: 81–93.6%), while the sensitivity and specificity of the NASBA-OC were 79.8% (95% CI: 67–87%) and 100% (95% CI: 96.3–100%), respectively.

CONCLUSION Our findings indicate high sensitivity of the *Leishmania* OligoC-TesT on blood while the NASBA-OC is a better marker for active disease.

keywords visceral leishmaniasis, diagnosis, polymerase chain reaction, nucleic acid sequence-based amplification, sensitivity, specificity

Introduction

Visceral leishmaniasis (VL) is a major health problem on the African continent, where it mainly affects rural populations (Hunt *et al.* 2007). In East Africa, VL is exclusively linked with the *Leishmania donovani* species, and parasites mainly infect the reticuloendothelial organs like the liver, spleen and bone marrow. Antibody detection tests such as the direct agglutination test (DAT) (Harith *et al.* 1988; Boelaert *et al.* 1999) and the rK39 immunochromatographic strip test (Chappuis *et al.* 2005; Ritmeijer *et al.* 2006; ter Horst *et al.* 2009) play a key role in diagnosis of the disease, but parasite demonstration by microscopic analysis of lymph, bone marrow or splenic aspirates is still frequently used in East Africa as a confirmation test.

Molecular techniques such as polymerase chain reaction (PCR) and nucleic acid sequence-based amplification

(NASBA) are powerful tools for parasite detection as they combine sensitivity and specificity (Antinori *et al.* 2007; Reithinger & Dujardin 2007) and can be applied to blood and other types of specimens. Hence, molecular detection of *Leishmania* parasites' DNA or RNA in blood might lead to less invasive diagnosis of VL than conventional parasite detection in bone marrow or spleen aspirates. Several test formats are available, which can be classified into 'high-tech', 'mid-tech' and 'low-tech' approaches. High-tech approaches are methods that require elaborate and rather expensive equipment such as real-time PCR. Mid-tech approaches are probably the most widely used and comprise conventional PCR assays, in which PCR amplicons are resolved by electrophoresis in agarose gels. Low-tech approaches refer to simplified amplification and/or detection methods for use in laboratory settings with minimal molecular biology equipment.

Recently, the *Leishmania* OligoC-TesT and nucleic acid sequence-based amplification coupled to oligochromatography (NASBA-OC) were introduced as standardised low-tech molecular diagnostics for leishmaniasis (Deborggraeve *et al.* 2008a; Mugasa *et al.* 2010b). These assays amplify a part of the 18S ribosomal DNA or RNA by PCR (OligoC-TesT) or NASBA (NASBA-OC) where after the amplification products are detected by a simple and rapid dipstick method based on oligochromatography. Both assays showed high sensitivity and specificity for *Leishmania* detection during the phase I evaluations (Deborggraeve *et al.* 2008a; Mugasa *et al.* 2010b) and satisfactory repeatability and reproducibility in a multi-centre evaluation study (Mugasa *et al.* 2010a). In this study, we subjected both tests to a phase II evaluation (Boelaert *et al.* 2007) on blood specimens collected from patients with confirmed VL and healthy endemic controls in Baringo district in Kenya.

Methodology

Ethical considerations

Ethical clearance for the study was obtained from the institutional ethical committee of Kenya Medical Research Institute (KEMRI). Informed consent was obtained from the patients or their guardians and from the healthy endemic controls. Patients with confirmed VL were referred to the Centre for Clinical Research (CCR) at KEMRI or to Kabarnet District Hospital, where medication was provided to them at no cost.

Study participants

This phase II diagnostic study was carried out between 2007 and 2008, during which patients with VL were recruited in villages of the VL endemic areas in Baringo district (Kenya). Healthy endemic controls were volunteers recruited from the same villages or other villages in the endemic areas.

Participant classification and reference tests

A participant was classified as a confirmed VL case if there was clinical suspicion for VL, if DAT titre on serum was 1:12 000 or more and if parasites were observed during microscopic analysis of splenic aspirate. A participant was classified as a healthy endemic control if there was no previous history of VL, if there was no clinical suspicion for VL and if DAT on serum showed a titre of 1:6400 or below. Clinical suspicion for VL was defined as a history of fever for 2 weeks or more and splenomegaly or lymph-

adenopathy. The reference tests were performed at the collection site as described in the WHO manual on VL (WHO 1996) except for splenic aspirate taking. Individuals suspected of VL and with a positive DAT titre were transported to the Centre for Clinical Research (CCR) at KEMRI in Nairobi for microscopic analysis of splenic aspirates.

Index tests

Two hundred microlitres of blood was collected from the patients and healthy endemic controls and mixed with the same volume of L3 buffer (University of Amsterdam, confidential). This guanidium-based buffer allows storage of the blood without loss of DNA and RNA quality. The stabilised blood specimens were shipped at 4 °C from the collection site to KEMRI and stored at 4 °C for a maximum of 2 weeks. The nucleic acids of the blood specimens were extracted by the method described by Boom *et al.* (1990). Elution was performed in 50 µl RNase-free water by incubating at 56 °C for 10 min, and the purified nucleic acids were stored at –80 °C until further analysis. The extracts were tested with the *Leishmania* OligoC-TesT and NASBA-OC as described by Deborggraeve *et al.* (2008a) and Mugasa *et al.* (2010b). Test kits were provided by Coris BioConcept (Gembloux, Belgium). In brief, the *Leishmania* OligoC-TesT amplifies a short sequence of the *Leishmania* 18S ribosomal DNA by PCR, and the NASBA-OC amplifies a part of the 18S ribosomal RNA by NASBA. For both assays, the amplification products are mixed with migration buffer pre-heated to 55 °C after which the dipstick is dipped into the solution. Migration is performed at 55 °C, and test results are read after 10 min. The two tests contain internal controls to validate the migration as well as the amplification reaction. Nucleic acid extraction was performed at KEMRI within 2 weeks of arrival of the specimen from the collection site. The extracts were analysed with the index tests between October 2008 and February 2009 at Khartoum University (Sudan) in the frame of a collaborative consortium. No external quality control confirming the reference test or index test results could be performed during the study. The executors of the index tests were not blinded to the participant classification and thus the results of the reference tests.

Statistical analysis

The sensitivity and specificity of the *Leishmania* OligoC-TesT and NASBA-OC were calculated from data entered into contingency tables. Differences in sensitivity and specificity between the two tests were estimated by the

McNemar test. Concordances between the two tests were determined using the kappa index. All calculations were estimated at a 95% confidence interval (95% CI).

Results

Eighty-four patients with VL and 98 endemic healthy controls were recruited in Baringo district in Kenya. An overview of the sensitivity and specificity of the OligoC-TesT and NASBA-OC on the study population is presented in Table 1.

Sensitivity of the *Leishmania* OligoC-TesT and NASBA-OC

The *Leishmania* OligoC-TesT and NASBA-OC on blood showed a positive test result in 81 and 67 of the patients with VL, respectively. Hence, the sensitivity of the *Leishmania* OligoC-TesT is 96.4% (95% CI: 90–98.8%) and of the NASBA-OC 79.8% (95% CI: 67–87%), which is significantly lower ($P < 0.05$).

Specificity of the *Leishmania* OligoC-TesT and NASBA-OC

Eleven out of 98 healthy endemic controls were positive with the *Leishmania* OligoC-TesT, while no positive test results were observed with the NASBA-OC, indicating a specificity of 88.8% (95% CI: 81–93.6%) and 100% (95% CI: 96.3–100%), respectively. The McNemar test indicated a significant difference in specificity of both tests ($P < 0.05$).

Test agreement

For the 182 blood specimens analysed, the kappa index was 0.73 (95% CI: 0.59–0.87), indicating substantial agreement in results of the two index tests.

Discussion

When analysing blood from the confirmed VL cases, we observed a significantly higher sensitivity of the *Leishmania* OligoC-TesT (96.4%) than the NASBA-OC (79.8%). As VL diagnosis was confirmed by microscopic analysis of spleen aspirates, the high sensitivity of the OligoC-TesT on blood is promising. Indeed, this means that the OligoC-TesT on blood could indicate the infection status of VL-suspected cases. Our findings are in agreement with the sensitivities shown by conventional PCR techniques for *Leishmania*, which generally range from 92% to 100% (Anderson *et al.* 1994; Salotra *et al.* 2001; Maurya *et al.* 2005). The lower sensitivity of NASBA-OC was unexpected because this test showed similar sensitivity as the OligoC-TesT in a multicentre evaluation study on *Leishmania* parasite-spiked blood (Mugasa *et al.* 2010a). The lower sensitivity observed in the current phase II study might be because of the fact that NASBA-OC targets the parasite's RNA, which is known to be more liable to degradation compared to DNA. Nucleic acid quality might have been decreased during specimen storage and transportation from the collection sites in the field to the reference centre in Nairobi.

In contrast, the NASBA-OC showed a significant higher specificity than the OligoC-TesT when analysing blood from the healthy endemic controls (100% *vs.* 88.8%). Because the controls taken along during specimen analysis did not indicate contamination of the PCR-based OligoC-TesT, the detection of *Leishmania* DNA in this control group is probably associated with asymptomatic *Leishmania* infections, which are known to be common in VL endemic areas (Deborggraeve *et al.* 2008b; Bhattarai *et al.* 2009). The inclusion of healthy parasite carriers in the control group can be explained by the low concordance between negative DAT status and PCR outcome on blood from endemic control persons, as described by Deborggraeve *et al.* (2008b) and Bhattarai

Table 1 Diagnostic accuracy of the *Leishmania* OligoC-TesT and NASBA-OC on blood from confirmed VL cases and healthy endemic controls from Baringo district, Kenya

| Participants | Number of specimens | OligoC-TesT | | NASBA-OC | | | |
|--------------------------|---------------------|---------------------|------------------------|------------------------|---------------------|------------------------|------------------------|
| | | Number of positives | Sensitivity % (95% CI) | Specificity % (95% CI) | Number of positives | Sensitivity % (95% CI) | Specificity % (95% CI) |
| Confirmed VL cases | 84 | 81 | 96.4 (90–98.8) | | 67 | 79.8 (67–87) | |
| Healthy endemic controls | 98 | 11 | | 88.8 (81–93.6) | 0 | | 100 (96.3–100) |

CI, confidence interval; VL, visceral leishmaniasis; NASBA-OC, nucleic acid sequence-based amplification coupled to oligochromatography.

et al. (2009). The observation that these healthy parasite carriers are not picked up by the NASBA-OC is probably because of its lower sensitivity, as reflected in the results on the confirmed VL cases. Patients with confirmed VL are individuals presenting symptoms and thus probably have higher parasite loads than asymptomatic parasite carriers. The sensitivity and specificity of both tests should also be evaluated in settings that do not need specimen storage and transport. It might be that in the present study, the sensitivity of the NASBA-OC is underestimated, and the specificity overestimated because of RNA degradation during specimen storage or transport.

The low specificity of the *Leishmania* OligoC-TesT in this study confirms that PCR-based diagnostic tests are a marker of infection rather than disease, as described by Deborggraeve *et al.* (2008b). As only diseased persons are treated, PCR alone is of less value for VL diagnosis in endemic regions. However, one could state the same for diagnosis based on antibody detection tests alone or on clinical data alone because none of them represent a gold standard for acute disease. Hence, laboratory tests should always be interpreted in combination with a standardised clinical case definition. Although molecular diagnostics are not yet applicable in most operational settings because of their complexity, they can be useful in hospitals with basic molecular biology facilities. Molecular diagnostics should not replace the existing immunodiagnoses but give an added value as a marker of infection.

It is important to emphasise that the *Leishmania* OligoC-Test and NASBA-OC are important steps forward to improved and standardised molecular detection of the parasite, but they are still restricted to use in reference centres with basic molecular biology facilities. Efforts towards further simplification of molecular diagnostics should therefore be strongly encouraged. In addition, although still to be confirmed by large-scale studies, molecular detection of the parasite might be useful for cure assessment. While antibodies remain detectable for years after successful treatment (Hailu 1990), the parasite's RNA and DNA is rapidly degraded following parasite death (Prina *et al.* 2007).

In conclusion, we successfully evaluated two innovative molecular diagnostics for VL in a phase II study on Kenyan patients with VL and healthy endemic controls. The *Leishmania* OligoC-TesT showed high sensitivity on blood but a rather low specificity for active disease. In contrast, the sensitivity of RNA detection by NASBA-OC in blood was lower but the specificity was 100%, indicating that it might be a better marker for active VL.

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