Cutaneous leishmaniasis: new developments in diagnosis and treatment evaluation
van der Meide, W.F.

Link to publication

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

UvA-DARE is a service provided by the library of the University of Amsterdam (http://dare.uva.nl)

Download date: 03 Jan 2019
Comparison between Quantitative Nucleic Acid Sequence-Based Amplification, Real-Time Reverse Transcriptase PCR, and Real-Time PCR for Quantification of Leishmania Parasites

Wendy van der Meide
Jorge Guerra,
Gerard Schoone
Marit Farenhorst
Leila Coelho
William Faber
Inge Peekel
Henk Schallig

J Clin Microbiol, 2008 in press
Comparison between QT-NASBA, qRT-PCR and qPCR

Summary

DNA or RNA amplification methods for detection of *Leishmania* parasites have advantages regarding sensitivity and potential quantitative characteristics in comparison with conventional diagnostic methods, but are often still not routinely applied. However, the use and application of molecular assays are increasing, but comparative studies on the performance of these different assays are lacking. The aim of this study was to compare three molecular assays for detection and quantification of *Leishmania* parasites in serial dilutions of parasites and in skin biopsies collected from cutaneous leishmaniasis (CL) patients in Manaus, Brazil. A serial dilution of promatigotes spiked in blood was tested in triplicate in three different runs by quantitative nucleic acid sequence-based amplification (QT-NASBA), quantitative real-time reverse transcriptase PCR (qRT-PCR), and quantitative real-time PCR (qPCR). In addition, the costs, durations and numbers of handling steps were compared, and 84 skin biopsies from patients with suspected CL were tested. Both QT-NASBA and qRT-PCR had a detection limit of 100 parasites/ml of blood, while qPCR detected 1,000 parasites/ml. QT-NASBA had the lowest range of intra-assay variation (coefficients of variation [CV], 0.5% to 3.3%), while qPCR had the lowest range of interassay variation (CV, 0.4% to 5.3%). Furthermore, qRT-PCR had higher $r^2$ values and amplification efficiencies than qPCR, and qPCR and qRT-PCR had faster time procedures than QT-NASBA. All assays performed equally well with patient samples, with significant correlations between parasite counts. Overall, qRT-PCR is preferred over QT-NASBA and qPCR as the most optimal diagnostic assay for quantification of *Leishmania* parasites, since it was highly sensitive and reproducible and the procedure was relatively fast.
Introduction

Cutaneous leishmaniasis (CL), a protozoan skin infection, is a major public health problem in the Amazonas state in Brazil, with almost 1,000 cases reported annually in the capital city, Manaus.\textsuperscript{1} The disease is primarily a zoonose, with a sylvatic cycle of transmission occurring between phlebotomine sandfly vectors of the species \textit{Lutzomyia umbratilis} and \textit{Lutzomyia anduzei} and wild animal hosts. CL was considered mainly as an occupational disease, infecting people involved in activities in forests such as wood and mineral exploitation or in building of roads in forests or of hydroelectric dams.\textsuperscript{2} Nowadays, CL seems to have become an important problem in the rural settlements around the urban areas of Manaus, which are habituated mainly by a poor population.

Almost half of the CL patients in the Amazonas state visit the large health institute in Manaus, Fundação de Medicina Tropical do Amazonas (FMTAM).\textsuperscript{1} At this institute, diagnosis relies predominantly on the visualization of amastigotes in Giemsa-stained smears with microscopy and on the clinical picture of the lesion. Alternative methods are histopathology, culture in Novy-MacNeal-Nicole medium, or inoculation into susceptible animals, such as hamsters.\textsuperscript{3} However, these methods are rather insensitive and/or time-consuming. The PCR method has proven to be an important approach to diagnose CL, since it can be highly sensitive and specific and has potential quantitative characteristics. Moreover, restriction enzyme analysis after amplification allows differentiation between different \textit{Leishmania} species.\textsuperscript{4,6} Currently, different DNA and RNA amplification methods have been established for detection and quantification of \textit{Leishmania} parasites, including quantitative real-time PCR (qPCR) and quantititative nucleic acid sequence-based amplification (QT-NASBA).\textsuperscript{7-10} Sensitive molecular tools can offer significant advantages, not only in diagnosis but also in studies requiring accurate and sensitive quantification of parasites, such as drug treatment efficacy studies.\textsuperscript{11}
Comparison between QT-NASBA, qRT-PCR and qPCR

A fluorogenic probe (e.g., TaqMan or fluorescent resonance energy transfer) specific for the target sequence is used in qPCR, allowing continuous monitoring of the amplified PCR products.\textsuperscript{12} The closed-tube format of the assay reduces the risk of contamination. An RNA amplification method, such as the 18S QT-NASBA\textsuperscript{10} has advantages since it is based on an isothermal reaction and thus circumvents the need for a thermocycler. Furthermore, it is much more sensitive than conventional PCR, since it targets rRNA copies, which are more abundant than ribosomal DNA (rDNA).\textsuperscript{10,13} As an alternative quantitative RNA amplification method, we used quantitative real-time reverse transcriptase PCR (qRT-PCR). This technique is an extended version of a qPCR, with a step involving treatment with reverse transcriptase enzyme (an RNA-dependent DNA polymerase) prior to a normal PCR reaction. This assay combines the advantages of both qPCR (closed-tube format) and QT-NASBA (detection of RNA copies). The 18S ribosomal sequence was chosen as the target gene because it is a multiple copy gene, targets all \textit{Leishmania} species, and has proven to be highly efficient for the diagnosis of leishmaniasis from human clinical material.\textsuperscript{10,13-15}

The present study compared three molecular diagnostic tools based on the amplification of small-subunit ribosomal 18S sequences for the detection and quantification of \textit{Leishmania} parasites in blood and clinical CL samples collected at FMTAM in 2006. Assay performance was described in terms of analytical sensitivity, intra- and interassay variations (within and between runs), costs, and time to perform the assays.
Materials and methods

QT-NASBA
The QT-NASBA, which targets a 170-bp region in the 18S rRNA, was performed using the Nuclisense BasicKit (bioMérieux) for amplification, following the procedures published previously. Primers and probes are listed in Table 1. In vitro quantitative RNA (Q-RNA, constructed by site-directed mutagenesis) is added to the sample prior to extraction and serves as competitor RNA for the QT-NASBA and as an internal control (IC). After extraction and amplification, the samples were detected by the electrochemiluminescence (ECL) detection method.

QRT-PCR
The primers and probes for qRT-PCR were based on the same 170-bp 18S rRNA and rDNA sequences as used for the QT-NASBA (Table 1). Since the same IC Q-RNA was used as in QT-NASBA, two probes with different reporter dyes (6-carboxyfluorescein [FAM] and 5-tetrachloro-fluorescein [TET]) were designed for the wild type target and Q-RNA sequence. Both probes were conjugated to a minor-groove binder (MGB) at the 5’-end (Applied Biosystems, Foster City, CA), causing a higher binding affinity of the probes to the target. For the reaction, 2.5 μl of isolated DNA/RNA sample was added to 22.5 μl amplification mix containing 1x buffer (Bio-Rad; 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.8 mM deoxynucleoside triphosphates, 3 mM MgCl2 and 0.6 U/μl iTaq DNA polymerase), 0.8 μM of each primer, 0.2 μM FAM-MGB probe, and 0.2 μM TET-MGB probe. Amplification and real-time measurement were performed in the iCycler iQ5 (Bio-Rad) with the following conditions: 10 min at 50°C, 5 min at 95°C, and then 45 cycles of 30 s at 95°C and 45 sec at 60°C. The number of parasites was calculated from the threshold cycle (Ct), i.e., the amplification cycle number at which the emitted fluorescence exceeded the set baseline of 100 reference fluorescence units ( = background emission plus 10 standard deviations).
Comparison between QT-NASBA, qRT-PCR and qPCR

QPCR

The qPCR was carried out according to an established protocol described by Wortmann et al.\textsuperscript{17} and targets a 60-bp region in the 18S ribosomal gene specific for all members of the \textit{Leishmania} genus. The protocol was adjusted by the incorporation of two primers and one probe to detect the IC plasmid pEntIC 2 (kindly donated by Richard Molenkamp, Clinical Virology Department, Academic Medical Center), which was originally developed for \textit{Enterovirus} species (members of the family \textit{Picornaviridae}).\textsuperscript{18} Primer and probe sequences are presented in Table 1. For the amplification reaction, 2.5 μl of isolated DNA/RNA sample was added to 22.5 μl amplification mix containing 1x Supermix buffer (Bio-Rad; 20 mM Tris-HCl [pH 8.4], 50 mM KCl, 0.8 mM deoxynucleoside triphosphates, 3 mM MgCl₂ and 0.6 U/μl iTaq DNA polymerase), 0.8 μM of each primer (18SF and 18SR), 0.2 μM FAM probe, 0.4 μM of each IC primer (Entero-1 and Bio-entero-2), and 0.1 μM enterovirus VIC-MGB probe (Applied Biosystems). Amplification and real-time measurement were performed in the iCycler iQ5 (Bio-Rad) with the following conditions: 5 min at 95°C followed by 45 cycles of 30 s at 95°C and 45 s at 60°C. The number of parasites was calculated from the \(C_T\), i.e., the amplification cycle number at which the emitted fluorescence exceeded the set baseline of 150 reference fluorescence units (= background emission plus over 10 standard deviations).

Standard curves for \textit{Leishmania} parasites

To assess the reproducibility of absolute quantification by the three assays, a standard curve was tested and used to determine if the absolute values of the parasite numbers amplified from the cultivated parasites were statistically similar. To perform this experiment, blood was spiked with parasites from one parasite serial dilution in order to obtain 10,000,000, 100,000, 1,000, 100, 10, and 0 parasites per ml blood.

RNA extracts of these samples were tested in the three assays in triplicate in three different runs. Before extraction, two ICs were added to each sample; 1,000,000 molecules of in vitro-generated Q-RNA\textsuperscript{10} and the IC plasmid pEntIC 2 at 5,000 molecules. Both controls were used as extraction and inhibition controls to check whether samples were
### Table 1. Primers and probes used in the three molecular assays for detection and quantification of *Leishmania* parasites

<table>
<thead>
<tr>
<th>Assay</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>QT-NASBA</td>
<td>5' -GATGCA AGG TCG CAT ATG</td>
<td>5' -AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GGC CCG TAA AGG CCG AAT AG-3'</td>
<td>5’ biotin -GAC CAT TGT AGT CCA CAC TG-3'</td>
</tr>
<tr>
<td>QT-NASBA IC</td>
<td><strong>5’ biotin</strong></td>
<td>5’ biotin -CTT AGG TCC ACT AAG GTA CC-3’</td>
<td></td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>5’ -C CAA AGT GTG GAG ATC</td>
<td>5’ -GGC CGG TAA AGG CCG AAT AG-3’</td>
<td>5’ -6FAM AC CAT TGT AGT CCA CAC TGC-NFQ-MGB</td>
</tr>
<tr>
<td>qRT-PCR IC</td>
<td>5’ -C CAA AGT GTG GAG ATC</td>
<td>5’ -GGC CGG TAA AGG CCG AAT AG-3’</td>
<td>5’ -TET CTT AGG TCC ACT AAG GTA CC- NFQ- MGB</td>
</tr>
<tr>
<td>qPCR</td>
<td>5’ -AAG TGC TTT CCC ATC GCA ACT-3’</td>
<td>5’ -GAC GCA CTA AAC CCC TCC AA-3’</td>
<td>5’ 6FAM CGG TTC GGT GTG TGG CGC C-NFQ</td>
</tr>
<tr>
<td>qPCR EV-IC</td>
<td>5’ -GTC CCC TGA ATG CGG CTA AT-3’</td>
<td>5’ -GGG ATT GTC ACC ATA AGC AGC C-3’</td>
<td>5’ -VIC CTT GAG ACG TGC GTG GTA ACC-NFQ-MGB</td>
</tr>
</tbody>
</table>

*The region in bold is for generic ECL detection.

*bThe underlined region is a T7-promoter sequence.

*cFAM, TET, and VIC code for the reporter fluorophores, and NFQ codes for a nonfluorescent quencher group.*
Comparison between QT-NASBA, qRT-PCR and qPCR

truly negative, while in vitro Q-RNA also served as quantitative RNA for accurate quantification of the parasites in the QT-NASBA.19

After extraction, each concentration of the standard curve was divided into three aliquots and stored at -70°C for a maximum of 4 days. One aliquot was used for each run. Quantification of *Leishmania* parasites was achieved by plotting the log input parasite concentrations against either the C_T values or the log (Wild type/Q × 1,000) values with linear regression. The slopes (a) and y intercepts (b) of the standard curves were used to calculate the corresponding log output parasite concentrations with the formula y = ax + b.

Intra- and interassay variations (i.e., variations within one run and between the three different runs, respectively) of the quantified data of the standard curve were measured as coefficients of variation (CV) for each input concentration, calculated as the standard deviation/average × 100% for each triplicate (intra-assay variation) and for all nine output data (interassay variation). Correlation coefficients (r² values) were calculated for each standard curve using a linear regression analysis. For the real-time assays the amplification efficiency was calculated as $E = [10^{(1/Slope)} - 1] × 100\%$, as described by Smith et al.20 Both the slope and the y intercept in the standard equation are important, because absolute parasite numbers are determined from a number of different standard curves and the r² value and amplification efficiency alone give no information that will uniquely describe any individual curve.20 The analytical sensitivities of the three assays were determined by the lowest consistent detected concentration of parasites per ml.

Costs, time use, and user-friendliness

For each assay the total costs of reagents per sample were calculated, as were the time (total time necessary to perform each assay, from preparing reagent mixes until obtaining results; RNA/DNA extraction thus was excluded) and number of handling steps (the number of pipetting steps for the complete procedure).

Patients

This study was approved by the Brazilian National Review Board of the Ministry of Health (Comissão Nacional de Ética em Pesquisa, Parecer no. 1142/2005). The study population
comprised patients with suspected CL visiting FMTAM in Manaus, Brazil. In total, 84 patients who met the following inclusion criteria were included in the study: age between 17 and 65 years, clinical suspicion of CL, no previous history of CL, and written informed consent provided. Parasitological diagnosis was done by direct microscopic identification of *Leishmania* amastigotes in Giemsa-stained skin smears. During each evaluation, one skin biopsy (2 mm in diameter) was collected with a sterile disposable skin biopsy puncher from the active edge of the lesion according to WHO recommendations. Skin biopsies were taken under local anesthesia with xylocaine. In the absence of a true gold standard for diagnosis of CL, patients were defined as CL positive when the skin smear was positive in microscopy or in one of two *Leishmania* PCRs (based on mini-exon and hsp70 genes, as described by Marfurt et al. and Garcia et al.) performed on skin biopsies. Patients were defined as having non-confirmed CL when samples were tested negative by all three diagnostic methods. In total, 75 out of 84 suspected CL patients were defined as having confirmed CL, while 9 patients were defined as non-confirmed.

Additionally, restriction fragment length polymorphism on the PCR products was applied to distinguish the infecting *Leishmania* species of the CL patient. In each PCR run, the following reference strains were included as positive controls: *Leishmania* (Viannia) guyanensis MHOM/BR/75/M4147, *Leishmania* (Viannia) braziliensis MHOM/BZ/75/M2903, *Leishmania* (Leishmania) mexicana MHOM/MX/85/Solis, *Leishmania* (Leishmania) amazonensis MHOM/BR/81/LTB16, *Leishmania* (Viannia) lainsoni MHOM/BR/86/M6426, and *Leishmania* (Viannia) naiffi MHOM/00/94/CRE58.

**Patient samples**

Skin biopsies (2 mm in diameter) were mixed with 950 µl L6 lysis buffer (50 mM Tris HCl, 5 M guanidinium isothiocyanate, 20 mM EDTA, 0.1% Triton X-100) and stored at –70°C at FMTAM. After transportation (under cold conditions) to KIT Biomedical Research in Amsterdam, The Netherlands, RNA and DNA were extracted from the samples as described previously by the Boom method. In each extraction series, 16 patient samples and a serial dilution of promastigotes (see “Standard curves for *Leishmania* parasites” above) were included with three negative controls (water or negative blood) in order to
Comparison between QT-NASBA, qRT-PCR and qPCR

assess carryover contamination. Before extraction, the two ICs (Q-RNA and plasmid pEntIC 2) were added to each sample. Each extraction series was tested by QT-NASBA, qRT-PCR, and qPCR. The parasite quantification results for the patient samples in each assay were compared with those for the samples in the other two assays using Spearman’s correlation.

Results

Standard curves

For each parasite concentration of the standard curve, the CV (standard deviation/average × 100%) was calculated for one run (intra-assay variation), and the results are presented in Table 2. Intra-assay variation for all parasite concentrations had a lower range in QT-NASBA (CV from 0.5% to 3.3%), than in qRT-PCR (CV from 0.2% to 23.1%) and qPCR (CV from 0.1% to 8.4%). Overall, only 5 out of the 33 intra-assay CV calculated were above 5%. Only for the lower parasite loads (1,000 and 100 parasites/ml) did the intra-assay CV values in QT-NASBA remained below 5%, while most of the CV in qRT-PCR at 100 parasites/ml and in qPCR at 1,000 parasites/ml were above 5%. Furthermore, the range of variability did not increase using replicated standard curves within separate runs (interassay variation) for qRT-PCR (1.8% to 14.3%) and qPCR (0.4% to 5.3%). However, in QT-NASBA the range of variability increased slightly within separate runs (CV from 3.3% to 8.3%). The test results are plotted against the different dilutions in Figure 1.

In Table 3 the mean \( r^2 \) values, amplification efficiencies, and standard equations for the standard curves are presented. The high \( r^2 \) values for all three assays confirm the linearity of the standard curves used for quantification of parasite concentrations (in log values) and validate the use of a linear regression analysis. The efficiency of a PCR reaction provides information on amplification linearity and the effect of dilution of template DNA on the PCR amplification. In a 100% efficient reaction, it takes approximately 3.32 cycles for 10-fold amplification of the target, a value that is equal to the slope of the standard curve.
Figure 1. *Leishmania* parasite quantification of one *in vitro* cultured parasite serial dilution spiked in blood. QT-NASBA, qRT-PCR and qPCR were performed in triplicate in three different runs (run 1, 2 and 3).
In our study, PCR efficiencies were lower for qPCR (62% to 71%) than for qRT-PCR (82% to 85%).

The QT-NASBA could detect 10 parasites/ml in some samples, which corresponds to 0.5 parasites per blood sample because only 50 μl blood is processed. In reality, a level of 10 parasites/ml results in samples with one parasite and samples with no parasites, which increases the risk of false-negatives. For this reason, the detection limit was set at 100 parasites/ml, which is equivalent to 5 parasites per 50 μl blood. The detection limit of qRT-PCR was 100 parasites/ml, while the detection limit for qPCR was 1,000 parasites/ml.

**Patients**

In total, 84 suspected CL patients were included, from which 75 patients were diagnosed with CL by Giemsa smear in microscopy (n = 69) and/or by skin biopsies tested in PCR (n = 75). The median age of the 75 CL patients was 26 years (range, 17 to 63), the median number of lesions was 2 (range, 1 to 13), and the median duration of CL symptoms before diagnosis was 4 weeks (range, 1.5 to 52 weeks). The two PCR-restriction fragment length polymorphism assays revealed that 68 patients were infected with *L. (V.) guyanensis*, 2 patients with *L. (L.) amazonensis* and 5 patients with *L. (V.) braziliensis*.

**Patient results**

All 75 confirmed CL patients were positive by QT-NASBA, qRT-PCR, and qPCR before treatment, with median parasite counts of 30,000 (range, 5 to 5,780,000), 6,130 (range, 5 to 109,000) and 94,100 (range, 38 to 12,800,000) parasites/biopsy, respectively. It was notable that in four out of five *L. (V.) braziliensis*-infected patients, parasites were not detected by microscopy.

Eight out of nine non-confirmed patients who were negative by both microscopy and PCR were also negative by QT-NASBA, qRT-PCR, and qPCR. Negative results were not due to inhibition, since all ICs were positive. One of the non-confirmed CL patients was positive in all three assays for the wild-type targets, with parasite counts of 22, 43, and 40 parasites/biopsy, respectively. This patient was treated on the judgment of the infectiologist.
**Table 2.** Intra- and inter-assay variation calculated as Coefficients of Variation (CV = SD/Mean × 100%) of a standard curve tested in QT-NASBA, qRT-PCR and qPCR in triplicate in three different runs

<table>
<thead>
<tr>
<th>Log input (parasites/ml)</th>
<th>QT-NASBA</th>
<th>qRT-PCR</th>
<th>qPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 3</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>2.7</td>
<td>1.7</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>2.7</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>2.5</td>
<td>5.7</td>
</tr>
</tbody>
</table>

\(^{a}\)ND, not detected.

**Table 3.** Regression coefficients ($r^2$), amplification efficiency [$E = (10^{slope} - 1) \times 100\%.$], and the equations of the standard curves tested in QT-NASBA, qRT-PCR, and qPCR in triplicate in three different runs

<table>
<thead>
<tr>
<th>Value in:</th>
<th>QT-NASBA</th>
<th>q RT-PCR</th>
<th>q PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
<td>Run 3</td>
</tr>
<tr>
<td>$r^2$</td>
<td>0.995</td>
<td>0.997</td>
<td>0.999</td>
</tr>
<tr>
<td>E (%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Equations</td>
<td>$y=0.921x$</td>
<td>$y=0.888x$</td>
<td>$y=0.896x$</td>
</tr>
<tr>
<td></td>
<td>+0.694</td>
<td>+0.814</td>
<td>+0.542</td>
</tr>
</tbody>
</table>

\(^{a}\)NA, not applicable.

\(^{b}\) $x = \log ({\text{Wild type/Q}} \times 1,000)$ for QT-NASBA results and the $C_T$ value for qRT-PCR and qPCR results; $y = \log$ calculated number of parasites/ml.
Comparison between QT-NASBA, qRT-PCR and qPCR

Overall, when parasite counts from the three assays were compared, significant correlations were found between QT-NASBA and qRT-PCR (Spearman’s $\rho = 0.961$; $P < 0.001$), QT-NASBA and qPCR (Spearman’s $\rho = 0.919$; $P < 0.001$), and qRT-PCR and qPCR (Spearman’s $\rho = 0.966$; $P < 0.001$).

Costs, time-use and user-friendliness

For each assay the cost of reagents per sample, duration of assay, and number of steps for sample handling were calculated. The qPCR was the least expensive assay (€ 1.55 per sample, in comparison with € 5.15 for QT-NASBA and € 2.20 for qRT-PCR). QPCR required the shortest assay time, of only 2 hours, while for QT-NASBA and qRT-PCR, 4 hours 30 minutes and 2 hours 20 minutes, respectively, were required. Overall, the qRT-PCR assay is less than half as expensive as the QT-NASBA assay and twice as rapid in use. In both qPCR and qRT-PCR the reaction takes place in a closed-tube format, which minimizes sample handling and avoids the risk of contamination. The total number of handling steps per sample for both qPCR and qRT-PCR was only two. In contrast, QT-NASBA has an extensive time-course between sample preparation and data collection and requires eight handling steps due to the ECL detection method.

Discussion

Only a few studies have compared the application of molecular diagnostic tools for leishmaniasis. These studies are needed in order to assess the best assay performance for implementation in areas where the disease either is or is not endemic. In the present study, two RNA-based amplification assays (QT-NASBA and qRT-PCR) were compared with one DNA amplification assay (qPCR). In all three assays, an IC was included and one Leishmania serial dilution was tested in triplicate and in separate runs.

Both QT-NASBA and qRT-PCR were 10-fold more sensitive than qPCR, detecting at least 100 parasites/ml. In a few samples the QT-NASBA detected parasite concentrations of
10 parasites/ml. Both QT-NASBA and qRT-PCR amplifies both parasite rRNA, while qRT-PCR also amplifies rDNA, since no DNase step prior to amplification is introduced. Parasite rRNA copy numbers have shown to be over 100-fold more abundant than the gene copy numbers\(^1\), which explains the lower sensitivity of the qPCR, in which only target DNA is amplified.

Overall, QT-NASBA had the most constant intra-assay variation over the whole range of parasite concentrations, with all CVs below 3.3%. For qRT-PCR and qPCR the variation increased for the lower parasite concentrations (100 parasites/ml and 1,000 parasites/ml). However, QT-NASBA had higher inter-assay variations over the whole range, except for the lowest parasite concentration of 100 parasites/ml. Furthermore, QT-NASBA and qRT-PCR performed better than qPCR, with higher regression coefficients and a higher reaction efficiency for the qRT-PCR. The lower performance of qPCR can be attributed to the incorporation of a noncompetitive IC (i.e., a DNA template with different primer sequences) with a different length than the target DNA. In the other two assays (QT-NASBA and qRT-PCR), the same competitive IC (Q-RNA is amplified with the same primers as the wild-type target) has been incorporated.

In this study the QT-NASBA amplification was detected by ECL, which makes the assay more laborious and expensive. By the incorporation of a molecular beacon as a fluorescent label, a real-time QT-NASBA format can be constructed.\(^2\) This real-time QT-NASBA assay is very promising, since it combines the isothermal characteristics, sensitivity, and the rapidity of the NASBA reaction with the simple, closed-tube format of a real-time assay.\(^3\) So far, no real-time QT-NASBA has been established for leishmaniasis. Another alternative for ECL detection is oligochromatography. With this method, the amplified products are visualized within 5 minutes on a dipstick through hybridisation with a gold-conjugated probe.\(^4\) For human African trypanosomiasis, this method was successfully combined with PCR and proved to be sensitive and specific.\(^5\) While such an assay would have no quantification purposes, NASBA in combination with oligochromatography would circumvent the need for a thermocycler and postamplification equipment and would be ideal for lower-tech laboratories. Currently, simplified detection methods are under development.\(^6\)
Comparison between QT-NASBA, qRT-PCR and qPCR

Several qPCRs for the detection of *Leishmania* spp. have been described, but in most cases the analytical sensitivity was not reported, an inhibition control was lacking, or the assays were based on detection of one specific species, limiting their use in clinical diagnosis.11,15,33 The 18S target is preferred, since it is almost completely homologous for all *Leishmania* species13, which increases its value for clinical diagnosis.10,14 When the three assays were applied to patient samples, quantitative results were significantly correlated between all three assays. No negative results were found, giving a sensitivity of 100% for this patient group. However, one patient in the CL-negative group was positive in all three assays. Based on the clinical symptoms and clinical improvement during treatment, this patient is still assumed to have had CL. This would also imply a higher sensitivity of these assays than microscopy and the PCR assays applied in this study. The other eight nonconfirmed patients who were tested negative probably had ulcerating pyoderma. The importance of a high sensitive assay is demonstrated in our results; as 4 out of 6 microscopy negative patients were infected with *L. (V.) braziliensis*, a species of which is known to cause low parasite loads in the lesions.34 When *L. (V.) braziliensis* infection remains undetected it can have serious implications for the patient since it can cause mutilating mucocutaneous leishmaniasis.35

In conclusion, all three molecular assays are reliable methods for the detection and quantification of *Leishmania* parasites in clinical samples. The RNA based methods (QT-NASBA and qRT-PCR) are the most sensitive assays generating reproducible results. The QT-NASBA is less convenient since the ECL detection involves more handling steps and procedure time. The development of an easy and rapid post amplification method, like oligochromatography can increase the value of QT-NASBA for endemic areas with no sophisticated laboratory. However, a disadvantage remains the high prices for these molecular assays, especially the QT-NASBA. For now, the use of qRT-PCR is recommended as a sensitive and most convenient assay, providing highly sensitive detection and quantification of *Leishmania* parasites. This method can be applied for diagnosis, treatment efficacy studies, epidemiological studies and vaccine trials.
Acknowledgments

We gratefully thank the patients for their participation in this study and the staff of the Leishmaniose Laboratory at FMTAM who helped with translations and registration of patients. Furthermore, we thank Patricia van Sambeek and Esther Pariama (AMC, Amsterdam, The Netherlands) for collection of data and patient samples and Fabio Eudes Leal for his help and efforts to send the patient samples to The Netherlands. We thank Marcel Beld and Richard Molenkamp (Department of Medical Microbiology, AMC Amsterdam, The Netherlands) for help in development of the qRT-PCR and for providing the IC. We thank Lisa Lambregts and Inge Versteeg (KIT Biomedical Research) for help in sample analyses. This work was supported by a grant from The Netherlands Foundation for the Advancement of Tropical research (WOTRO contract 96-210).
Comparison between QT-NASBA, qRT-PCR and qPCR

References

Comparison between QT-NASBA, qRT-PCR and qPCR


Chapter 4