Cutaneous leishmaniasis: new developments in diagnosis and treatment evaluation

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Treatment assessment by monitoring parasite load in skin biopsies from cutaneous leishmaniasis patients with QT-NASBA

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Summary

Background. Currently diagnostic methods for cutaneous leishmaniasis (CL) have a low sensitivity or are not useful for treatment follow-up. We previously described the QT-NASBA method as a sensitive and specific assay for detection and quantification of *Leishmania* parasites in skin biopsies. This assay could be a valuable instrument for monitoring therapy response of CL and identify treatment failures at an early stage.

Aim. QT-NASBA results of skin biopsies at the end and 6 weeks after therapy from proven CL patients treated with various treatment schedules were compared with clinical outcome.

Methods. The QT-NASBA assay measured the parasite load in skin biopsies before, at the end and 6 weeks after treatment. The results were compared with therapy outcome (clinical cure, delayed healing response or treatment failure) up to 6 months after treatment.

Results. In total, 137 skin biopsies were obtained from 53 patients. A positive QT-NASBA result 6 weeks after treatment was significantly associated with treatment failure/delayed healing up to 6 months (P<0.001). The Positive Predictive Value (PPV) was 100% and the Negative Predictive Value NPV 92% (95% CI = 82%-100%). QT-NASBA results at the end of treatment and clinical outcome showed a less significant association (P<0.05), PPV was 46% with a 95% confidence interval of 16% - 75% and NPV was 89% (95% CI = 79% – 99%).

Conclusions. The QT-NASBA assay is an useful instrument to monitor parasite loads in skin biopsies of CL patients 6 weeks after treatment and can help predicting clinical outcome.
Introduction

Cutaneous leishmaniasis (CL) is an infectious disease of the skin affecting between 1.0-1.5 million people worldwide every year. The disease is caused by protozoan parasites of the genus *Leishmania* and is endemic to most tropical and subtropical countries. CL comprises several distinct clinical manifestations; from single skin ulcers to more disseminated forms. Most forms are self-healing within months, but this process can also take several years leaving visible atrophic scars. In the mucocutaneous form (MCL) the parasites spread to the nasopharyngeal mucosa, causing extensive destruction. MCL can emerge even after many years following the initial skin lesion. Therefore, early diagnosis and effective treatment would aid in the clinical management and eventual outcome of CL.

The incidence of leishmaniasis is increasing worldwide, partly due to treatment failure. The occurrence of treatment failures may be predicted when sensitive diagnostic tools are applied to monitor the progress of a disease. In most countries where CL is endemic, diagnosis is based on clinical and epidemiological criteria only. Where facilities are present, clinical practice often combines microscopy of stained scrapings, histopathology and/or culture. However, these tools lack sensitivity. Nowadays, molecular techniques offer tools for CL diagnosis as well as treatment evaluation. Several polymerase chain reaction (PCR) methods have been developed offering improved sensitivity over microscopy and culture.

Although these assays have become well-established diagnostic tools, the value of the PCR for treatment evaluation of CL seems to be low. DNA of *Leishmania* parasites can be detected in scars of CL patients even months after clinical cure. Up to this date, the clinician depends basically on clinical criteria to evaluate the therapy outcome and clinical cure of the lesions. A sensitive method that can quantify the parasite load in CL lesions could be an useful determinant to evaluate treatment success and could help to predict the occurrence of treatment failures. Moreover, such an assay would be relevant to apply in randomised controlled studies for the implementation of new drugs, or judgement of treatment schedules.
Treatment assessment

Currently, no reports are available to date that applied quantitative molecular techniques to evaluate treatment of CL. Recently, we developed a quantitative nucleic acid sequence-based amplification (QT-NASBA) for leishmaniasis. The QT-NASBA has shown both increased sensitivity and accurate quantification for the detection of *Leishmania* parasites in skin biopsies, and may be an important assay for the follow-up of CL treatment. The *Leishmania* QT-NASBA is based on amplification of single-stranded 18S ribosomal RNA sequences. Since parasites contain a larger number of 18S rRNA copies (> 10⁴) than rDNA (~10²), the assay is more sensitive than PCR for the same sequence. Furthermore, the 18S rRNA target is present in all *Leishmania* species, allowing quantification of all species in a similar manner. QT-NASBA has already been successfully applied to monitor the response to miltefosine in a patient treated for visceral leishmaniasis. In this study we addressed the following question: Do QT-NASBA results obtained at the end and 6 weeks after treatment correlate significantly with the clinical outcome of CL?

Materials and methods

Patients

The study protocol was reviewed and approved by the Medical Ethical Committee of the Academic Medical Center (AMC) in Amsterdam (MEC 03/228). This study was set up as a non-randomized prospective hospital outpatient based study. Fifty-three CL patients who visited the outpatient clinics of the Departments of Dermatology and Tropical Medicine of the AMC between 2004 and 2006 were included. No patients were included who were younger than 18 year or had lesions confined to the face. All patients were diagnosed with CL by at least one of four conventional diagnostic methods; Giemsa staining of biopsy smears, histopathological examination of biopsies, culture and/or PCR on DNA extracted from lesion biopsies. After initial diagnosis, all patients were treated by first choice treatment schedule based on clinical picture and geographical exposure area. Three patients received sodium stibogluconate (Pentostam®, Glaxo Wellcome) 20 mg/kg/day intravenously (i.v.) for 20 days, while one patient received Pentostam® i.v. treatment for 16 days (4 days less than the intended 20 days because of severe side-effects). Twenty-two
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patients received 1-2 doses of cryotherapy in combination with 2-8 injections of Pentostam® intralesionally (i.l.) (150-250 mg per lesion) with few days interval, one patient was treated with 5, and another patient with 6 intermittent injections of Pentostam® i.l. without cryotherapy. Five patients received 4 intermittent intramuscular (i.m.) injections of 250 mg pentamidine (Pentacarinat®, Aventis Pharma) spread over 10 days. One patient was treated with fluconazol (Diflucan®, Pfizer), 200 mg per day for 6 weeks in combination with topical 15% paromomycin cream (locally prepared) b.i.d. for 20 days. One patient was treated with itraconazol (Trisporal®, Janssen-Cilag) 400 mg per day for 6 weeks, and in one patient the lesion was excised. Seventeen patients were treated with miltefosine (Impavido®, Zentaris) 50 mg t.i.d. (1.3 – 2.1 mg/kg/day) for 28 days. All treatment regimes were applied as primary therapy, except for 16 patients treated with miltefosine, who had been treated with Pentostam® i.l. as primary therapy in a field setting (n=15) or had been treated with cryotherapy combined with Pentostam® i.l. (n=1).

Patients were evaluated before, at the last day of treatment, 6 weeks and 6 months after treatment. In total 137 skin biopsies were collected: before (n = 53), on the last day (n = 49) and 6 weeks (n = 35) after treatment. Before treatment all biopsies were collected from patients with active lesions. Only those biopsies were included which were taken within a 14 day interval before or after the scheduled time-points. Biopsies (2 mm in diameter) were taken by an experienced dermatologist from the active edge of the lesion according to the recommendations of the WHO. Biopsies collected after treatment were taken from the same lesion as the biopsies collected at the start of treatment.

Clinical criteria
Assessment of clinical cure of the patient was done 6 months after treatment by an experienced dermatologist. A patient was defined as clinically cured if all lesions completely reepithelialized in the absence of induration, lymphangitis, lymphadenitis and satellite lesions, with only presence of (an) atrophic scar(s). In case the lesion was completely epithelialized, without lymphangitis or lymphadenitis but still indurated, it was defined as delayed therapy response. A treatment failure was defined as incomplete
epithelialization and persistent induration in combination with lymphangitis or lymphadenitis or presence of new satellite lesions.

**QT-NASBA and PCR-RFLP**

Skin biopsies (2-mm in diameter) were stored in 950 μl L6 lysis buffer (50 mM Tris HCl, 5 M GuSCN, 20 mM EDTA, 0.1% Triton- X-100) at -20°C. Skin biopsies were processed and the RNA/DNA extractions were tested in the QT-NASBA as described previously. Briefly, NASBA reaction was performed using NucliSens Basic Kit for amplification according to the manufacturer’s instructions (bioMérieux) in a 10 μl total reaction volume at a final KCl concentration of 70 mM. The reaction mixture was incubated with 2.5 μl RNA extract at 65 °C for 5 min and subsequently at 41 °C for 5 min. The isothermal amplification took place for 90 min at 41 °C. The next step was detection with the electrochemiluminescence (ECL) detection method. QT-NASBA results with a parasite count of > 0.1 P/μl were defined as positive, which is equivalent to 5 parasites per biopsy. Samples collected before treatment were also tested in the small subunit (SSU) – internal transcribed spacer (ITS) and Heat Shock Protein 70 (hsp70) genes PCR-RFLP, as described by Rotureau et al. and Garcia et al. respectively to identify the infecting species.

**Statistical analysis**

To quantify the number of parasites a best-fit regression analysis was performed as described previously. Binary logistic regression analysis was performed to assess if initial parasite load was a predictor for treatment outcome at 6 months. A Fisher’s Exact test was used to compare QT-NASBA results and clinical outcome. A p<0.05 was considered statistically significant. The positive and negative predictive values of the QT-NASBA for treatment failure or delayed response were calculated in the present study as follows: Positive predictive value = TP / (TP + FP) x 100% and negative predictive value = TN / (FN + TN) x 100%. Where TN represents true negative, TP true positive, FN false negative and FP false positive. Confidence intervals of 95% were calculated.
Results

Patients
In total, 53 patients were included with a median age of 27 (19-78) years and a median number of 2 lesions (1-31). Forty-nine (92%) patients were male. Thirty seven patients were infected with *L. major* (35 patients infected in Afghanistan, one patient in Morocco and one patient in Israel), 10 patients were infected with *L. (L.) mexicana* and one patient with *L. (V.) braziliensis* who visited Belize and 5 patients were infected with *L. (V.) guyanensis* who visited Suriname.

<table>
<thead>
<tr>
<th>Table 1. QT-NASBA results and clinical outcome at 6 months evaluation of 53 cutaneous leishmaniasis patients assembled according to Old (Afghanistan, Israel and Morocco) and New World (Belize and Suriname) Leishmania infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leishmania infection contracted in the Old or New World</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Old World (n=37)</td>
</tr>
<tr>
<td>New World (n=16)</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>P biopsy$^{-1}$ = number of parasites per biopsy sample</td>
</tr>
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</table>

QT-NASBA results
In total 137 biopsies were tested (53 before, 49 at end of treatment and 35 six weeks after treatment). In Table 1 QT-NASBA results are presented for the Old (Afghanistan, Morocco and Israel) and New World group (Belize and Suriname) separately. All skin biopsies
collected at start of treatment were positive with a median parasite count of 6,500 ranging between 5 – 274,000 P biopsy\(^{-1}\) (parasites per biopsy sample).

Twenty-four of the 26 patients with a negative biopsy at 6 weeks were not further treated and were clinically cured at 6 months. In one patient additional intralesional therapy was given on clinical grounds and was cured at 6 months, another patient developed a clinical relapse 2.5 month later and was still not cured at 6 months. The 3 patients with inconclusive NASBA results 6 weeks after treatment were cured at 6 months. Of the 6 patients with a positive NASBA result at week 6, 3 needed further treatment (1 i.v. Pentostam® and 2 i.l. Pentostam®) between 2.5 to 3.5 months after ending the first treatment course. They were further observed and finally cured. The other 3 still had some activity with infiltrated lesions at 6 months but were not further treated. While the end-point of this study was 6 months, the 3 patients with delayed response needed further evaluation after 6 months up to 1 year. They were finally judged as cured.

Overall, 7 out of 53 (13%) patients were defined as treatment failures, and three patients had a delayed response (6%). Binary logistic regression analysis showed that the number of parasites (parasite load in skin biopsies) at start of treatment was no predictor of treatment outcome \((P = 0.536)\). The 43 patients who were cured at 6 months had a median parasite count of 3,850 P biopsy\(^{-1}\) (range 5 to 274,000) at start of treatment, while the patients who were not cured had a median parasite count of 4,280 P biopsy\(^{-1}\) (range 217 to 83,500).

**Association between QT-NASBA results and clinical outcome**

In Table 2A and 2B QT-NASBA results are compared with clinical outcome of the disease. From 48 patients, from whom skin biopsies were collected on the last day of treatment (Table 2A), 6 patients developed a treatment failure and needed a second course of treatment. Three other patients still had infiltrated lesions at 6 months of follow-up and could not be defined as clinically cured. Out of this group of 9 patients 5 had positive QT-NASBA results with a median parasite count of 128 P biopsy\(^{-1}\) (range 5 to 105,000 P biopsy\(^{-1}\)). Six out of 39 patients of the cured patient group had positive QT-NASBA results at the end of treatment, with a median parasite count of 290 P biopsy\(^{-1}\) (range 5 to 6,350).
A significant correlation was found between QT-NASBA result at the end of treatment and clinical outcome (P<0.05). In addition, positive and negative predictive values were calculated. The positive predictive value (PPV) or the possibility that a positive QT-NASBA result predicted a treatment failure or a delayed treatment response up to 6 months follow-up was 46% with a 95% confidence interval (CI) of 16% - 75%. The Negative Predictive Value (NPV) was 89% (95% CI = 79% – 99%).

In Table 2B, QT-NASBA results are presented of 32 skin biopsies collected 6 weeks after treatment in conjunction with the clinical outcome of the patient. In total, 8 patients were not clinically cured after 6 month follow-up. Of this group 6 patients had positive QT-NASBA results with a median parasite count of 2,850 P biopsy−1 (range 14 to 53,400 P biopsy−1). Furthermore, no positive QT-NASBA results were found within the cured patient group. However, 2 patients with a negative QT-NASBA, were given a second treatment course on clinical grounds. One of these 2 patients received a second treatment regime with cryotherapy and il. Pentostam ® 2.5 months after therapy only in one remaining papule adjacent to the target lesion. The target lesion, from where the biopsies were collected, was already clinically healed at this moment. The other patient had a positive QT-NASBA result in a skin biopsy collected 9 weeks after treatment (data not shown). Overall, a strong significant correlation was found between QT-NASBA result 6 weeks after treatment and clinical outcome (P<0.001). The PPV of the QT-NASBA at week 6 for treatment failure or
Treatment assessment

delayed response was 100%. The NPV was calculated as 92% with a 95% CI between 82% - 100%.

Table 2.B Clinical outcome at 6 months follow-up in relation to QT-NASBA results (P<0.001) of skin biopsies from cutaneous leishmaniasis patients collected 6 weeks after treatment (n = 32). Three samples were excluded due to inconclusive results.

<table>
<thead>
<tr>
<th>Evaluation point</th>
<th>NASBA 6 weeks after treatment No. positive</th>
<th>NASBA 6 weeks after treatment No. negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not cured 6 month after treatment*</td>
<td>6</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Cured 6 month after treatment</td>
<td>0</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>26</td>
<td>32</td>
</tr>
</tbody>
</table>

* including patients with treatment failure and delayed healing response

Discussion

Our study demonstrates a highly significant correlation between QT-NASBA results 6 weeks after treatment and clinical outcome of disease. QT-NASBA results obtained at the end of treatment were also significantly associated with clinical outcome, albeit the association was lower compared with the 6 weeks time-point.

Since in many endemic countries patients do not return for treatment follow-up, an end-point measure at the end of the treatment would be highly beneficial. In this way, not responding patients can be identified early and can be given prolonged treatment immediately or carefully evaluated. This may prevent the development of new lesions, and decreases the chance for transmission. Unfortunately, in this study we only found a weak association between NASBA results at the end of treatment and clinical outcome. The reason for this may be the temporarily decrease of the parasites until below detection level of the assay caused by the medication. This can be supported by the fact that three patients of our study group who were negative in NASBA at the end of treatment had indeed positive NASBA counts in later follow-up. It is also known that CL lesions may demonstrate a delayed clinical response after 3-4 weeks, and may not entirely heal until
several weeks after ending treatment. This partly depends on an inadequate Th-1 immune response of the host. This would explain the positive NASBA counts at the end of treatment of patients who were eventually cured at 6 months.

Thus far, our results show that 6 weeks after treatment is a relevant time point to re-evaluate parasite loads in the lesions of patients. A positive QT-NASBA result 6 weeks after treatment seems to predict treatment failure or delayed response, whereas a negative QT-NASBA result is a good indication for a clinically cured patient. Unfortunately, only 35 skin biopsies were tested 6 weeks after therapy, while 53 patients were included at start of treatment. While most patients did return to the clinic for follow-up, they returned on another time point than was prescribed by the study protocol. For this reason, 18 samples at week 6 after therapy are missing.

CL is a complicated spectral disease. Some forms present with self-healing lesions, but leave scars with may lead to strong social stigma. Other forms are non-healing or chronic with sequels leading to disfigurement or mutilation of the face, especially for New World species as *L. (V.) braziliensis*. The most optimal time-point to collect a sample for QT-NASBA will probably depend on different factors as infecting species, applied treatment, parasite loads and host factors. In the present study, we did not find an association with parasite load quantified in QT-NASBA before treatment and treatment outcome. However, it is recognized that time to cure can be different in CL patients infected with the same species, but treated differently. This initial study included different methods of treatment and different species. To study more reliable the influence of different factors larger and well-defined study groups are necessary. In future studies we suggest to test variable time-points after treatment for different treatment schedules and different *Leishmania* species. However, at the moment overall 6 weeks seems to be a valid universal end point.

The widely diverse responses to treatments emphasizes the need for a reliable monitoring tool. QT-NASBA has already proven to be a valuable tool for monitoring parasite load and even predicting treatment failure in other infectious diseases. We believe that the assessment of treatment outcome can be improved by the addition of QT-NASBA method, because the assay is able to predict treatment failure or delayed responses.
Treatment assessment

In conclusion, this study shows that QT-NASBA can be applied as a reliable assay to monitor parasite loads after treatment in an outpatient setting and helps the clinician in decision making for further clinical management.

Acknowledgments

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