Cutaneous leishmaniasis: new developments in diagnosis and treatment evaluation
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Citation for published version (APA):

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Evaluation of treatment for cutaneous leishmaniasis in Suriname with pentamidine

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Submitted
**Summary**

**Background** In Suriname Pentamidine Isethionate (PI) is the only available drug for treatment of cutaneous leishmaniasis (CL). Recently, local dermatologists have observed an increase in CL patients not responding adequately to the standard doses.

**Methods** In this study patient compliance to PI treatment was assessed and the efficacy was evaluated by comparing clinical criteria and parasitological load in week 3 of treatment. Skin biopsies were collected before, during and at the end of therapy and tested in QT-NASBA, an RNA amplification method.

**Results** In total, 67 CL suspected patients were enrolled during recruitment period, of which only 23 CL confirmed patients could be followed until the end of treatment. All 23 patients were found to be infected with *L. (V.) guyanensis*. A lower cure rate (76% - 78%) was estimated than before (90%) and only 50% of the recruited CL patients finished the complete treatment schedule.

**Conclusions** Since the half of CL patients were treated insufficiently, a much shorter treatment protocol should be considered to improve the inadequate follow-up.
Chapter 5

Introduction

Cutaneous leishmaniasis (CL), a protozoan skin disease, presents a public health problem in Suriname, a country in the northern Amazon region of South America. The disease is endemic in the forested interior of the country and is locally known as Boschyaws or Boessie-Yassi.1 Updated incidence numbers of CL in Suriname are not available. In Suriname, CL is primarily a zoonotic disease transmitted by the bite of a female sand-fly of the genus Lutzomyia. Humans are considered to be incidental hosts only, while the animal reservoir for leishmaniasis has not yet been identified.2 L. (V.) guyanensis has been reported to be the only Leishmania species present.1 Most people who contract the disease live and/or work in the forested interior of Suriname (gold miners, soldiers, woodcutters) or visit it for recreation or hunting.1

CL is a disease with a wide spectrum of clinical manifestations, from localized to mucocutaneous and diffuse forms. Several treatment options are available and choice of treatment depends on clinical manifestation and infecting species.3 Pentavalent antimonials remain the first choice treatment for CL, with primarily meglumine antimoniate (Glucantime ®) as first option for treatment in the New World.4 Currently, the diamidine pentamidine isethionate (PI) has become the first line treatment specifically for L. (V.) guyanensis infection.5 PI is a safe and effective drug for CL with a comparable cure rate to that of pentavalent antimonials.6,7 Moreover, a short course PI has advantages in respect of duration and costs.1

Currently, PI is the only available drug for CL patients in Suriname. With a standard treatment regime of 3 to 4 doses of 300 mg PI (once every week) a cure rate of 90% was achieved between 1994 and 2000.1 However, dermatologists in Paramaribo (the capital city of the country) recently observed that an increasing number of CL patients do not adequately respond to the standard treatment (personal communication Prof. dr. R.F.M. Lai A Fat, Department of Dermatology, Academic Hospital, Paramaribo). Worldwide a decline in the efficacy of pentamidine has only been reported for patients with visceral leishmaniasis (the systemic form of leishmaniasis) in India.8 In the past few years, two
studies performed in the New World reported high cure rates (84% - 95%), while only one study reported a low cure rate of 35% with pentamidine treatment against CL infection.\textsuperscript{6,9,10}

Inefficacy of antileishmanial therapy may be due to different factors including subtherapeutic doses of the drug and insufficient duration of treatment, variable sensitivities of \textit{Leishmania} species or an inadequate immune response of the host.\textsuperscript{8,9} Little is known about the relative therapeutic efficacy of pentamidine against different \textit{Leishmania} species. So far, pentamidine seems to be more effective against \textit{L. (V.) guyanensis} and \textit{L. (V.) panamensis} than against \textit{L. (V.) braziliensis} infection.\textsuperscript{9} In Suriname, \textit{L. (V.) guyanensis} has been reported to be the only species present.\textsuperscript{1} However, patients with diverse clinical forms of CL have been encountered suggesting that other \textit{Leishmania} species do occur.\textsuperscript{11} This could influence the efficacy of pentamidine treatment.

Since no updated reports are available on the current efficacy of and compliance to PI treatment in Suriname, a study was conducted in Paramaribo between 2005 and 2006. To monitor decrease in parasite loads during and after treatment we applied QT-NASBA for follow-up. QT-NASBA, an RNA amplification method, is a very sensitive and specific method for the detection and quantification of \textit{Leishmania} parasites, and was already successfully applied in skin biopsies of CL patients.\textsuperscript{12} We calculated the decrease of parasite loads in lesions before, during, at the end and after treatment and compared it with clinical response.

\section*{Materials and methods}

\subsection*{Patients}

The study was conducted at the Dermatology Service (department of Ministry of Health) and Dermatology Department (Academic Hospital) in Paramaribo, Suriname between April 2005 and July 2006. The study population comprised cutaneous leishmaniasis (CL) suspected patients visiting these Dermatology clinics. Cases were included in the study if they met the following inclusion criteria: age between 17 and 65 years, clinical suspicion of CL and written informed consent to participate in the study. One exception was made for a
patient younger than 17 years who wanted to participate in the study and after informed consent was given by one of his/her parents, this case was also included in the study. The study protocol was reviewed and approved by the Medical Ethical Committee of the Academic Medical Center (AMC) in Amsterdam (MEC 03/228) in 2003.

Parasitological diagnosis

CL was confirmed by either the direct microscopic identification of *Leishmania* amastigotes in Giemsa stained smears, histopathological examination in the departments of Pathology and Dermatology in Paramaribo or with QT-NASBA in The Netherlands.

Treatment and follow-up

All patients received standard treatment of 3 to 4 doses 300 mg PI salt (corresponds with 172.5 mg pentamidine drug; PHARM-InterSprl Brussels Belgium) by intramuscular (IM) injection once every week. Patients were evaluated before, at week 2, week 3 (= end of therapy for schedule with 3 injections), week 4 (= end of therapy for schedule with 4 injections) and when possible 6 weeks after the last injection. During each evaluation two 2-mm skin biopsies were taken 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, and collected from the same lesion before, during and after treatment.

Clinical criteria

Two experienced dermatologists (Dr. Henry J.C. de Vries and Prof. dr. Wiliam R Faber) of the Academic Medical Center (Amsterdam, The Netherlands) evaluated the clinical response of the patient by comparing photos of the target lesion at start and in week 3 (last day of 3 injections schedule, when patient received the last dose of PI). Both dermatologists were blinded for patient characteristics and results of QT-NASBA. The target lesion was judged on the following three clinical criteria: re-epithelialization and degree of contraction of the lesion, decrease of necrotic tissue and decrease in border activity. For each criteria a degree of healing was given between 1 to 5 (1 = very bad, 2 = bad, 3 = no change, 4 = good
clinical response, 5 = excellent clinical response), counted up and divided by the number of judged criteria (n = 3). A total score of ≤ 3 was defined as initial therapy unresponsiveness.

**Nucleic acid extraction**
Skin biopsies (2-mm in diameter) were mixed with 950 µL lysis buffer (50 mM Tris HCl, 5 M GuSCN, 20 mM EDTA, 0.1% Triton-X-100) and stored at −70 °C at the Central Laboratory (Bureau of Public Health, Paramaribo, Suriname). After transport (under cold conditions) to KIT Biomedical Research (Amsterdam, The Netherlands) samples were stored at −70 °C until processing. RNA and DNA were extracted as described by Boom.14 In brief, skin biopsies were disrupted by shaking the samples with a 5-mm stain-less steel bead in the Mixer Mill MM 301 (Retsch GmbH & Co. KG) for 300 seconds at 30 Hz. Next, RNA and DNA were extracted with the GuSCN-silica procedure.14 In each extraction serie, a standard curve was included (10,000,000; 100,000; 1,000 and 100 parasites per ml blood) and three negative controls (water or negative blood) in order to assess carry-over contamination. The samples were stored at −20 °C until further analyses.

**PCR-RFLP (mini-exon and hsp70)**
Two Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) assays were performed to identify the infecting *Leishmania* species of the patient. RNA and DNA extractions of the patient samples, collected before treatment, were analysed with the spliced leader RNA gene PCR-RFLP (mini-exon) and Heat Shock Protein 70 (hsp70) gene PCR-RFLP, as described by Marfurt et al.15 and Garcia et al.16 In each PCR run of both assays the following reference strains were included as positive controls: *L. (V.) guyanensis* MHOM/BR/75/M4147, *L. (V.) braziliensis* MHOM/BZ/75/M2903, *L. (L.) mexicana* MHOM/MX/85/Solis, *L. (L.) amazonensis* MHOM/BR/81/LTB16, *L. (V.) lainsoni* MHOM/BR/86/M6426 and *L. (V.) naiffi* MHOM/00/94/CRE58. After amplification, the PCR amplicons were cut by HaeIII (New Englands Biolabs) for 2 hours at 37 °C. Next, PCR fragments were detected by UV light on a 3% pronarose (Sphaero Q, Burgos Spain) gel, stained with ethidium bromide. A 100-bp DNA ladder (Amersham Biosciences, Buckinghamshire, UK) was used as a marker.
QT-NASBA
All samples were analysed in QT-NASBA following the procedures published previously. After extraction and amplification, the samples were detected with 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.

Parasite quantification and statistical analysis
To quantify the number of parasites with QT-NASBA in biopsy samples, the wild-type (WT) RNA of the parasite was co-amplified with in vitro Q-RNA by competitively binding of the same primer set. ECL generated two signals for each sample by the hybridisation of a WT and a Q capture probe; a WT and Q count, respectively. Final parasite counts in each sample were obtained by comparing the WT and Q ECL ratio by best-fit regression analysis in comparison with a standard curve.

A Wilcoxon Signed Ranks Test was used to compare the differences between the results of the two biopsies in QT-NASBA collected from the same lesion at the same time-point. A Wilcoxon-Mann-Whitney test was used to test for significance (P<0.05) between patients with a good and poor clinical response for initial parasite loads.
Results

Patient characteristics and compliance
During the recruitment period, 67 patients with suspicion of CL were included in the study. Sixty-four (96%) patients were diagnosed with CL and in three patients the diagnostic tests (4%) were negative. Out of 64 CL confirmed patients, 32 patients (50%) received 3 to 4 injections with PI, 9 patients (14%) received 2 injections, 18 patients (28%) received 1 injection and one patient (2%) received 7 injections. From 4 patients (6%) the treatment status is unknown.

In total, only 23 out of 64 CL patients received at least 3 injections with PI, and could be followed until the end of treatment. The patient’s characteristics are presented in Table 1. Most patients contracted the disease in the Brokopondo region, in the south east of Suriname (n=8) and during work-related activities (n=13). Five out of 23 patients were gold miners. Other main activities were hunting (n=6) and recreation (n=4). PCR-RFLP revealed that all patients were infected with L. (V.) guyanensis (n= 23). The number of lesions per patient ranged from 1 to 16 with a median of 2 lesions. Most patients had their lesions located on the extremities (33% arms and 38% legs). The duration of the clinical symptoms ranged from 2 - 33 weeks, with a median duration of 7 weeks. Four patients, who were included had a Leishmania recurrence 1 month to 4 years after first treatment.

Nineteen patients were treated with 3 injections and 3 patients received 4 injections of 300 mg PI once every week. One patient received an extended schedule of 7 injections. Only three patients returned 4 to 6 weeks after ending treatment for clinical and parasitological evaluation. Three patients returned to the clinic for additional treatment (one month and 6 months after ending therapy).
### Table 1. Patient characteristics of 23 patients

<table>
<thead>
<tr>
<th>Id. no.</th>
<th>Sex</th>
<th>Age</th>
<th>Area</th>
<th>District</th>
<th>Activity when patient got infected</th>
<th>No. of lesions</th>
<th>Location</th>
<th>Duration to start treatment (wks)</th>
<th>Giemsa pathology</th>
<th>Histopathology</th>
<th>Score at wk 3</th>
</tr>
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<tr>
<td>01</td>
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<td>Hunting</td>
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<tr>
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<tr>
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<td>Sipalawini</td>
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<tr>
<td>05</td>
<td>F</td>
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<td>Sipalawini</td>
<td>Coking gold miners</td>
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<td>L</td>
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<td>06</td>
<td>M</td>
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<td>T</td>
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<td>Saramacca</td>
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<td>L</td>
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<td>33</td>
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<td>M</td>
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<td>Nassau</td>
<td>Sipalawini</td>
<td>Working (soldier)</td>
<td>1</td>
<td>L</td>
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<td>L</td>
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<td>n.d. positive</td>
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<td>A</td>
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<td>Brokopondo</td>
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<td>M</td>
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</table>
QT-NASBA results

In total 168 skin biopsies were analyzed with QT-NASBA, with two biopsies for each time-point. One out of 168 skin biopsies was excluded from further analysis, due to amplification inhibition. When QT-NASBA results of two biopsy samples from the same patient collected at the same time point were compared, no significant difference were found in parasite counts (P = 0.063).

In Table 2 and Figure 1 QT-NASBA results are presented for four relevant time-points (before, week 2 [day of 2nd injection], week 3 [day of 3rd injection] and week 4 [day of 4th injection or one week after 3rd injection]). For each patient and time-point the mean QT-NASBA result of two biopsies was calculated. The results are divided into three categories: high (> 1,000 parasites/biopsy), low (1 to 1,000 parasites/biopsy) or negative (0 parasites/biopsy) parasite counts. All skin biopsies collected at start of treatment (n= 22; from one patient no pre-treatment biopsy was collected) were positive with a median parasite count of 14,600, ranging from 66 to 194,000 parasites/biopsy. In week 2 [day of 2nd injection] a median was found of 5,830, ranging from 0 to 464,000 parasites/biopsy, with only 2 out of 22 patients (9%) negative in both skin biopsy samples. In week 3 (day of 3rd injection) a median parasite count of 16 was found, ranging from 0 to 36,000 parasites/biopsy. Twelve patients (52%) had positive QT-NASBA results. In week 4 (day of 4th injection or one week after 3rd injection), a median was found of 0, ranging from 0 to 85,900 parasites/biopsy with only 6 out of the 15 patients (40%) with positive parasite counts.

Table 2. QT-NASBA results (duplicate biopsies from 23 patients) before, at end of therapy and one week after.

<table>
<thead>
<tr>
<th>QT-NASBA (parasites/biopsy)</th>
<th>Three to four doses of pentamidine (300mg/week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>1-1000</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 1000</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
</tr>
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</table>

* For 20 patients last day of treatment
** For 3 patients last day of treatment
Clinical criteria

From 21 patients the photos were assessed by dermatologists in week 3 (from two patients the photos could not be evaluated). Five out of 21 patients (24%) showed a therapy unresponsiveness based on the 3 clinical criteria of their target lesion. When initial parasite loads calculated in QT-NASBA were compared between patients with a good clinical response (n=17, from one patient the initial samples were not collected) and a poor clinical response (n=5), no significant differences were found in QT-NASBA (P= 0.784). Median parasite count in QT-NASBA in week 3 was higher in the patients with a poor clinical response than with a good clinical response (Figure 2). One of these patients received an extended injection schedule after the three standard injections, and additional treatment in a later follow-up. Two patients did develop a treatment failure and were treated 3.5 to 5.5 months later with another course of PI. It is unknown if the other two patients with a poor clinical response also developed a treatment failure.

Three patients with a good clinical response at the end of treatment returned 4 to 6 weeks after treatment and their lesions showed further improvement. Additional skin biopsies collected on these time-points were negative in QT-NASBA (data not shown).
Median QT-NASBA results with ranges in log values before treatment and in week 3 according to clinical response

Figure 2. Median QT-NASBA results with ranges before and in week 3 of 21 patients (clinical response of two patients could not be assessed). Clinical response in week 3 was assessed by two dermatologists based on three criteria (degree of epithelialization and contraction, necrotic tissue and border activity). Patients with a score $\leq 3$ had a poor clinical response, and $> 3$ had a good clinical response.

1 = Before treatment
2 = Good clinical response in week 3
3 = Poor clinical response in week 3
* Pre treatment biopsies are missing for one patient

Discussion

A worrying result of this study is the high inclusion rate, but very low follow-up rate of the CL confirmed patients. Only 23 out of 64 patients could be followed until the end of treatment. In contrast to other studies in which evaluation time points of 6 and 12 months are included, the present study was adjusted to the practical situation in Suriname with an insufficient follow-up, and clinical response was evaluated at end of therapy of the 3rd or 4th injection.

Most patients live and work in the forested interior. Some patients stay and receive their complete injection schedule in Paramaribo, while others buy the pentamidine ampoules at the pharmacy and return to the interior where they administer the medicines. Other patients only receive part of their injection schedule, because they simply do not have money for
further treatment. In our study only 50% (n=32) of the patients received the complete therapy of at least 3 injections at one of the two clinics in the city. The very low compliance of patients to the treatment and lack of control is a major problem in Suriname. This situation could lead to incomplete therapy and sub-therapeutic pentamidine blood levels, contributing to development of resistant parasites. In the end the emergence of drug resistant parasites could limit the usefulness of the drug. It is not clear how many patients are receiving the complete treatment or develop treatment failures. During the inclusion period, four patients were encountered with persisting CL for over 4 years with several unsuccessful pentamidine isethionate (PI) treatment periods (observation during this study).

In the present study a wide variation in parasite loads was observed after the same PI treatment schedule. Negative QT-NASBA results were found for 12 patients at the end of therapy of 3 or 4 injections (in both skin biopsy samples). It has been previously reported that QT-NASBA has a Negative Predictive Value (NPV) of 89% and a Positive Predictive Value (PPV) of only 46% at end of therapy [Chapter 3]. This suggests that in principle almost all 12 QT-NASBA negative patients at end of therapy will be clinically cured at 6 months after end of treatment, but 5 out of 11 QT-NASBA positive patients will develop a treatment failure. Based on these numbers (18 patients cured vs 5 patients developing a treatment failure) the cure rate of 3 to 4 injections of 300 mg PI is estimated as 78%. This number is even lower if the 3 patients who were also treated for CL one month to one year before the study period are not included. These 3 patients started with lower QT-NASBA counts at start of treatment, and turned already negative in week 2 of their treatment. Furthermore, assuming that a low clinical response in week 3 would predict a treatment failure in a later stage (for at least 3 out of 5 patients this is true) a cure rate of only 76% would be found. If these numbers are valid, this would imply a reduced efficacy from 90% to 76% - 78% for PI treatment in Suriname with the standard doses.

The outcome in this study was not related to infecting parasite species, since all patients were infected with L. (V.) guyanensis. However, not only genetic variation between species, but also within Leishmania parasites of the same species may be the base of different clinical characteristics such as virulence, pathogenicity and sensitivity to certain drugs. Molecular characterization of Leishmania New World isolates show tremendous
diversity within this genus, which could produce different phenotypes associated with different clinical characteristics.\textsuperscript{19} In French Guiana and in Amazonas state in Brazil antigenically distinct populations of \textit{Leishmania (V.) guyanensis} have been encountered.\textsuperscript{20,21} Two distinct \textit{L. (V.) guyanensis} populations in French Guiana were described related with different parasite loads in the lesions, need for different doses of pentamidine and linked to two different ecologically regions.\textsuperscript{21} These \textit{L. (V.) guyanensis} populations could also be present in Suriname. In a future study larger patient groups from different parts of the country are needed to find out if distinct \textit{L. (V.) guyanensis} strains are linked with different clinical characteristics.

Since there is a major concern about the small number of drugs currently under exploitation for leishmaniasis and no alternative medicines are available which are more simple in use and lower in cost, all measures should be undertaken to avoid the development of resistant parasites to existing drugs.\textsuperscript{22} This study emphasizes the need for a reformulated treatment regime with pentamidine in Suriname to increase patient compliance to treatment. Following promising results obtained in French Guiana with a shorter pentamidine schedule, but with higher doses per injection, the implementation of the same protocol in Suriname should be considered.\textsuperscript{10} A single or two injections (given 48 hours apart) with higher doses of 7 mg/kg PI per injection (corresponds with 4 mg pentamidine drug) cured 78.8\% or 83.6\% of the patients, respectively. While the single injection treatment has a similar cure rate as the estimated cure rates (76\%-78\%) in our study, a single injection protocol would still increase treatment compliance and save the patients from the interior time and money when visiting Paramaribo.
Acknowledgements

Most importantly we thank all patients for their time, patience and willingness to contribute to this study. We gratefully acknowledge Ricardo V.P. Hu, Gert-Jan Stolting (Dermatology Service, Paramaribo), Ramon M. Soetosenjo (Department of Dermatology, AZ, Paramaribo) for collection of samples and Martinus A. Vrede (Department of Pathology, AZ, Paramaribo) for performing histopathology. We thank Tilotmadebie Soebhag-Ramlal and Haidi Tjon-Kon-Fat-Bronstein (Central Laboratory, Bureau of Public Health, Paramaribo) for their help with storage and transport of the samples to The Netherlands. Furthermore, we thank Henry de Vries (AMC, Amsterdam) for evaluating the clinical response of the patients, Lisa Lambregts (KIT Biomedical Research, Amsterdam) for the differentiation of Leishmania species and Natasha Tjon Kiem Sang and Karin Sewpersad (Department of Dermatology, AZ Paramaribo) for help with inclusion patients.

This study was supported by a grant from The Netherlands Foundation for the Advancement of Tropical research (WOTRO contract 96-210).
References


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