Cutaneous leishmaniasis: new developments in diagnosis and treatment evaluation

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General introduction
1. Leishmaniasis

Leishmaniasis is caused by single cellular, hemoflagellate protozoan parasites of the genus *Leishmania* (family *Trypanosomatidae*). The parasites are transmitted by the bite of an infected female sand fly of the genus *Phlebotomus* and *Lutzomyia*. Leishmania infection can give rise to several distinct clinical manifestations, ranging from single to many large skin ulcers in case of cutaneous leishmaniasis (CL) to systemic disease (visceral leishmaniasis). This broad clinical spectrum indicates the complexity of leishmaniasis pathogenesis; around 20 *Leishmania* species are described as human pathogens and 31 sandfly species as vectors of the disease. Figure 1 presents the most important *Leishmania* species. Geographically, the disease can be divided into Old World (Southern Europe, Africa, the Middle East, West and Central Asia) and New World (Southern USA to Northern Argentina) leishmaniasis. The two subgenera, *Leishmania* and *Viannia*, are discriminated on the basis of their position in the vector’s intestine, with *Leishmania* being present in both Old and New World, while *Viannia* is restricted to the New World.

Annual incidence is estimated at 1 - 1.5 million cases of CL and 500,000 cases of VL. The overall prevalence of leishmaniasis is 12 million cases and 350 million people from 88 countries (from which 72 developing countries, and 13 belong to the least developed) are at risk of contracting the disease. In most countries the incidence numbers are probably highly underestimated, since many cases are not recognized and there is no obligation to report the disease. DALYs are calculated as the sum of the years of life lost due to premature mortality in the population and the years lost due to disability adjusted for severity. The disease burden expressed in Disability Adjusted Life Years (DALYs) is estimated at 2,357,000 (946,000 for men and 1,410,000 for women).
### Figure 1.

Taxonomy of *Leishmania* species infecting humans; underlined species are or have been questioned. (Bañuls et al.88)
2. cutaneous leishmaniasis

The most prevalent clinical manifestation of leishmaniasis is CL, and 90% of CL cases occur in seven countries: Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. The disease is characterized by one or more (nodular) ulcer(s) on exposed parts of the body and in some cases with the presence of nodules along the draining lymphatic vessels, so-called nodular lymphangitis. Old World *Leishmania* species, as *L. (L.) major* and *L. (L.) tropica* often cause benign and self-limiting ulcers. New World species, as *L. (V.) guyanensis, L. (V.) braziliensis* or *L. (L.) amazonensis*, cause more variable manifestations, of which mucocutaneous (MCL) and diffuse or disseminated CL (DCL) are the most severe. MCL, also called *espundia*, leads to progressive and mutilating destruction of the nasopharynx and invasion of the respiratory tract and is mainly caused by *L. (V.) braziliensis*. It is estimated that between 2 to 10% of patients with CL due to *L. (V.) braziliensis* will develop MCL. DCL is a rare form of the disease and is associated with a defective, cell-mediated immune response to the *Leishmania* parasite, allowing uncontrolled propagation. The disease often occurs as isolated cases and has been encountered in Venezuela, the Dominican Republic, Brazil, Mexico, Bolivia, Colombia, Peru. In the New World the disease is mainly caused by *L. (L.) amazonensis*. So far, there is no effective treatment for DCL.

2.1. Increasing incidence

CL is a growing public health problem worldwide. In some areas, there is a clear and disturbing increase in the number of cases (e.g. Brazil, Afghanistan, Colombia and Syria), while in other areas new endemic foci are reported where the disease was not previously known to be endemic (e.g. Iran, Israel and Ghana). Also, in the three main regions, where the studies of this thesis were conducted, medical doctors are confronted with a rising increase of CL patients; i.e. in a non-endemic country (Amsterdam, The Netherlands) as well as in two endemic regions (Paramaribo, Suriname and Manaus, Brazil).
The increase in CL incidence is related to environmental and behavioral changes and individual risk factors:

- Urbanization and deforestation. For example in Manaus, the disease was traditionally confined to rural areas, but in the last few years there is an increasing number of CL patients on the outskirts of urban zones, which were previously non-endemic areas;
- Movement of non-immune persons (seasonal workers, refugees, travelers) to endemic regions. For example, Dutch forces on military mission in Afghanistan, travellers visiting South American countries and Dutch citizens from Suriname origin visiting their family in Suriname;
- Decline in social and economic circumstances in the poor suburbs of cities, where poor people concentrate due to massive rural-urban migration, facilitating outbreaks. For example, in Kabul in Afghanistan;
- Inadequate vector or reservoir control;
- Increased detection of CL associated with opportunistic infections (e.g., HIV/AIDS) or immuno-suppressive medications;
- The emergence of anti-leishmanial drug resistance.

While CL disease is not lethal, it can cause significant morbidity and the course of the disease is often accompanied by psychological and social repercussions. Therefore, CL control is very important. Because there is still no candidate vaccine with a sufficient and long-lasting effectiveness for public health, CL control depends mainly on early and accurate diagnosis and effective treatment.

### 2.2. Diagnosis

#### 2.2.1. Conventional methods

Accurate diagnosis is needed to exclude patients with cutaneous lesions with other causes (e.g. fungal and mycobacterial infections, skin cancers and super-infected insect bites). In most endemic countries the diagnosis of CL is often based on clinical symptoms and
history of exposure, in combination with microscopic visualization of the parasite in Giemsa-stained skin smears or aspirates. *Leishmania* amastigotes can be differentiated from other intracellular pathogens by visualizing the nucleus and kinetoplast (a typical rod-shaped structure found in the cytoplasm of the parasite) surrounded by a cell membrane. Oil-immersion microscopy is required for proper identification. However, the sensitivity of microscopic examination is low. Different studies reported sensitivities ranging from 54% to 77%. In well-equipped laboratories parasites can be isolated by culturing of biopsies or aspirates. However, contamination is a constant risk in culturing and can sometimes affect 30% of the samples. Additionally, it takes days to weeks until parasites can be visualized, while some species are very difficult to culture and the quality of the growth medium preparation can be highly variable. Sensitivity of culturing parasites has been reported to vary from 42% to 70%. Histopathology on stained sections of biopsies, which is based on visualization of amastigotes with the routine hematoxylin and eosin stain, can be used as an alternative diagnostic approach, but has a comparable low sensitivity ranging from 33% to 69%. Although Giemsa-stain is theoretically better, adequate staining may be difficult to obtain on paraffin sections. Overall, all conventional methods are dependent on the performance and experience of laboratory technicians or pathologists and none of them is accepted as gold standard for diagnosis of CL.

### 2.2.2. Immunodiagnostic methods

Because of the above-mentioned limitations, a number of alternative diagnostic methods are commonly used. Immunodiagnostic methods assays include serological tests to detect *Leishmania* antibodies or antigens, and assays to detect *Leishmania*-specific cell-mediated immunity, in particular the delayed-type hypersensitivity to leishmanial antigens. The Montenegro skin test (MST), is based on this last type of immune reactivity and is based on a crude parasite mixture. However, different parasite preparations and disease duration affects test sensitivity, and is therefore highly variable. Furthermore, the test can not distinguish between past and present infection. Because CL is often a localized disease, systemic antibody responses in patients tend to be low. However, many different ELISA assays have been established to measure antibody levels, often based on purified crude
leishmanial antigens. In most studies these assays are developed for diagnosis or epidemiological purposes, but also to follow the course of the disease, especially in patients with MCL. However, there is no consensus on about the role of these assays as diagnostic or follow-up tools in CL.

2.2.3. Polymerase Chain Reaction methods
Currently, Polymerase Chain Reaction (PCR) based methods are considered to probably be the best approach to diagnose CL. Many different assays have been established based on variable gene targets, different primer sets and reaction conditions. The selection of these criteria, and others (e.g. nucleic acid extraction, quality of reagents, equipment, proper training of technicians), are critical for both PCR sensitivity and specificity. Often multi-copy sequences are used as PCR target, including kinetoplast DNA, ribosomal RNA genes, internal transcribed spacers, spliced leader RNA (mini-exon) gene repeats and regions of the gp63 gene locus. Although PCR is a very sensitive tool in detecting Leishmania parasites, there is still no agreement on one universal Leishmania PCR. Many institutes use their own “in house” PCR assay, which has its own specific DNA target and primer pair. To establish which method is most optimal, comparative studies are needed. However, only few comparative studies have been published, and only one specifically for CL disease. Furthermore, PCRs are based on DNA amplification, which does not provide information on the viability of the pathogen. The test may remain positive for a long time after pathogen death which limits its value as a diagnostic tool for active disease. A sensitive tool to detect viable parasites would be very useful to improve diagnosis of leishmaniasis.

2.2.4. Species differentiation
For choosing an appropriate treatment and designing control measures, not only rapid diagnosis, but also identification of the infecting Leishmania species is mandatory. With the traditional diagnostic methods species identification is not possible. The infecting species can to a certain extend be identical on the basis of geographical distribution and clinical symptoms. However, this approach is inadequate, since in many endemic regions multiple
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*Leishmania* species exist together. Currently, identification of *Leishmania* species is made by electrophoretic analysis of isoenzymes (Multilocus Enzyme Electrophoresis [MLEE]), which is considered as the gold standard.\(^5\) Parasite isolates are differentiated by their enzymatic profile and can be grouped in taxonomic units, so-called zymodemes. However, this method is very laborious and time-consuming, and only few laboratories have the required expertise to perform this method. Other complicated procedures involves the use of monoclonal antibodies, DNA hybridization techniques or Random Amplified Polymorphic DNA (RAPD).\(^5\) In recent years, many PCR assays have been combined with Restriction Fragment Length Polymorphism (RFLP) to characterize parasites, combining amplification of specific targets with digestion of the PCR product with specific isoenzymes.\(^5\)\(^,\)\(^5\)\(^5\)\(^,\)\(^6\)\(^3\)\(^,\)\(^6\)\(^4\) Probably the best approach is to use a Multi-Locus PCR (MLP) approach, thus applying different PCR-RFLPs exploring different *Leishmania*-specific targets.\(^6\)

2.3. Treatment

While CL is considered as a self-healing disease, rapid treatment remains important to avoid unattractive scars and parasite dissemination (i.e. nodular lymphangitis and MCL). At present no single optimal treatment exists for CL.\(^6\)\(^6\)\(^7\) Current first-line therapies are intravenous (i.v.) or intralesional (i.l.) administration of pentavalent antimony compounds (Sodium stibogluconate [Pentostam ®] or Meglumine antimoniate [Glucantime ®]). Alternative treatment regimes include pentamidine, amphotericin B (especially for MCL), aminosidine (paromomycin), a variety of antifungal agents and heat- or cryotherapy. However, these therapies may cause severe side effects, are variable effective for different *Leishmania* species, or have a low cure rate.\(^6\) Moreover, the lengthy course of intramuscular or intravenous injections of standard treatment protocols implies that patients fail to complete the full course of treatment.\(^6\)\(^9\)

Low compliance appears to be the principal reason behind the emergence of drug-resistant parasite strains.\(^7\)\(^0\) In various regions in South America 7% to 39% of the patients fail to respond to first-line treatment.\(^7\)\(^1\)\(^-\)\(^7\)\(^3\) In Suriname, only one drug for CL patients is
available and depends on a standard treatment regime with weekly intramuscular injections.\textsuperscript{74} Recently, dermatologists observed that an increasing number of CL patients does not adequately respond to the standard treatment (personal communication Prof. dr. R.F.M. Lai A Fat). Therefore, monitoring the efficacy of treatment schemes is very important. Sensitive and quantitative assays could be of value. While PCR is a highly sensitive method, the assay seems to be unsuitable for treatment follow-up, as DNA of \emph{Leishmania} parasites can be detected in scars of CL patients even months after clinical cure.\textsuperscript{75,76} However, no alternative assays are available which allow to decide whether a patient is cured. Up to this date a patient is declared cured on the basis of clinical criteria, which may delay the detection of a treatment failure.

2.4. New developments

2.4.1. RNA amplification-based assays

At present, there is no sensitive assay for detection of active disease, which also gives information on parasite load for evaluation of treatment. A sensitive tool to detect and quantify viable parasites would be very useful to improve diagnosis of leishmaniasis. Furthermore, such an assay would make it possible to monitor the effectiveness of treatment schemes. One technique that may fulfil the above mentioned requirements is the Quantitative Nucleic Acid Sequence-Based Amplification (QT-NASBA) assay. QT-NASBA is based on the amplification of specific RNA sequences and is also known as “self-sustained sequence replication”.\textsuperscript{77,78} The NASBA amplification reaction is isothermal at 41 °C (no need for a thermo-cycler) and rapid (90 minutes). Three enzymes are involved: Avian Myeloblastosis Virus Reverse Transcriptase, RNase H and T7 dependent RNA polymerase.\textsuperscript{79} NASBA is very sensitive, as little as 10 to 100 target RNA molecules (e.g. mRNA or rRNA) in a sample can be amplified, without the interference of DNA.\textsuperscript{79} The technology has proven to be highly sensitive and specific and has been applied for several infectious diseases, like HIV, mycobacterial diseases and malaria.\textsuperscript{80-83} The QT-NASBA assay has also been successfully applied in studies to assess the efficacy of drug therapies for \emph{Plasmodium falciparum} and to predict treatment outcomes.\textsuperscript{84,85}
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2.4.2. Serological-based assays

Molecular techniques are not readily applicable under field conditions as they often require equipment, cold chain and electricity. In many infectious diseases a simple serological test is sufficient as a basic diagnostic tool. At present, there is still no agreement on the value of serologic tests for diagnosis in CL. ELISAs in current use are difficult to compare with each other, because great variability exists in antigen use and validation criteria. It can be argued that the use of a homologous antigen improves the performance of a serological assay, but there is a lack of studies comparing antigens of specific species with identified Leishmania infections. For CL most serological assays are based on one specific Leishmania species, while the infecting Leishmania species is different or unknown. In the northeastern region of South America, including the regions focused in this thesis, L. (V.) guyanensis is responsible in >90% of the CL cases. However, so far there are no serological assays based on L. (V.) guyanensis antigens reported.


2.5. Background information on study areas

The Dermatology and Tropical Medicine Departments of the Academic Medical Center (AMC) in Amsterdam see approximately 10 to 20 patients with various forms of CL each year and this number is increasing. The majority of the patients are travellers, and acquire the disease in South and Central America. The diagnostic procedure depends on four methods; Giemsa-stained smears, histopathological examination, culture and PCR. The majority of the patients are treated systemically with pentavalent antimony, pentamidine isethionate or itraconazole. Patients are also treated locally, the majority with a combination of cryosurgery and intralesional pentavalent antimony.

In contrast to The Netherlands, CL is highly endemic in Brazil and Suriname. The Brazilian Ministry of Health reported a total of 22,590 cases in 2004. The disease is mainly caused by \( L. (V.) guyanensis \), \( L. (V.) braziliensis \) and \( L. (L.) amazonensis \). At Fundação de Medicina Tropical do Amazonas (FMTAM) in Manaus (capital of the federal state of Amazonas), over a thousand CL cases are diagnosed annually, of which the majority are inhabitants of the city. CL is especially problematic in the rural settlements around the urban areas of Manaus, mainly habituated by the poor. Diagnosis at FMTAM relies predominantly on Giemsa-stained smears and clinical picture. Alternative methods include histopathology, culture or inoculation into susceptible animals, like hamsters. First-line therapies are meglumine antimoniate (Glucantime®) and pentamidine isethionate.

In Suriname, an endemic country in the northern Amazon region of South America, CL affects mainly people in the forested interior during the rainy seasons. Every year, 200 - 300 patients are seen in the capital, Paramaribo, at the Department of Dermatology, Academic Hospital and the Dermatology Service (Ministry of Health). These numbers are probably an underestimate as many patients (e.g. immigrant gold miners) buy medicines in Paramaribo and apply self medication. Hardly any data are available, including updated incidence numbers of CL in Suriname. So far known, only \( L. (V.) guyanensis \) is identified as causative agent of CL. The diagnosis at the Dermatology Service (Ministry of Health), relies predominantly on Giemsa-stained smears and clinical picture; diagnosis at the Department of Dermatology, Academic Hospital depend on histopathological examination.
2.6. Outline of this thesis

The burden of cutaneous leishmaniasis (CL) is increasing worldwide and clinicians in endemic as well as non-endemic countries are more and more confronted with CL patients. CL control is currently depending on early and accurate diagnosis and treatment, because a prophylactic vaccine is not available. Current diagnostic assays for leishmaniasis have several shortcomings and many cases are unnoticed. As leishmaniasis mainly affects poor, research and development of new diagnostic tools and drugs have been neglected. Moreover, many, mostly uncontrolled, treatment schemes are employed with varying success. Up to this date the duration of treatment and definition of cure are based on pure clinical criteria.

The overall aim of this thesis was the development and evaluation of new tools for the diagnosis of CL, and for the determination of the duration and efficacy of treatment.

The specific objectives of this thesis were:
1. Development and evaluation of a quantitative nucleic acid sequence based amplification assay (QT-NASBA) for diagnosis and quantification of *Leishmania* parasites.
2. To determine the number of parasites in a lesion before, at the end and six weeks after treatment with QT-NASBA in order to assess the outcome of treatment.
3. Comparison of QT-NASBA with two other quantitative molecular assays; real-time Reverse-Transcriptase PCR and real-time PCR.
4. To evaluate the standard treatment regime and treatment compliance for CL in Suriname
5. To estimate annual detection rate and identify infecting *Leishmania* species in Suriname
6. To compare homologue and heterologeous antigens in an Enzyme-linked Immunosorbent assay (ELISA) in Brazilian and Suriname patients infected with *Leishmania (Viannia) guyanensis.*
The studies presented in this thesis were performed in Suriname, Brazil and The Netherlands and were part of the project “Development and evaluation of new tools for the diagnosis cutaneous leishmaniasis, and for the determination of the duration and efficacy of treatment, performed in endemic (Brazil and Surinam) and non-endemic (The Netherlands) regions.” The work was supported by Netherlands Foundation For the Advancement of Tropical Research (WOTRO, W96-210).

This thesis describes the development and evaluation of quantitative Nucleic Acid Sequence-Based Amplification (QT-NASBA), a specific sensitive molecular tool that allows for the detection and parasite quantification in skin biopsies [Chapter 2]. The assay was applied as instrument for monitoring therapy response of CL in a non-endemic setting [Chapter 3] and compared with two other techniques which allows for parasite quantification [Chapter 4]. Chapter 5 to 7 describes part of the study conducted in Suriname. Standard treatment and treatment compliance were evaluated [Chapter 5], one case was presented with a rare manifestation of the disease and a newly identified Leishmania species for Suriname [Chapter 6] and an epidemiological study on CL was performed in 2006 [Chapter 7]. In Chapter 8 an Enzyme-linked immunosorbent assay (ELISA) was presented for the serodiagnosis of Leishmania (Viannia) guyanensis infected patients from the northeastern Amazon region. The results presented in this thesis are summarized and discussed in Chapter 9 and recommendations for further research are given.
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