General discussion
This thesis presents a laboratory part with two newly developed molecular assays (QT-NASBA and real-time RT-PCR [qRT-PCR]), a comparison between different antigens for a serological assay for cutaneous leishmaniasis (CL) and a clinical / epidemiological part with new data on treatment and epidemiology of CL in Suriname. The 18S rRNA QT-NASBA is a highly sensitive and specific method [Chapter 2], which can be used to predict treatment outcome [Chapter 3]. The assay was compared with two other molecular assays, the qRT-PCR and real-time PCR; the qRT-PCR gave better results in terms of cost, procedure time and user friendliness [Chapter 4]. Furthermore, Leishmania (Viannia) guyanensis and L. (Leishmania) donovani antigens performed better than five antigens from other species in a serological assay for CL in patients infected with L. (V.) guyanensis [Chapter 8]. Studies performed in Suriname [Chapter 5, 6 and 7] showed that treatment compliance in Suriname is low, efficacy of treatment seems to be diminishing and the presence of Leishmania species not previously identified.

Results and technical considerations on the QT-NASBA are discussed in the first part of this chapter. In the second part results are discussed and potential directions for future research are given.

1. Results and technical considerations

1.1. 18S rRNA QT-NASBA; gives it information on viability?
For the diagnosis of CL a sensitive and specific method, that could demonstrate viable Leishmania parasites, would be highly beneficial. PCR assays meet in part these specifications, i.e. they exhibit a high sensitivity and specificity, but are based on detection of DNA, rather than intact viable cells. A disadvantage of using DNA as a target for amplification may be the persistence of DNA after the death of the pathogen.1 In CL patients DNA can be detected months to years after clinical cure in scars of healed lesions.2,3 In contrast, the presence of intact ribosomal RNA was found to be a valuable indicator of viability in Mycobacterium smegmatis.4 Therefore, an RNA amplification method, like QT-NASBA, appears to be preferred above PCR. However, Uyttendaele et al.5
showed that 16S rRNA was quite stable and resistant to heating at 100 °C and could not be used for differentiating between viable and non-viable Campylobacter jejuni. An alternative method would be to develop a NASBA targeting messenger RNA (mRNA). mRNA is a highly labile molecule, associated with metabolic activity and a short half life, and could therefore serve as a better target to discriminate between viable and dead cells. Cools et al. developed a real-time NASBA assay to amplify a mRNA target for the detection of viable C. jejuni. However, they found that mRNA remained detectable up to 48 hour after lethal treatment, and the assay could not be used to determine the viability of C. jejuni cells.

We conducted few experiments with Leishmania promastigotes (L. (L.) donovani, L. (V.) guyanensis, L. (L.) chagasi) in culture and with Leishmania amastigotes (L. (V.) guyanensis) in in vitro cultured macrophages (human monocyte cell line U-937 and human monocytes isolated from Peripheral Blood Mononuclear Cells). Lethal treatment of the parasites was performed with pentavalent antimony (Pentostam®). Experiments were conducted in culture medium (RPMI supplemented with 15% foetal calf serum) and human blood at 37 °C. Our results showed that 18S rRNA copies remained detectable 15 days after parasite death (data not shown). As the 18S rRNA QT-NASBA could not be used to determine viability of Leishmania parasites, a mRNA QT-NASBA assay was considered. As target the major surface glycoprotein 63 (GP63) multiple-copy gene locus was chosen. However, preliminary experiments showed that the transcript of this gene was also stable and persisted after parasite death. However, other mRNA targets should be further exploited, which are expressed in all the physiological states of a Leishmania parasite and of which the transcripts are less stable. Such genes could be more suitable candidates for the development of a QT-NASBA assay for the detection of viable Leishmania parasites.

1.2. High analytical sensitivity; an important feature of the assay
Currently, many different PCR methods are used for the diagnosis of CL. Most of these assays target genomic or kinetoplast DNA and are described as highly sensitive. However, sensitivity is correlated with the copy number of the amplified region. The kDNA PCR is considered to be the most sensitive method for diagnosing leishmaniasis since there are ~10,000 minicircles per parasite. However, these kDNA PCRs generally either amplify
subgenus-specific conserved regions or require separate primer pairs for each species of *Leishmania*.\(^9\)\(^,\)\(^10\) In contrast, the QT-NASBA is based on the 18S rRNA gene sequence and this target has been found to detect all *Leishmania* species equally. While each parasite contains ~ 160 copies of the 18S rRNA gene (DNA), it is assumed that the number of rRNA copies in the cytoplasm is \(>10^4\)\(^11\). Our experiments showed that one *Leishmania* parasite contains around \(10^{4.8}\) copies of 18S rRNA [Chapter 2]. The high number of template molecules significantly increases the sensitivity of the QT-NASBA and decreases required sample volume. Furthermore, NASBA generates the same number of copies in a shorter period of time than PCR because it results in an exponential increase every cycle due to the simultaneous action of three enzymes, whereas PCR progresses in a binary fashion. The higher sensitivity was confirmed in our experiments when *L. (L.) donovani* promastigotes dilutions were compared in QT-NASBA and a conventional PCR targeting 18S rDNA. The *Leishmania* QT-NASBA detected at least 100-fold lower levels of parasites than the PCR (data not shown).

Sensitive detection methods are especially important in patients at risk for mucocutaneous leishmaniasis (MCL). MCL is primarily caused by *L. (V.) braziliensis*, and skin or mucosal lesions caused by this species, are often characterized by very low parasite loads. Our results showed that four out of five patients identified with *L. (V.) braziliensis* infection in Manaus, were tested positive in QT-NASBA, but negative with microscopy [Chapter 4].

### 1.3. Possible improvements in detection methods

#### 1.3.1. Real-time NASBA

At present, the detection of NASBA amplification products takes place at the end of the process using electro-chemiluminescence (ECL) [Chapter 2]. This method can be adapted by the incorporation of a molecular beacon during the amplification reaction. Molecular beacons are single-stranded, fluorophore-labeled nucleic acid probes capable of forming a stem-loop structure with at one end of the stem a quencher and at the other end a fluorescent reporter dye \(^12\). They are capable of generating a fluorescent signal in the
presence of target, but do not fluorescence in the absence of it. Molecular beacons allow concurrently amplification and detection in one run, so-called amplification in “real-time”. By removing the need for post-amplification manipulation of products, the contamination risk for subsequent reactions is greatly reduced, and also procedure duration. Furthermore, inaccurate quantification of high parasite densities can be avoided, which may occur with endpoint detection due to depletion of reagents during the amplification reaction. Several successful NASBA assays based on molecular beacon technologies have been developed 13-15, but not (yet) for leishmaniasis.

1.3.2. Oligochromatography
An alternative detection method is oligochromatography (OC). While such a method has no quantification purposes, the simplicity and speed of membrane chromatography allows the amplified products to be visualized within 5 minutes by the naked eye.16 PCR products are visualized by hybridization with a gold-conjugated probe and two internal controls are integrated, one for PCR amplification and one for the chromatographic migration. In contrast to conventional post-amplification methods, OC requires no equipment other than a dry heating block and a pipette. Currently, an OC-PCR has been developed for the diagnosis of sleeping sickness with 100% sensitivity and specificity.17 NASBA in combination with oligochromatography could circumvent the need for a thermocycler and would be ideal for low-tech laboratories in resources poor countries. The combination of NASBA and OC should be further explored for leishmaniasis.

1.3.3. Multiplex NASBA
The QT-NASBA assay was developed to quantify all *Leishmania* species capable of infecting humans in patient samples by using the highly conserved 18S rRNA gene. However, species identification is also important because different species may require different treatment regimes.18 The detection of multiple targets within one reaction for various *Leishmania* species would increase the value of a real-time assay for clinical use. Furthermore, it would circumvent the need for additional post-amplification procedures and
considerably decrease costs of analysis. Differentiation of *Leishmania* parasites by real-time PCR has been described to the level of *Leishmania* complexes or specifically for Old World species.\textsuperscript{19,21} So far, no real-time PCR formats have been developed to differentiate the clinically important *L. (V.) braziliensis* from other closely related species within the *Viannia* genus. Depending on the number of starting copies of the target, NASBA may be preferred over PCR in real-time format [section 1.2. and Chapter 4]. Real-time multiplex NASBA assays have been developed for various pathogens, but not for leishmaniasis.\textsuperscript{22,23}

## 2. Results in Suriname

### 2.1. Treatment evaluation

Pentamidine isethionate is the only available treatment for CL in Suriname. In Chapter 5 we showed that the very low compliance of patients to the treatment and lack of control is a major problem. In Paramaribo, the capital city of the country, only 50% of the patients receive the complete therapy of at least 3 injections. Therefore, we could only follow 23 out of 64 patients until the end of treatment. This study was more descriptive and not an adequately controlled therapeutic trial since it was not comparative (i.e. without control), not randomized or double-blind. Currently, many studies aiming to determine efficacies of treatments are based on uncontrolled, small series and case reports, what makes it difficult to assess the true efficacy.\textsuperscript{24} Nevertheless, these studies provide often important observations, and draw attention to otherwise unnoticed issues.

More accurate evaluation of the standard pentamidine treatment is needed in Suriname. Patients need to be randomized in study groups and treated with the standard 3-week pentamidine regime and our recommended 3-day short course pentamidine treatment or another alternative [Chapter 5]. A longer follow-up period is required of at least 6 weeks and 6 months after therapy and decrease in parasite load should be measured by QT-NASBA, because this assay was also able to predict occurrence of relapses [Chapter 3].

However, it will be very difficult to accomplish a well-designed clinical trial in Suriname. Most CL patients live in poor socio-economic conditions, mostly in the remote forested hinterland, and cannot easily reach Paramaribo. Treatment costs are high for the
patients, and there are the added cost for transportation to the health clinics and loss of income due to inability to work. Furthermore, gold miners, who constitute a high risk group, work and live in different areas in the hinterland, and are difficult to follow-up in clinical studies. Even in more developed countries as The Netherlands follow-up of patients can be problematic, because patients simply return on other time points than was appointed by the study protocol [Chapter 3].

2.2. Possible drug resistance?
Our data show that treatment compliance in Suriname is very low, therefore patients are treated inadequately [Chapter 5]. It is known that sub-optimal doses can contribute to development of drug resistant parasites. An increasing number of patients are encountered not responding adequately to standard treatment of pentamidine in Paramaribo. Up till now the mechanism of action of the drug, and also the possible drug resistant mechanism(s) is unknown. Recent studies have shown that pentamidine accumulates in the mitochondria, while in pentamidine resistant mutants, the drug does not accumulate. One candidate that is probably involved is the ABC transporter PRP1. Studies of resistance will permit the identification of intracellular targets and parasite defence mechanisms, allowing researchers to develop molecular assays that could rapidly detect resistance genes within the parasite. These assays are important in therapy management, because it may limit the use of possible ineffective and toxic agents. Robust drug resistant markers have not (yet) been found for leishmaniasis.

2.3. Different L. (V.) guyanensis subspecies?
Earlier studies indicated that L. (V.) guyanensis isolates from the region of the Amazon river in Brazil are a homogeneous group similar to the reference strain MHOM/BR/75/M4147. Currently, variability within this species is observed based on MLEE in agarose gel, monoclonal antibodies or RFLP analysis of the ribosomal small subunit (rSSU) and the internal transcribed spacer (ITS). Rotureau et al. found two distinct populations in French Guiana. Genotype group 1 comprised homogeneous samples
who were closely related and seemed to have originated from the northern part of the country and a heterogeneous genotype group II, which seemed to have originated in the southern part of French Guiana and from neighbouring countries (Suriname and Brazil). Patients of genotype I needed more additional injections to the standard pentamidine protocol than genotype II and this group were likely to present higher parasite densities. Atypical clinical forms, such as disseminated presentations and chronic disease, were mostly found in Genotype II. When we sequenced the ITS region from six Suriname patients, we found three heterozygous samples, what could imply that these samples belonged to the same Genotype II group that Rotureau et al. described. Patients in Suriname are seen with different atypical clinical forms, suggesting that they are infected with other Leishmania species [Chapter 6 and 7]. However, these patients may also be infected with a different subspecies of L. (V.) guyanensis as Genotype II. Several techniques are available for use in genetic typing of individuals or larger populations, of which microsatellites (also known as short tandem repeats or STRs) are probably the most useful molecular markers. Currently, only studies have been published in a few Leishmania taxa as L. (L.) donovani complex, L.(L.) tropica and the subgenus Viannia. Schwenckenbacher et al. showed that this technique reveals more intraspecific genetic polymorphism and higher frequencies of heterozygous loci than other techniques. Further discrimination among L. (V.) guyanensis isolates is needed, not only in Suriname but for the whole Amazon region, to find out which clinical implications these differences have.
References


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