Characterisation of *Leishmania* amino acid permease 3 (AAP3) coding sequences and flanking regions as a target for detection and diagnosis of the leishmaniases

Karl Erik Müller

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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UNIVERSITY OF BERGEN

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Contents

Let us not take it for granted that life exists more
fully in what is commonly thought big than in
what is commonly thought small.

> - The Common Reader (1925), Virginia Woolf

The role of the infinitely small in nature is infinitely large.

> - Louis Pasteur

1. Scientific environment

Throughout this PhD, I have been affiliated with the Research Group for Infection and Microbiology at the Department of Clinical Science, University of Bergen, and the Laboratory of Trypanosomatid Physiology (BatLab) at the Institute of Biosciences, University of São Paulo. During the Medical Student Research Programme, I also worked at the Laboratory for Seroepidemiology at the Institute of Tropical Medicine, University of São Paulo. Professor Audun Helge Nerland, Professor Are Næss, and Professor Haakon Sjursen gave me supervision during the Medical Student Research Programme. Professor Audun Helge Nerland was my main supervisor and Professor Lucile Maria Floeter-Winter and Dr Juliana Ide Aoki were co-supervisors for my doctoral work.

2. Acknowledgements

In the attic at Lensmannsbakken in Brunlanes, where I grew up, my father had stored away his microscope. With this, as a young boy, I studied whatever I could manage to magnify! As a haematologist, my father had slides stored around, and I stole some of them to look at. What a fantastic world I discovered. As the years went by, natural sciences intrigued me, but I lost fascination for them during high school. I found the teaching dull and not open for curiosity. I found a new home in the social sciences. However, after completing a degree in economics, I became convinced that I had to get back to my old dream – to study medicine, and if possible, to study the infinitely small things in life.

I am eternally grateful to my wise guide throughout the years, Professor Audun Helge Nerland. Your endless curiosity, numerous ideas and in-depth knowledge of immunology, microbiology and molecular biology intrigues me. You lured me into multiple projects: producing antibodies in fish, trying to make vaccines, digging deep into molecular biology. Suddenly we had three masters students at our lab and Audun, you thought it wise that I co-supervised all of them. Although I was doubtful, you somehow knew that I could do it. You have always been positive and supportive; and your mentorship has been invaluable to me. Without your collaboration and supervision, this thesis would never have existed. Thank you!

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My co-supervisor, Dr Juliana Ide Aoki – thank you. Although our initial project didn´t work out, I have not given up. Several new projects are already up and running. Your example of hard and dedicated work has been a true inspiration!

I am grateful for Professors Are Næss and Haakon Sjursen for including me in their research group in the beginning of my research days. I am also grateful to fellow PhD-student Karen Rebbestad for welcoming me with open arms to the research field.

Dr Ricardo Andrade Zampieri – muito obrigado amigo! I think we have the world-record in the number of Skype meetings! Thank you for sharing your vast knowledge of molecular biology – especially all the hidden tips and tricks! I have been amazed by how you welcomed me and how perfectly we have been working together – asking questions and sharing our curiosity, while at the same time being very result-oriented. Parabéns pra nós!

I also wish to thank Dr Sandra Marcia Muxel and all the students at the BatLab for making my research stays there so welcoming and nice. Vamos que vamos!

The Medical Student Research Programme at the University of Bergen – without it, I doubt I would ever have got into research. A special thanks go to Marianne Stien and Professor Anne Berit Guttormsen.

As a medical student, I was also able to take part in the excellent laboratory of Professor Hiro Goto at the Institute of Tropical Medicine at the University of São Paulo. I was inexperienced; despite this, Hiro and her team welcomed me and taught me the basics of working with *Leishmania*. Muito obrigado Hiro, Magnus, Chris, Célia, Luiza, Eduardo and the rest of the team at the Instituto de Medicina Tropical de São Paulo (IMT). During my year at Hiro´s lab, I also had the opportunity to go to the Emilio Ríbas hospital twice a week to attend to patients with leishmaniasis. The

clinical experience was made possible by the excellent and kind mentorship of Dr José Angelo Lauletta Lindoso. Thank you, Angelo, for giving me that opportunity.

At the infectious disease lab in Bergen, I had the great fortune of working closely with two fantastic engineers who have taught me a lot. Marit Gjerde Tellevik and Steinar Sørnes – thank you!

I am also grateful to the staff and students at the Gades group of infection and immunity, in particular, Professors Rebecca Jane Cox Brokstad and Vidar Bakken who continuously encouraged me and gave helpful comments to my work.

During my medical and doctoral studies, I have had the great fortune to work with some great colleagues in various academic settings, although somewhat outside my research field. Dr Carl Tollef Solberg, we shared tips and tricks for the PhD, but also extensively discussed various philosophical issues and we had great fun writing together. And now, good friends! I look forward to the numerous collaborations we will have in the future. Ingrid Neteland, always enthusiastic, but also clear-sighted and rigorous – it has always been a true inspiration to work with you. Dr Jan Reinert Karslen, thank you for inviting me to the Centre for the Study of Sciences and the Humanities, your courses and seminars were magnificent, and helped me always keep the critical eye open for my own research projects and their limitations while being open to criticism of your own work. Professor Edvin Schei, thank you for inviting me into the Filosofisk Poliklinikk at an early phase, a place for *Bildung* and thank you for greeting my curiosity with great enthusiasm and directing it into something productive. It has been fascinating to work with you to develop research and new inspiring courses for medical students. Dr Anna Therese Bjerkreim and Ingeborg Eskerud, it has been a true pleasure to uncover the secrets of the success and challenges to the Medical Student Research Programme in Bergen.

Thank you, Anders Tangen, for your continuous friendship and putting a lot of things in my life into perspective!

Without my parents-in-law, I would never have been able to complete this PhD-thesis. They opened up their home to me and always supported me. Muito obrigado meus queridos e minha familia brasilieira, Babate, Bie, Ana, Gabriel, Silvia. Maria e Manu! E Luzia – e sopa e meu sonho!

My family in Norway. My dear mother and my dear father. They have been and remain an inspiration. You fostered my curiosity, taught me the value of hard and dedicated work, but also social engagement. You taught me from the very beginning that no one road holds the truth, and I think you can see this reflected in my work today. Thank you! Thank you also to my dear siblings for teasing me when I deserved it, for making fun of me when my arrogance was too much and for showing and telling your love.

Last, but not least Julia – the rock in my life. Eu te amo! Our lives crossed each other in London, and since then we have been inseparable. Your dedication to your own work has been important to me – your level of concentration, clear sightedness and result orientation remain an inspiration. Furthermore, without you, this thesis would never have existed. There were times I had to stay long in the lab. There were times I had to work in the evenings at home and I had to ask not to be disturbed. You were supportive, you were understanding – but importantly, you also put clear limits. I am incredibly proud of you and of what we have been able to build together. And now Therese is here, exploring life! I hope that some of the exploration I did in this work, can be an inspiration to you Therese.

This work is dedicated to Therese and Julia.

3. Abstract

The protozoan parasites of *Leishmania* genus are the etiological agents of the leishmaniases, diseases whose clinical manifestations range from being asymptomatic, self-healing cutaneous lesions, via mutilating muco-cutaneous lesions, to a potentially lethal visceral form. The World Health Organization defines the leishmaniases as neglected tropical diseases (NTDs). The aim of this thesis was to explore a new potential target, the amino acid permease 3 (*aap3*) coding regions and flanking nucleotide sequences for parasite detection and species identification. In four papers, the findings from research on the general and specific aims are presented. In **paper I**, the *aap3* coding sequence was investigated for its potential as a target for parasite detection. Using real-time polymerase chain reaction (real-time PCR) the developed assay was useful for qualitative purposes, and when run in duplex with a host specific gene-assay, it was also able to quantify parasites in the mammal host. In **paper II**, the *aap3* coding sequence was investigated for its potential as a target for species discrimination. Using high resolution melting (HRM) analysis, *aap3* was found to be a specific and sensitive target for *Leishmania* species-identification. The method was validated on samples from humans, from experimentally infected mice, as well as from naturally infected sand flies. In **paper III**, a multi-centre prospective clinical study on the occurrence, diagnosis, treatment of the leishmaniases in Norway was performed. Biopsy material was collected to validate *aap3* as a target in clinical samples. In this material, the small subunit ribosomal RNA coding sequence (SSU rDNA), a molecular target routinely used in *Leishmania* detection tests, was found to be more sensitive in conventional PCR assays. Skin biopsy was the most appropriate material for diagnosis in cutaneous leishmaniasis. In **paper IV**, the *aap3* gene region from several *Leishmania* species was sequenced using single molecule real-time (SMRT) sequencing. In all species analysed, two copies of the encoding gene sequences organized *in tandem* were found. These sequences are conserved within the species and between species of the same subgenera. In conclusion, this thesis shows that *aap3* with appropriate technology can be a sensitive and specific target for

both genus detection and species discrimination. Furthermore, precise sequencing of *aap3*, as described here, can be a very useful tool in subsequent gene editing studies for a better understanding of the physiology and genetics of these parasites.

Resumo (Portuguese)

Os protozoários parasitas do gênero *Leishmania* são os agentes etiológicos das leishmanioses, doenças cujas manifestações clínicas variam de formas assintomáticas com lesões de cura espontânea, lesões muco-cutâneas mutilantes, até a forma visceral, potencialmente fatal. A Organização Mundial da Saúde define as leishmanioses como uma doença tropical negligenciada (NTD). O objetivo desta tese foi explorar o potencial de um novo alvo, as sequências nucleotídicas que codificam a amino acid permease 3 (aap3) e as regiões que as flanqueiam, para a detecção e identificação das espécies do parasita. As conclusões de pesquisas sobre os objetivos gerais e específicos estão apresentadas em quatro artigos e manuscritos. **No artigo I**, foi investigado o potencial da sequência codificadora do *aap3* como alvo de detecção do parasita. O ensaio desenvolvido, baseado em PCR em tempo real (real-time PCR), foi capaz de detectar o parasita e, quando combinado em "duplex" com um gene alvo específico do hospedeiro, foi capaz de quantificar o parasita em amostras de hospedeiro mamífero. **No artigo II**, foi investigado o potencial da sequência codificadora do *aap3* como alvo de discriminação das espécies do parasita. Utilizando como metodologia a dissociação de DNA em alta resolução (HRM - *High Resolution Melting*), foi demonstrado que o *aap3* pode ser um alvo específico e sensível para a identificação de espécies de *Leishmania*. O método foi validado em amostras obtidas de casos humanos, de camundongos experimentalmente infectados e de flebotomíneos naturalmente infectados. No **artigo III**, foi realizado um estudo multicêntrico prospectivo da ocorrência, diagnóstico e tratamento de leishmaniose na Noruega. Biópsias foram coletadas para validar o *aap3* como alvo diagnóstico em amostras clínicas. Nesse material, a sequência que codifica a subunidade menor do ribossomo (SSU rDNA), rotineiramente utilizado como alvo em ensaios de detecção de *Leishmania*, se mostrou mais sensível na detecção do parasita por PCR convencional. As biópsias de pele foram o material mais apropriado para o diagnóstico de leishmaniose cutânea. No **artigo IV** as regiões codificadoras de *aap3* de várias espécies de *Leishmania* foram sequenciadas pela metodologia SMRT (*Single Molecule Real-Time*). Em todas as espécies analisadas foram encontradas duas cópias das sequências codificadoras organizadas *in tandem*. Essas sequências

são conservadas nas cepas da mesma espécie e entre as espécies do mesmo subgênero. Concluiu-se nesta tese que o *aap3* pode ser um alvo sensível e específico tanto para a detecção diferencial de subgêneros quanto para a discriminação de espécies. Além disso, o sequenciamento preciso do *aap3*, como descrito aqui, pode ser uma ferramenta extremamente útil em posteriores estudos de edição gênica para o melhor entendimento da fisiologia e da genética desses parasitas.

Abstrakt (Norwegian)

Parasitter av slekten *Leishmania* fører til sykdomsgruppen leishmaniasis, sykdommer hvis kliniske manifestasjoner spenner fra å være asymptomatiske, selvhelende kutane lesjoner, mutilerende muko-kutane lesjoner, til potensielt dødelig visceral form. Verdens Helseorganisasjon (WHO) definerer leishmaniasis som en av de glemte tropiske sykdommer (NTDs). Målet med denne avhandlingen var å utforske aminosyre permease 3 (*aap3)* genet og omkringliggende nukleotidsekvenser for parasittdeteksjon og artsidentifikasjon. I fire artikler presenteres funnene fra forsking på de generelle og spesifikke målene i avhandlingen. I **artikkel I** blir den *aap3* kodende sekvens undersøkt for dens potensial for parasittdeteksjon. Ved å bruke sanntids polymerase kjede reaksjon (real-time PCR) var den utviklede metoden nyttig for kvalitative formål, og når den ble brukt sammen med vertsspesifikk gen, kunne den også bli brukt til kvantifisering i verten. I **artikkel II** ble den *aap3* kodende sekvensen undersøkt for dets potensial som mål for artsdiskriminering. Ved å bruke høy-resolusjon smeltepunktsanalyse (HRM), viste det seg at *aap3* var et spesifikt og sensitivt mål for *Leishmania* arts-identifikasjon. Metoden ble validert på prøver fra menneske, eksperimentelt infiserte mus og fra infiserte sandfluer. **Artikkel III** var en multi-senter prospektiv klinisk studie på forekomst, diagnose, behandling av leishmaniasis i Norge. Biopsimateriale ble samlet inn for å validere *aap3* som et mål på kliniske prøver. I dette materialet fant vi at det mer vanlige brukte målet small subunit ribosomal RNA coding sequence (SSU rDNA), var mer sensitivt ved brukt av konvensjonell PCR. Hudbiopsi var det mest passende materialet for diagnostikk ved kutan leishmaniasis. I **artikkel IV** ble *aap3* gen-regionen fra flere *Leishmania* arter sekvensert ved å bruke «single molecule real-time» (SMRT) sekvensering. I alle artene analysert, ble det funne to kopier av gensekvenser organisert i tandem. Disse sekvensene var bevart innenfor arten og mellom arter av ulike subgenera. Denne avhandlingen viser at *aap3* kan være et sensitivt og spesifikt mål for både parasittdeteksjon og artsidentifisering. Videre, presis sekvensering av *aap3*, som beskrevet her, kan være et meget nyttig verktøy i påfølgende genredigeringsstudier for å bedre forstå parasittens fysiologi og genetikk.

4. List of publications

I. **Detection of a broad range of *Leishmania* species and determination of parasite load of infected mouse by real-time PCR targeting the arginine permease gene AAP3**.
Tellevik MG, <u>Müller KE</u>, Løkken KR, Nerland AH.
Acta Tropica 2014; 137: 99–104. doi: 10.1016/j.actatropica.2014.05.008

II. **Amino acid 3 permease (*aap3*) coding sequence as a target for *Leishmania* identification and diagnosis of leishmaniases using high resolution melting analysis.**
<u>Müller KE</u>[*], Zampieri RA[*], Aoki JI, Muxel SM, Nerland AH, Floeter-Winter LM.
Parasites and Vectors 2018; 11: 421. doi: 10.1186/s13071-018-2989-z
*Contributed equally

III. **Leishmaniasis in Norway.**
<u>Müller KE</u>, Blomberg B, Tellevik MG, Jensenius M, Lier T, Sand G, Hannula R, Langeland N, Mørch K.
Under review

IV. **Characterization of Amino Acid Permease 3 gene organization in *Leishmania* spp. by long amplicon single molecule real-time (SMRT) sequencing.**
Zampieri RA[*], <u>Müller KE</u>[*], Aoki JL, Muxel SM, Laranjeira-Silva MF, Sundaram AYM, Floeter-Winter LM, Nerland AH
Manuscript.
*Contributed equally

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Related publications not included in this thesis

Paper related to phagocytosis of *Leishmania*:

I. **Flow cytometry technique for analysing *Leishmania* promastigote phagocytosis by human polymorphonuclear leucocytes and monocytes.**
    Rebbestad K, Herredsvela S, Sornes S, Eide GE, Müller KE, Spriet E, Sjursen H, Naess A.
    Parasite Immunology 2012; 34: 528–535. doi: 10.1111/j.1365 3024.2012.01385.x

Papers related to arginine, arginase and *aap3* in *Leishmania*

II. **RNA-seq transcriptional profiling of *Leishmania amazonensis* reveals an arginase-dependent gene expression regulation.**
     Aoki JI, Muxel SA, Zampieri RA, Laranjeira-Silva MF, Müller KE, Nerland AH, Floeter-Winter LM.
     PLoS Negl Trop Dis 2017; 11: e0006026. doi: 10.1371/journal.pntd.0006026

III. **Arginine and polyamines fate in Leishmania infection.**
      Muxel SA, Aoki JI, Fernandes JC, Laranjeira-Silva MF, Zampieri RA, Acuna SM, Müller KE, Vanderlinde RB, Floeter-Winter LM.
      Front. Microbiol. 2017; 8: 2682. doi: 10.3389/fmicb.2017.02682

Other papers

IV. **Developing a vaccine for leishmaniasis: how biology shapes policy.**
     Müller KE, Solberg CT, Aoki JI, Floeter-Winter LM, Nerland AH.
     Tidsskr. Nor Legeforen 2017. doi: 10.4045/tidsskr.17.0620

V. **Differential immune response modulation in early *Leishmania amazonensis* infection of BALB/c and C57BL/6 macrophages based on transcriptome profiles.**
    Aoki JI, Muxel SM, Zampieri RA, Müller KE, Nerland AH, Floeter-Winter LM. Sci Rep 2019; 9: 19841. doi: 10.1038/s41598-019-56305-1

5. List of figures and tables

6. Abbreviations[1]

AAAP	Amino acid/auxin permease
AAP3	Amino acid permease 3 (the protein)
aap3	aap3 (the coding DNA sequence)
ACL	Anthroponotic cutaneous leishmaniasis
AVL	Anthroponotic visceral leishmaniasis
bp	Base pairs
CAT	Cationic amino acid transporter
cDNA	Complementary DNA
CDS	Coding sequences
CL	Cutaneous leishmaniasis
DAT	Direct agglutination test
DCL	Diffuse cutaneous leishmaniasis
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucletoide triphosphate
dsDNA	Double stranded DNA
ELISA	Enzyme linked immunosorbent assay
HIV	Human immunodeficiency virus
HRM	High-resolution melting
IFA	Indirect immunofluorescence
INF-γ	Interferon – gamma
IST	Immunochromatopgraphic strip test
kDNA	Kinetoplastid DNA
MCL	Mucocutaneous leishmaniasis
MFL	Median fluorescence intensity
ML	Mucous leishmaniasis
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MY	Million years
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription PCR
qPCR	Quantitative polymerase chain reaction
SL	Splice leader
SMRT	Single molecule real-time sequencing

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[1] In the *Leishmania* literature it is common to divide the world into the Old (Eastern hemnisphere) and New World (Americas). In this doctoral thesis, this distinction has been avoided as it does have colonial and imperialistic connotations.

ssDNA	Single stranded DNA
SSU rDNA	Small subunit ribosomal RNA coding sequence
tfrc	Transferrin receptor gene
TM	Melting temperature
TNF-α	Tumor necrosis factor - alfa
UTR	Untranslated region
VL	Visceral leishmaniasis
WHO	World health organization
ZCL	Zoonotic cutaneous leishmaniasis
ZVL	Zoonotic visceral leishmaniasis

7. Introduction

(Literature review completed December 2019)

> "On making smear preparations from the spleen pulp, I was struck by the curious appearance, among the spleen cells and red corpuscles, of enormous number of small round or oval bodies, 2 to 3 μ in diameter, which corresponded to nothing I had seen figured or described."(1)

In 1903 an army physician, W.B. Leishman described what he could see in the microscope in patients with so-called Dum-dum fever (1). He further explained some of the classical signs of visceral leishmaniasis (VL):

> "Clinically, these cases presented no very definite features distinguishing them from other and commoner forms of tropical cachexia, the chief symptoms being an irregularly remittent type of fever, grave anemia, progressive muscular atrophy, and great enlargement of the liver." (1)

Since *Homo sapiens* emerged in Africa and before, parasites have been part of our history. The first written records of parasites are from Egyptian, Chinese, Indian and Arabic physicians (2). Without doubt, the helminths would have been known to our early ancestors but it was not until the development of the microscope that we could identify the protozoans, one of which is the theme for this thesis. And it was a protozoa, namely *Giardia lamblia*, that Antonie van Leeuwenhoek – the man to whom we credit the development of the microscope – first saw in his microscope at the end of the 17th century (2).

The *Leishmania* parasite was likely seen before W. B. Leishman described it (1). That is to say, the parasite was seen by David Cunningham in 1885 and P.F. Borovsky in 1898 (3). The parasite was first described by William Leishman and Charles Donovan independently (4). It was, however, not until 1921 that the sand fly was established as the vector for the disease, demonstrated by Edouard and Etienne Sergent (5).

Besides fossil findings indicating the presence of *Leishmania* in ancient times, there are a few accounts of *Leishmania*. Naturally, the earliest descriptions are of cutaneous leishmaniasis (CL). There are descriptions of lesions that could be CL on tablets from the library of the Assyrian King Ashurbanipal (2). A study of mummies from Egypt (2050-1650 BCE) found *L. (L.) donovani* DNA in several of the mummies (6). A skin condition called "Nile Pimple", compatible with CL is mentioned as early as 1500 BCE in the Ebers Papyrus scriptures (2). Furthermore, early ceramics from Columbia show facial conditions that could be mucocutaneous leishmaniasis (MCL) (7).

Today, leishmaniasis[2] remains a neglected tropical disease – underreported, underestimated, underfunded and underprioritized. The World Health Organization (WHO) currently classifies 20 communicable diseases and conditions as neglected tropical diseases (NTDs) (8). Worldwide, the NTDs affect more than a billion people and cause high levels of morbidity and mortality (8,9). For the leishmaniases there are challenges on all levels; understanding the parasite itself, its interaction with the vector, reservoir(s) and host. More knowledge about these are essential for the development of new treatment options and vaccines. Furthermore, there are no standard techniques for diagnosis in endemic nor non-endemic regions.

Arginine is a key amino acid for macrophage defence against *Leishmania*, but also essential for *Leishmania* replication (10). The uptake of arginine is largely mediated by the amino acid permease (AAP3) in *Leishmania*. In this thesis, *aap3* is explored as a diagnostic target using molecular diagnostic techniques (paper I-II), one of the diagnostic techniques is also explored in samples from real patients (paper III), and the coding sequence is explored further by third generation sequencing (paper IV).

This introduction aims to give the naïve reader a broad introduction to the field before going into the particular issues that this doctoral work has concerned itself with. This

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[2] Leishmaniasis is also referred to as the leishmaniases, to highlight the plethora of clinical symptoms the various strains of the parasite can cause. This thesis will use both terms. If a specific clinical condition is referred to, it will be made clear in the text.

thesis proceeds as follows: I begin with a general introduction to the *Leishmania* parasite and the leishmaniases; thereafter I will discuss amino acid permease 3 (AAP3) followed by a deeper discussion of diagnosis, detection and identification. The aims of this thesis are then presented, and I subsequently discuss materials and methods used, give a brief summary of the papers and a more in-depth presentation and discussion of the results, followed by a general conclusion and proposals for future studies.

7.1 The parasite, vector, reservoir and host

Figure 1. The taxonomy of *Leishmania*
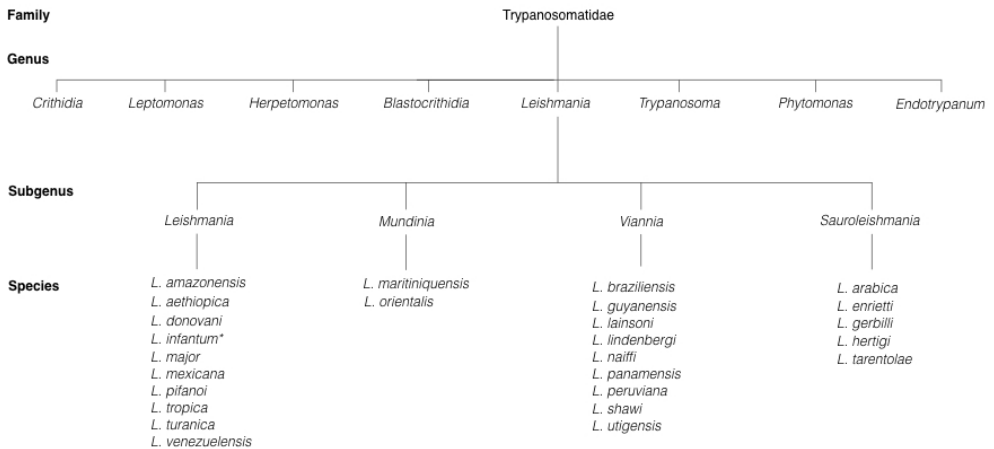
Figure 1. The taxonomy of *Leishmania*. The genus is divided into four subgenera, where *Leishmania (Sauroleishmania)* is not considered pathogenic to humans. The subgenus *Leishmania (Mundinia)* has recently been described (11–13). The list of species in the figure is not exhaustive. The figure is adapted from (11,14). *L. (L) chagasi has been found to be identical to *L. (L.) infantum* (15,16).

Leishmaniasis is caused by the protozoan parasite from the genus *Leishmania* of the order Kinetoplastida and family Trypanosomatidae. The genus is further divided into three sub genera, *L. (Leishmania)*, *L. (Viannia)* and *L. (Sauroleishmania)*[3]. Twenty one different species are known to be pathogenic to humans (14). The taxonomy of

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[3] A further subgenus has been proposed, namely *Leishmania Mundinia*(11).

Leishmania is debated. See figure 1 for an overview of a suggested taxonomy for *Leishmania*.

Leishmania is a protozoan that cycles between the sand fly vector and the macrophages of the mammalian host, where it is an obligate intracellular parasite (17) (see figure 2). There are two major morphological forms in the life cycle of *Leishmania:* the promastigote and amastigote. The extracellular and flagellated promastigote (approximately 15-20 µM long and 1.5-3.5 µM in width) is found in the digestive tract of the sand fly. The amastigote (approximately 2-4 µM in diameter) has no apparent flagellum and resides, in general, in cells of the reticuloendothelial cell line of the mammalian host. The amastigotes multiply within phagocytes, in particular macrophages, where they scavenge for essential nutrients, like purines, polyamines, vitamins, iron, magnesium and more (10,18).

Figure 2. The life cycle of *Leishmania*
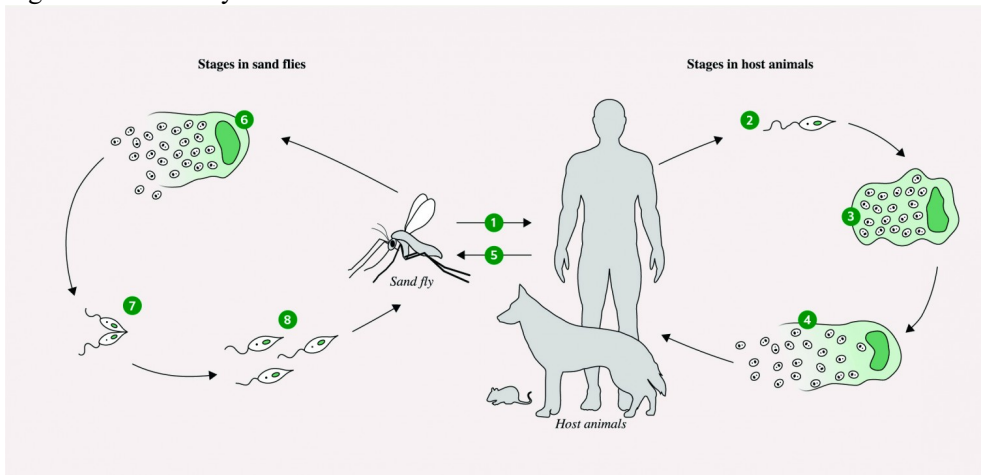
Figure 2. The life cycle of *Leishmania*. (1) The parasite is transmitted to the animal host through the bite of a sandfly. (2) Promastigotes infect cells of the reticuloendothelial systems, most commonly macrophages. (3) Promastigotes differentiate into amastigotes in the host cell, where they multiply (4). (5) sandflies are infected through a blood meal from an infected host. (6) Amastigotes are released into the gut of the sandfly. (7) Amastigotes differentiate into promastigotes and multiply. (8) Metacyclic (infective form) of promastigotes move to the sandfly proboscis, ready to be transmitted to another animal host. Source: (19) and with permission to reprint.

7.1.1 Transmission

Sandflies of the genera *Phlebotomus* and *Lutzomyia*[4] are the invertebrate host of *Leishmania,* and are the vectors for human leishmaniases. They are found worldwide in tropical and subtropical regions, from 50°N to 40°S (21,22). As one can expect, there are differences in the species of sandflies, where some feed on a wide range of mammals, while others prefer humans. This is reflected in human leishmaniases, which can be both zoonotic and anthroponotic in transmission.

In Europe, North Africa, the Middle East and Asia, sandflies of the *Phlebotomus* spp. transmit the parasite, while in the Americas, sandflies of *Lutzomyia* spp. transmit the parasite. *Leishmania* spp. can be transmitted by sandflies in two ways: (i) from animals (zoonotic) or from other humans (anthroponotic). Zoonotic is the most common form of transmission. Anthroponotic transmission has been reported for *L. (L.) tropica* and *L. (L.) donovani* in some endemic regions, but the exclusivity of the human-to-human transmission has been debated (23,24). The transmission in Africa and Asia is wide, from urban centres to rural villages, it is likely that animal reservoirs exist, and have also been reported for *L. (L.) tropica* (25). Infected animals have been found in Nepal and Bangladesh, although their role in the transmission cycle remain debated (26,27).

Non-vector transmission, such as accidental needle injection, is rare (28), as well as transmission by blood transfusion (29). Although some *Trypanosoma* species present vertical transmission, from human to human (30,31), vertical transmission of *Leshmania* has not been proved. Of the approximate 800 sand fly species, around 30 of them are proven vectors, with more than 40 involved in transmission (21,28).

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[4] Other sandfly species have been implicated in transmission in Brazil, but the results have yet to be confirmed (20).

7.1.2 Sandflies

Sandflies are small, approximately 3 mm long, bloodsucking insects. They mostly rest during day-time hours in cool and humid places, although there are considerable differences in the preferred resting sites (21). Their habitat is usually geographically limited, and their dispersal is usually less than 1 km (22). Sandflies live in remarkably diverse ecological habitats. In Europe, Africa and Asia the habitat is more common in semi-arid to desert conditions. While in South America, it is most commonly related to forests and has a sylvatic cycle.

Sand flies are primarily nocturnal, and biting during daylight is thought to be limited to a few species (22). Sand flies will take their blood-meal from a range of hosts. Thus it is not surprising that deforestation, urbanisation and changing agricultural practices affect the feeding pattern and thereby also the transmission cycle in several regions (28) . Furthermore, in most species, females bite most commonly outdoors (exophagic) and they usually rest outdoors while their eggs mature (exophilic). This observation limits the value of indoor insecticide spraying as a control measure for leishmaniasis. After attempts to eradicate malaria by house spraying with dichlorodiphenyltrichloroethane (DDT), for example, in India, leishmaniasis prevalence fell in several endemic regions but returned when spraying stopped (30,31). However, such a strategy will not work where the transmission cycle is sylvatic. While both males and females feed on carbohydrates, only females feed on blood. The number of blood meals during the day and for each batch of eggs differs between species and this also affects the efficiency of transmission of *Leishmania.*

When the female sand fly bites, she injects saliva into the skin. The saliva has, in numerous studies, been found to affect the outcome of the infection (32–34). The saliva consists of proteins, enzymes and nucleic acids (35). Its main function is vasodilation and counteraction of haemostasis (32,36). Saliva has been reported to induce expression of Th2 cytokines by activated macrophages (37) (see section 7.1.5), and, moreover, it has been shown to have chemotactic activity on the same

cells, potentially exacerbating an infection (38). Moreover, sand fly saliva has also been shown to inhibit the ability of dendritic cells to present antigens and increase apoptosis of neutrophils, also contributing to infection (39,40). Previous exposure to the saliva has been reported to lead to protection, by potentially limiting the effects of the saliva on the immune system (32,41). The magnitude of this effect in humans is still not clear.

7.1.3 Host and reservoir

The terminology around host and reservoir can at times be confusing and it is used variably in the literature (42,43). A reservoir can be defined as " an ecological system in which an infectious agent survives indefinitely"(44). Incidental hosts, on the other hand, are organisms that become infected but are not essential for the maintenance of the transmission cycle of that pathogen. Although a range of different accidental hosts has been reported, their precise role in the transmission cycle remains to be elucidated (26,27,45). What a host is, is possibly even less clear than what a reservoir is. A host can be defined as "the entity that houses its associated microbiome/microbiota, interacts with microbes, and responds to them in a way that results in damage, benefit, or indifference, thus producing the states of symbiosis, colonization, commensalism, latency, and disease"(43). A range of animals can be hosts and reservoirs for the *Leishmaia* parasite, and rodents and carnivores are among the most common. Animals in the same order can serve as both reservoir and host, and this is partly dependent on the parasite species (46). Limited knowledge of the true ecology of *Leishmania* transmission can lead public health authorities to ill-informed measures and initiation of policies that are less likely to be successful. An example is for *L. (L.) infantum*, where dogs are seen as the main reservoir in the European-Mediterranean area. However, several other animals have been reported as both hosts and reservoirs, such as cats and rabbits (47,48). Thus, dog culling may not have the expected effect on *Leishmania* transmission in such an area (49).

7.1.4 Clinical presentation

Leishmaniasis can present with a wide spectrum of clinical manifestations. The clinical manifestations are commonly in the skin, mucous membranes and/or visceral organs. Cutaneous leishmaniasis (CL) is characterized, most commonly, by localised skin lesions at the site of the sand fly bite (LCL), multiple non-ulcerative nodules (Diffuse cutaneous leishmaniasis (DCL)), mucosal affection (MCL) and visceral leishmaniasis (VL).

See table 1 for an overview over the clinical forms, their aetiology and main geographical distribution. The range of clinical forms is dependent on several factors, such as; parasite species, host age, nutritional state and host immune response which can be affected by genetic polymorphisms (50).

Table 1. *Leishmania* species, clinical forms, transmission and main geographical distribution.

Species	Clinical form	Transmission cycle	Main geographical distribution
Leishmania (Leishmania)			
L. (L.) amazonensis	LCL, DCL	Zoonotic	South America
L. (L.) aethiopia	LCL, DCL	Zoonotic	Ethiopia, Kenya
L. (L.) donovani	VL, LCL	Anthroponotic, zoonotic	Africa, Central and South East Asia
L. (L.) infantum	VL, LCL	Anthroponotic, zoonotic	Europe, North Africa, Central and South America
L. (L.) major	LCL	Zoonotic	Central Asia, North and East Africa, Middle East
L. (L.) mexicana	LCL, DCL	Zoonotic	Central America, Mexico, USA
L. (L.) pifanoi	LCL	Zoonotic	South America
L. (L.) tropica	LCL	Anthroponotic	Central Asia, North and East Africa, Middle East
L. (L.) venezuelensis	LCL	Zoonotic	South America

Species	Clinical form	Transmission cycle	Main geographical distribution
Leishmania (Mundinia)			
L. (M.) martiniquensis	LCL, DCL, VL	Unknown	West Indies, Thailand
L. (M.) orientalis	DCL, VL	Unknown	Thailand
Leishmania (Viannia)			
L. (V.) braziliensis	LCL, MCL	Zoonotic	South America, Central America, Mexico
L. (V.) colombiensis	LCL	Zoonotic	Northern South America
L. (V.) guyanensis	LCL	Zoonotic	South America
L. (V.) lainsoni	LCL	Zoonotic	South America
L. (V.) panamensis	LCL, MCL	Zoonotic	Central and South America
L. (V.) peruviana	LCL	Zoonotic	Peru
L. (V.) shawi	LCL	Zoonotic	South America

Table 1: Species causing human leishmaniasis, their main clinical forms, transmission cycle and main geographical distribution. DCL: diffuse cutaneous leishmaniasis, LCL: local cutaneous leishmaniasis, MCL: mucocutaneous leishmaniasis, VL; visceral leishmaniasis. The table is partly adapted from (28). References regarding *Leishmania (Mundinia)*: (12,13,51,52)

CL is caused by several strains of both *L. (Leishmania)* and *L. (Viannia)* subgenera. The cutaneous manifestation is the most common, where ulcers most commonly occur at the site of the sand fly bite (53). The ulcers usually heal spontaneously over months to years (17). They often leave an atrophic and disfiguring scar. The scar may cause great morbidity by social isolation and stigma (54). CL, like VL, probably remains symptomless in most cases. Symptomatic CL typically starts with erythema, which develops into a papule, then nodule that ulcerates over weeks to months. The severity and time to self-heal varies between the lesions and seems to be related to the species involved. Typically, healing occurs within a year, but can be as rapid as 2 months, but also take several years. Lymphatic spread and involvement is common, also before clinical disease develops (28). After spontaneous healing, it is thought that the person has a lifelong protection, possibly also against other species, although this point is debated. Resolution of the disease is, however, connected to scarring.

DCL is a rare form, especially reported from Africa and parts of South America, is characterized by non-ulcerative papules that cover the entire body. This is a form that is difficult to treat and does not usually self-heal (28).

Mucosal leishmaniasis (ML) indicates the involvement of mucous membranes, most commonly nasopharynx, larynx and the oral cavity. Mucocutaneous leishmaniasis (MCL) can manifest itself long after CL[5](17). The mucosal lesions can be severely disfiguring, where septum perforation is not uncommon. CL precedes MCL in 5-20% of the cases (55). However, isolated MCL has been described in 17-18% of patients with *L. (V.) braziliensis* (56). MCL caused by viscerotropic strains has also been reported, especially in immunocompromised patients, but not restricted to this patient group (57,58)*.* How the parasites reach the mucous membranes is still under discussion and is thought to be dependent on the species causing the condition. Commonly cutaneous species, like *L. (L.) major* are thought to spread to mucous membranes directly from the skin lesion. On the other hand, for *L. (V.) braziliensis* it is more commonly thought that the spread is lymphatic or haematological (59). Direct inoculation of parasites by sandflies is also probable for oral and nasal sites, but less likely for laryngeal affections (60).

The most common early manifestations are in the upper respiratory tract, nose and oral cavity. In the early phases, MCL manifests with signs of inflammation, such as congestion, erythema, edemia, rhinorrhea, and epistaxis which may develop into dysphagia and dysphonia as the disease progresses.

Both subgenera may cause MCL, but in the Americas, MCL is predominantly caused by species of the *L. (Viannia)* subgenus (see table 1). In South America, MCL occurs most commonly in Brazil, Bolivia and Peru. In South America it is common to use the term American tegumentary leishmaniasis (ATL) to include CL, DCL and MCL (59). The strain involved in MCL in South America is commonly *L. (V.) braziliensis*, but *L. (V.) panamensis*, *L. (V.) guyanensis* and *L. (L.) amazonensis* may also cause the condition (59,60). Although ML/MCL is most common in South America, it has been reported for all the endemic regions for leishmaniasis (60). Outside of South-

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[5] For practical purposes, and although it might not be accurate in all cases, this thesis will treat MCL and ML as the same.

America, ML/MCL is caused by different strains of the *L. (Leishmania)* subgenus (57,61).

VL is mainly caused by *L. (L.) infantum* in Europe and South America, while in Africa and the Indian subcontinent, it is mainly caused by *L. (L.) donovani*. In Europe and South America, it is a zoonosis, where dogs are the main reservoir. On the Indian subcontinent it is thought to be mainly anthroponotic, but as mentioned in section 7.1.1, this is debated. Differential diagnosis includes mycobacterial infections, haematological malignancies, like lymphoma, disseminated histoplasmosis, and rheumatological conditions like Feltys syndrome. Biochemically, VL is characterized by pancytopenia (anaemia, thrombocytopenia and leukopenia), increase in inflammatory markers, like C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), hypergammaglobulinemia and hypoalbuminemia. In addition, production of anti-nuclear antibodies, anti dsDNA, anti-smooth muscle, anti-cardiolipin, anti-cyclic citrullinated peptide and IgM rheumatoid factor antibodies can sometimes also be detected (62).

7.1.5 Immune response

The clinical outcome of the infection depends on both the species of the parasite and the host immune response (63). See figure 2 for a graphical depiction of the *Leishmania* life cycle. This thesis does not give an in-depth analysis of general immunology, nor of the immunopathology of *Leishmania* spp. in humans. For a good introduction to immunology, the reader is directed to the many excellent books and other publications in the field (such as (64)). The outcome of infection with *Leishmania* is thought to be dependent on several factors, including the host immune response, host genetics, nutritional status and parasite virulence (63,65). Most studies focus on one species, and as is evident through the several clinical outcomes, caution should be taken when drawing general conclusions from studies on one species to another.

At the time of entry into the mammal, the parasites immediately encounter the complement system. The immediate effect of the complement system is activation of the membrane attack complex, which will puncture the membrane of the parasite. In addition, components of the complement system will act as opsonins thereby stimulating phagocytosis. GP63, a known virulence factor expressed by *Leishmania* promastigote is able to inactivate complement by binding C3b and increase the conversion of C3b to C3bi (66,67). *L. (L.) major* deficient of GP63 has been shown to be susceptible to complement-mediated lysis (68). GP63 is also able to suppress the proliferation of IL-2 activated NK-cells (69). NK-cells, in turn, are central in the mounting of the Th1 response through production of IFNγ. It was for *L. major* that the Th1/Th2 dichotomy response (cure/not cure) was based (70). In a Th1 response, Th1 cells produce IFN-γ and TNF-α. On the other hand, the exacerbation of leishmaniasis is in general thought to be related to a Th2 response, producing IL-4, IL-5 and IL-10. Caution should however be taken when interpreting a disease in such a strict dichotomy, as this is based on animal studies which may have limited applicability for the leishmaniases, both cutaneous and visceral (71).

The first cells to migrate to the infected site are neutrophils, and after that professional antigen presenting cells, such as macrophages and dendritic cells migrate. The migration of these cells is stimulated by molecules from the sand fly saliva (72). Saliva from *Lutzomyia longipalpis* decrease TNF-α, IL-10, and increase IL-6, IL-8 and IL-12 in macrophages, reducing inflammatory response of the host towards the parasite (73). Furthermore, pre-exposure to sand fly saliva has been shown to be protective against *Leishmania* infection (74)

Neutrophils are professional killer cells. Despite this, it has been suggested that neutrophils could work to the benefit of *Leishmania* as a "Trojan horse" (75). It has been shown *in vitro* that *Leishmania* can survive within the neutrophil phagosome. Further, the parasite may induce apoptosis, which, when the neutrophil is digested by a macrophage, will not induce an inflammatory response (76). The role of neutrophils has also been shown *in vivo*, where they rapidly migrate to the site of inoculation in

mice (77). Apoptotic neutrophils are in turn internalized by macrophages and dendritic cells, where the parasites may multiply.

After phagocytosis, promastigotes in phagosomes fuse to lysosomes forming the parasitophorous vacuole that can harbour individual or several parasites, in a species-specific way (78,79). In addition, once inside the macrophage, the parasite may modulate cytokine secretion. The aforementioned virulence factor, GP63 is able to activate several tyrosine phosphatases that are important in several cytokine pathways (72). GP63 is also able to cleave CD4, a central co-receptor in T-cells, and may inhibit cross-presentation by DC and macrophages. Cross-presentation is central in the immune response, as microbial antigens are presented to CD8+ T-cells.

Cytokines are small signalling molecules that play a critical role in directing the immune response. In particular, INF-γ, TNF-α and IL-10 are thought to be central for leishmaniases. Some authors argue that asymptomatic individuals are able to control the infection by balancing the immunoregulatory cytokine IL-10 with the effector IFN-γ (71,80). It is thought that TNF-α, IL-1α, IL-10 and TGF-β are significantly increased in patients with chronic disease (81). Furthermore, the frequency of CD8+ IFN-γ+ cells after stimulation with soluble *L. (V.) braziliensis* antigen was found to be higher for asymptomatic than for CL patients – also highlighting the central role of T-cells in fighting the pathogen (82).

As *Leishmania* is an intracellular pathogen, an adaptive immune response, in particular a strong T-cell response, is central to keep the infection at bay. CD4+ T-cells are important to regulate and mediate antigen presentation. A central cytokine, INF-γ, stimulates macrophages to produce NO, which is important for *Leishmania* killing. INF-γ, secreted by CD4+ T-cells has a dual role in infection with *Leishmania*, where it is central in activating the macrophage to keep the pathogen at bay, but it is also central in the tissue damage seen in CL (83). The other central cytokine, TNF-α also activates the leishmanicidal activity of macrophages and is central in fighting infection with *Leishmania*. TNF-α is important for granuloma formation. Anti-TNF-

α drugs, commonly used in several autoimmune diseases, do indeed increase the risk of developing infections, including leishmaniasis, especially intracellular organisms (84). IL-10 is an important regulatory cytokine in *Leishmania*, but there have been conflicting results as to the precise role for infections with different strains of parasites.

The pathogenesis of MCL is only partly understood (60). The immune response seems to play a central part in directing the *Leishmania* infection towards mucosal leishmaniasis (ML) or CL. Whereas IL-10 levels are equal in both ML and CL, IFN-γ and TNF-α are higher in ML. The important role of the immune system is strengthened by the fact that immunocompromised patients (such as HIV patients) have a higher rate of ML compared to immunocompetent *Leishmania* patients (85). HIV enhances the undesirable Th2 response.

7.1.6 L-Arginine and the amino acid permease

L-arginine is an essential amino acid for both *Leishmania* amastigotes and promastigotes (10). L-arginine has to be taken up by the parasite from the environment as the parasite does not have the ability to synthesize it *de novo*. Within promastigotes, there is a pool of several amino acids with alanine, ornithine, glutamate and glycine as the most abundant (10,86,87). Control of L-arginine depends on both uptake and intracellular concentrations. L-arginine is further used in synthesis of a range of important substrates. Polyamines are synthesized from L-arginine by arginase. L-arginine can also be decarboxylated to agmatine, that through hydrolysis is turned to putrescine and urea (88) Arginase is found in glycosomes (88). Amino acids can be transported over cell membranes by a variety of permeases. The amino acid permease 3 (AAP3) belongs to the amino acid/auxin permease (AAAP) group (89).

For *L. (L.) amazonensis* and *L. (L.) donvani* it has been shown that the parasite can sense the external pool, or availability, of the amino acid, and thereby regulate the expression of transporter proteins (87,90). Furthermore, the level of arginase activity also affects the intracellular pool of L-arginine. And lastly, it has been shown that the amastigotes can scavenge the phagolysosome for essential amino acids (91,92).

The amino acid permeases have been described as transporters with high affinity for L-arginine in several *Leishmania* species (86,90,93–97). Amino acid permease 3 (AAP3) is a high affinity transporter of L-arginine, but can also mediate uptake of other amino acids, such as lysine, histidine, phenylalanine of citrulline (87,94). AAP3 expression is upregulated in response to L-arginine starvation (98). Another study found that temperature also affected the transcription of one of the AAP3 copies, as well as protein expression and localisation (96). Furthermore, AAP3 has been found to be localised in both the cell membrane and the glycosome, indicating direct transport into this organelle (96).

7.1.7 Regulation of gene expression

Leishmania belongs to the Kinetoplastida, of which *Leishmania* spp. and *Trypanosoma* spp. are known to cause human disease. They share the common feature of being flagellated during parts of their lifecycle, and also share the common organelle, known as the kinetoplast. The kinetoplast contains several copies of mitochondrial DNA. The kinetoplast is organized in mini- (~ 1 kb) and maxi-circles (~25 kb). Mini-circles code for guide RNAs and maxi-circles for pre-mRNAs. The size of the genome in *Leishmania* varies from 29 Mb to 33 Mb (99). *Leishmania spp.* have 34–36 chromosomes. *Leishmania (Leishmania)* spp. in the Eastern hemisphere have 36 chromosomes (100). Species belonging to *L. (Viannia)* subgenus have 35 chromosomes where chromosome 20 and 34 are fused. While species in the Americas belonging to *L. (Leishmania)* subgenus have 34 chromosomes where chromosomes 8 and 3, and 20 and 36 are fused (101). Although they cause different clinical

manifestations the genomes are, in general, very conserved, and there are few species specific genes (102,103).

In eukaryotes gene expression can be regulated at several levels, such as the availability of chromatin, how the RNA is processed, the stability of mRNA and the rate of translation of mRNA. However, the control of transcription is central, often through transcription factors. In bacteria, on the other hand, transcription is commonly regulated by binding of proteins to an operon (regulatory sequence and genes). However, the gene expression in the trypanosomatids is regulated in a curiously different way. *Leishmania* lacks introns, and transcription itself is initiated at a few regions on each chromosome. The genome contains polycistronic gene clusters, where the genes are organized head-to-tail (104). The genes are thought to be constitutively transcribed into mRNA. The genes are transcribed in clusters (polycistrons) of 10-100 genes (105), but unlike polycistronic transcription in bacteria, the genes within a given pre-mRNA do not necessarily belong to the same pathways. Through trans-splicing a splice leader sequence (SL) is added to the 5´-end of the gene (106), and the 3´-end is polyadenylated. *Leishmania* have relatively short 5´-untranslated regions (UTRs), while the 3´-UTRs are longer, which is thought to be important for the stability of the RNA transcript (107,108). Polycistronic transcription and trans-splicing is not unique to *Leishmania*, but that the whole genome is transcribed this way is thought to be unique to the kinetoplastids (105).

So, how can *Leishmania* adapt to such different environments as the digestive tract of the anthropod vector and the different environments in the mammalian host? There seem to be relatively few differentially expressed genes in a comparison of amastigotes and promastigotes. However, there are studies which show that the set of differentially expressed genes are different when comparing promastigotes from the sand fly digestive tract to *in vitro* cultured amastigotes (109). Regulation of protein level is through other mechanisms, such as mRNA processing and stability (110). It has also been suggested that the plasticity between and within strains (mosaic aneuploidy) and gene copy number is key to differential gene expression (102,111).

This might be advantageous as beneficial mutations may spread more rapidly in haploid populations. On the other hand, diploid populations evolve more slowly, but are more stable (112).

7.2 Epidemiology

The neglected tropical diseases (NTDs) are a group of diseases and conditions that are among the most common diseases affecting the world´s poor (113). The number of deaths related to these diseases is lower than for diarrheal diseases, HIV-AIDS or malaria, but the disability and poverty related to them are considerable. According to the 2010 Global Burden of Disease study, the NTDs accounted for approximately 26.06 million disability adjusted life years (DALYs) (114). The NTDs can be important obstacles for economic development and constitute large burdens on fragile health systems (113).

The population in endemic regions for the NTDs are often infected with multiple NTDs simultaneously, furthering the disability. This highlights the importance of a coordinated approach where both rapid diagnosis and treatment is of paramount importance (113). For example, drug treatment packages have been suggested as a rapid initial response (113), but, as Hotez et al. point out, while preventive chemotherapy may be effective for some NTDs, it may not be effective against Chagas disease, human African trypanosomiasis and visceral leishmaniasis.

As highlighted by numerous reports, there is a severe shortage of reliable epidemiological data for the NTDs, including for the leishmaniases. The Global Burden of Disease study – a critical tool for monitoring global health and prioritizing between health programmes – systematically undervalues the NTDs (115,116). For the leishmaniases, there are problems related to the exact number of people affected because of a complex disease cycle and epidemiology, lack of simple diagnostic tools, and the fact that the diseases often exist in the poorest regions of the world. This also makes the extrapolation from official data, as international organizations

sometimes have to do, difficult, if not impossible (116). The accuracy of estimated impact measures, such as the disability adjusted life years (DALYs), which depend partly on the accuracy of incidence and mortality data.

The leishmaniases are endemic in 98 countries and 3 territories. See figure 3 below for the world distribution of cutaneous and visceral distribution of leishmaniasis. One of the most comprehensive analyses of the available data estimated the annual incidence for CL to be 0.7-1.2 million and for VL to be 0.2-0.4 million (116). The actual number of cases is not known in most of the endemic regions (116–118).

CL is endemic in 98 countries, but 75% of the cases are found in 10 countries: Afghanistan, Algeria, Brazil, Columbia, Costa Rica, Ethiopia, Iran, Peru, Sudan and Syria (116,119). 90% of VL is found in six countries: Bangladesh, Brazil, Ethiopia, India, Sudan and South Sudan (116). There are over 500 000 cases of VL reported annually, and the annual mortality estimates range from 20 000 to 60 000 (116,120). VL is mainly found in the poorest parts of society, and children, young adults and women are disproportionally affected (121).

There has been a marked increase in prevalence for both CL and MCL in recent years in the Middle East, Central Asia and East Africa. This is probably due to war and conflicts leading to increase of reported cases (114). Armed conflict has proven to be a major risk for an upsurge in leishmaniasis. Destruction of housing, health infrastructure and usually already weak leishmaniasis control programmes alongside major population movements has had devastating effects in several areas. Cutaneous leishmaniasis surged in Syria after the war (122). CL has been known in Syria for centuries and was widely known as the Aleppo boil. After the outbreak of the war, over 100 000 cases have been reported, as opposed to 25 000 to 40 000 before (122).

Figure 3. Map of the distribution of cutaneous and visceral leishmaniasis.
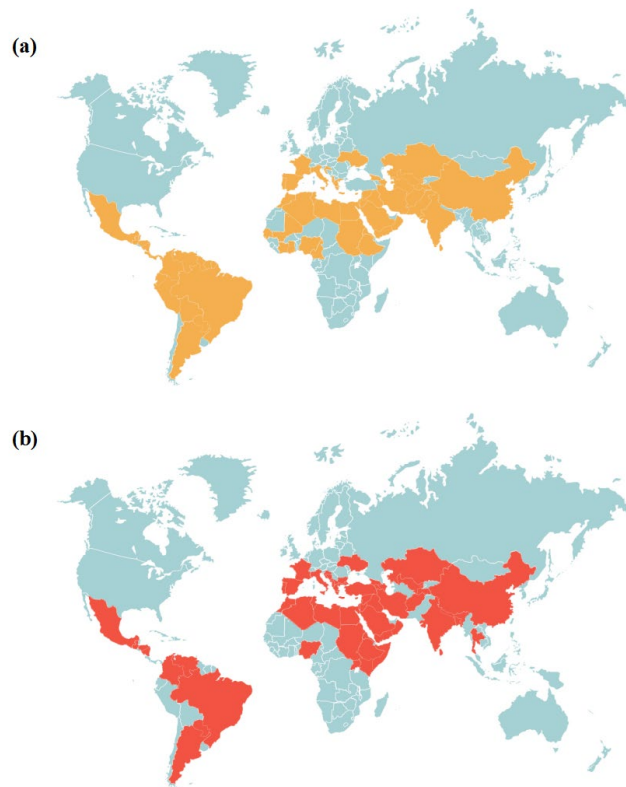
Figure 3. World distribution of cutaneous (a) and visceral leishmaniasis (b). Source (116)

The war not only escalated transmission, but CL was spread to parts of the country previously not thought to be endemic, and to neighbouring countries (123). Furthermore, in Sudan and South Sudan there have been several epidemics, where an outbreak between 1984 to 1994 reported over 100 000 deaths attributable to VL among a population of approximately 280 000 (124). India has the largest number of VL cases at 62 000, followed by South Sudan and Sudan.

In Europe, leishmaniasis is endemic around the Mediterranean basin. Some countries report a high prevalence of asymptomatic carries (125). There have also been recent outbreaks in several regions, such as the Madrid region in Spain (126), or Bologna in Italy (127). Imported leishmaniasis is reported regularly due to travellers'

transmission and also an increase in patients on immunosuppressant drugs (84). In a recent retrospective study from Sweden on imported leishmaniasis in the period 1993-2016, including cases from both primary health care and hospitals, 182 laboratory confirmed cases were found of which 96 were diagnosed in the last two years of the study (128). Leishmaniasis, on the other hand, has been reported sporadically in Norway (129,130). This could be due to a lack of tools to diagnose correctly and rapidly. This thesis aims to give more insight into the occurrence of leishmaniasis in Norway, to promote better treatment and clinical management (paper III).

It is believed that asymptomatic infection is the most common outcome of inoculation with *Leishmania* spp. (71). That leishmaniasis can cause asymptomatic infection is not extraordinary, this is true for many infections. People who are not aware of their situation, or have not been diagnosed yet, may be able to work as reservoirs and transmit the disease to others. Thus, people who are infectious, but who have no signs and symptoms are called asymptomatic carriers. The problem of asymptomatic carriers is a great challenge for the elimination of the disease on the Indian subcontinent (131). However, there is no consensus on how asymptomatic infections should be diagnosed. Normally a person with a positive serological test, PCR or leishmanin skin test (LST) but otherwise healthy is considered to be an asymptomatic carrier (131). The true epidemiology of asymptomatic infection is difficult to estimate, but the ratio of asymptomatic to clinical cases (incident) ranges from 50:1 in a study from Spain, 4:1 in Kenya, 18:1 to 1:2.4 in Sudan, with no obvious reason to these differences (131). Of note is, however, that in most endemic areas, a large part of the population are able to establish an effective immune response keeping the parasite at bay. It is not entirely clear what mechanisms are involved in controlling the pathogen and causing no or few clinical signs and symptoms. This knowledge is of particular importance for several reasons – maybe the most obvious one is for vaccine development. To date, there are no good immunological markers to identify asymptomatic patients.

Immunosuppression is an important risk factor for clinical disease, but may also alter how leishmaniasis presents and the response to treatment (53). In Southern Europe there was a re-emergence of VL in 2000 with a great number of cases related to HIV-co-infection. The parasites were transmitted by sharing of needles and syringes by intravenous drug users (132). During that outbreak in France, it was also shown that dermatotropic strains could visceralise. This problem was resolved in Europe with the introduction with highly active antiretroviral therapy (HAART). However, the problem has persisted in other parts of the world, especially South America and Africa (133,134). In Ethiopia a large number of people are immunosuppressed through HIV and it is also a highly endemic area for VL (53). In Ethiopia, between 20-40% of the individuals with VL are co-infected with HIV (132).

7.3   Treatment

"From what I observed, it is infinitely better to apply nothing, than any of the numberless medicines they make use of." (135)

As Alexander Russel points out in the quote above, CL may be treated with observation therapy. However, the more serious MCL and VL need treatment, and a late diagnosis could often be lethal if untreated.

Current treatment options for leishmaniasis can be defined as limited, and they have all been developed for other indications (120). The pentavalent antimonials, used for centuries, remain the first option in many parts of the world, including Brazil. Their mode of action is not clearly understood (136). The treatment regime is lengthy (30 days of injections), and several toxic side effects are reported, such as cardiotoxicity, renal failure, pancreatitis and anaemia. Resistance has been reported in several endemic regions against the pentavalent antimonials (120,137–139). This resistance is most likely due to its widespread, uncontrolled and inappropriate use, especially in India (137,140).

Amphotericin B and liposomal amphotericin work by targeting the ergosterol pathway in the membrane of *Leishmania* (121). In human cells, on the other hand, the main membrane sterol is cholesterol. Development of resistance against these drugs is more unlikely as the parasite would need to develop new pathways to replace surface ergosterols (120). To improve treatment, but also to eliminate or at least greatly reduce the incidence of transmitted VL, new dose regimens for treatments are necessary as today's treatment is lengthy and prone to toxic side effects. The WHO have described the use of a single-dose approach as safe and highly effective in Bangladesh, India and Nepal (141). Amphotericin B and paromomycin must also be given over lengthy periods, 21 days and 30 days, respectively. Paromomycin is an aminoglycoside, the mode of action against *Leishmania* is unknown (121).

Miltefosine has been used, especially in India, to treat VL. This treatment, although attractive as it is an oral therapy, remains unattractive as it still requires lengthy treatment (28 days) and strict birth control is necessary due to it being teratogenic.

For VL, combination therapy has increasingly been advocated, because of emergence of drug resistance, to shorten administration time of the drugs, affordability, and ease of administration (142). For elimination purposes, single-dose liposomal amphotericin B seems to be the best option, as it ensures high compliance, but also reduces the cost to families and decreases time off work. Another reason for introducing single-dose liposomal amphotericin B in some regions is its attractiveness, because it is relatively safe, effective and 100% compliance is guaranteed (143). The single dose approach has been recommended by the WHO as the first line treatment option in India (120,144). Treatment regimens have to differ from region to region, between various patient groups (145) and between the different *Leishmania* spp. For example, a combination therapy including liposomal amphotericin B and miltefosine might be more attractive for males, while a combination of liposomal amphotericin B and paromomycin might be more attractive for women.

In a study from Bangladesh, the efficacy and safety of single-dose liposomal amphotericin B for VL were investigated (146). Out of 300 patients included, 90% achieved final cure. The authors therefore concluded that a single dose liposomal amphotericin B (10 mg/kg bodyweight) could be adopted in an elimination programme in Bangladesh. However, the problem with liposomal amphotericin B is its requirement for a cold chain, which might be problematic in remote locations, which are also typically endemic for VL. Despite this, the authors found that the implementation of a single-dose liposomal amphotericin B treatment strategy is feasible in remote locations (146). Another study in rural Bangladesh confirmed the feasibility of such an approach, given proper training of health-care staff (147). There are however several problems related to single dose regimens: (i) what is the efficacy and safety in selected patient groups outside the studied group, (ii) extrapolation of the results to other regions endemic for leishmaniasis is problematic because of geographical heterogeneity in VL (such as Africa or other agents, like *L. (L.) infantum)* (142). Liposomal amphotericin B (AmBisome) is costly, but a price reduction for all low- and middle-income countries where leishmaniasis is endemic, has made it more feasible (148).

7.4 Prevention

Leishmaniasis, given the complex interplay between host, vector and reservoir, probably requires a multifaceted approach for control and prevention. Elimination of leishmaniasis is highly unlikely. In general, to eliminate an infectious disease requires that every infection leads to clinical disease and is only present in humans. For CL, most commonly a zoonosis, often with a sylvatic reservoir, the environmental cost of any preventive strategy would be too high. Furthermore, in VL the role of asymptomatic individuals in transmission is unclear, and it is likely that they contribute to the transmission (24,130). In addition, domestic animals might have a more prominent role as reservoirs, including in areas previously thought to be only anthroponotic transmission areas (26). That the sand fly in the Indian subcontinent should only feed on humans would be an exceptional finding, as the sand fly vector is

an opportunistic feeder and one could expect them to feed on wild and domestic animals in addition to humans.

Vector control can however work in certain areas. An example is from the European Mediterranean area, where an important reservoir for CL, *Phlebotomus obesus*, is controlled by zinc phosphide tablets in burrow entrances or clearing of vegetation in a 500 meter barrier zone around the habitat (119).

For humans, potential control strategies could include early detection, drugs and vaccine. A vaccine is probably the best way forward, especially since infected people are known to acquire long-lasting immunity against infection from parasites of related strains (49,148–150). Ideally, a vaccine for leishmaniasis should be broadly protective against all the species of the parasite causing the different variants of clinical disease, but this has so far proven difficult. However, in theory, it should be possible. When selecting antigens for a recombinant, vector or nucleic acid vaccine, antigens that are highly conserved among different species of the pathogen are typically selected. Even if two species are relatively distant in evolutionary terms, a vaccine based on conserved antigens could give protection against several species. Intuitively, it may be most pressing to develop a vaccine for the potentially fatal visceral form, commonly caused by only two species – *L. donovani* or *L. infantum*.

It is not entirely clear what type of vaccine is most likely to be effective against leishmaniasis, but as it is an intracellular pathogen, a strong T-cell response has to be induced. Vaccines based on killed or live attenuated pathogens have the potential to mimic natural infection closely. In the Middle East, so-called "leishmanisation" has been practised, whereby pus from cutaneous lesions and parasites from culture were used to induce a local infection (150). Leishmanisation was discontinued because of problems with reproducibility and safety issues. Furthermore, a leishmanisation strategy involving a viscerotropic strain, such as *L. donovani,* is highly unlikely, and probably unethical. Vaccines consisting of purified or recombinant proteins expressed in bacteria or eukaryotic cells usually require an adjuvant to induce a good T-cell

response (151). The Infectious Disease Research Institute in Seattle has developed a vaccine where several proteins conserved across several species of *Leishmania* are expressed in a single vaccine using a toll-like-receptor agonist as an adjuvant to induce a strong T-cell response (18, 19). This vaccine has shown promising results (152). Nucleic acid vaccines work by introducing expression vectors encoding for a protein antigen into the recipient. Here not the antigen itself, but the information required to produce it, is introduced. The recipient's cells produce and present the antigens to immune cells (153). This vaccine strategy has obvious attractions, such as the fact that the vaccine can be rapidly and cheaply produced. The DNA-vaccine approach has been tested with several antigens against *Leishmania*-infected mice, but its effect remains unconvincing. Vectors such as Adenovirus, expressing recombinant antigens, can also be used as vaccines. A novel vaccine uses this approach by expressing a gene encoding for two *Leishmania* proteins, and initial results are promising (154). But this is only in phase I clinical trial stage, and there are significant challenges ahead.

Over the years, several vaccine targets and vaccination routes have been proposed and tested (155). Some of them have shown great potential in protecting animals against leishmaniasis (156). However, remarkably few have been able to proceed to a clinical trial. This lack of progress is partly due to lack of a small-animal model that reflects human disease, and to the fact that many vaccines are tested with cutaneous strains where the testing has been undertaken by injections instead of sand fly bites (151).

7.5 Diagnosing the leishmaniases

In this section, I will discuss the common diagnostic approaches more in detail. Particular attention will be given to the molecular application with a discussion of potential targets and methods.

For diagnosing leishmaniasis, a range of diagnostic methods can be used, starting with history taking and symptoms, and - when leishmaniasis is suspected - microscopy, serology and molecular methods. Unfortunately, lesion characteristic and probable geographic area where the infection happened are not sufficient for diagnosis or species identification for CL. The latter is of particular importance in the Americas, where species from the subgenus *L. (Viannia)* may cause mucocutaneous lesions years after CL.

Clinical diagnosis of VL relies on non-specific symptoms like prolonged fever, weight-loss and splenomegaly. Biochemically, the patients often have pancytopenia (anaemia, leukopenia and thrombocytopenia), elevated inflammatory markers and hypergammaglobulinemia. However, although these symptoms and findings raise the suspicion of the diagnosis in the experienced physician, they are not sensitive and specific enough for diagnosis alone (162).

There is no standard protocol for diagnosis, and the methods used vary in endemic and non-endemic regions. Leishmaniasis should ideally be diagnosed using several approaches to improve the likelihood of a correct diagnostic result. The latest diagnostic recommendations from the Infectious Disease Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH) suggest that leishmaniasis should be diagnosed to the species level for all the clinical forms to guide treatment and clinical management (144).

In brief, leishmaniasis can be detected by seeing (clinical, microscope), by the measurement of the immune response (e.g. ELISA), or detection of protein, RNA or

DNA. All diagnostic approaches and targets come with their strengths and limitations. In non-endemic regions, the greatest challenge is probably the first step, suspecting the parasitic disease in the first place. Parasitological diagnosis involves the demonstration of the parasite in tissue or culture. Anti-leishmanial antibodies can be detected using several methods, such as enzyme linked immunosorbent assay (ELISA), direct agglutination test (DAT), indirect immunofluorescence (IFA) and immunochromatographic strip tests (IST). Assays have also been developed to detect antigen in urine (157). Molecular approaches, like PCR, real-time PCR and associated methodologies are increasingly being used.

Diagnosing leishmaniasis can be a challenge because of the wide spectrum of clinical manifestations. There are different diagnostic approaches for the main clinical forms of the disease. Also, a range of differential diagnoses should be considered, and accurate diagnosis is therefore important. In addition, in some endemic areas, co-infection with other trypanosomatids may cause problems with the diagnosis if the approach or assay used is not specific enough (158). Accurate diagnosis is also crucial for surveillance and epidemiological studies. Several important questions need to be answered when choosing a diagnostic strategy. Peeling and Mabey, highlighted the ASSURED-criteria when choosing diagnostic tests for the developing world (159). The ASSURED criteria are specifically made for rapid tests listing: Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment-free or minimal equipment and Deliverable to those who need them. So far, few, if any molecular methods meet these criteria.

According to Shaw et al. (160) a diagnostic tool should differentiate *Leishmania* species, be able to measure the parasite load in host and vector as well as being able to monitor the efficacy of treatment. This seems somewhat unrealistic and most, if not all tests, are unable to meet these criteria. Furthermore, the criteria seem somewhat biased towards molecular methods. Besides, the demand for monitoring the efficacy of drug treatment remains questionable as it is, so far, not included in recent guidelines (144). Some authors include further requirements that all seem

unrealistic and more research oriented, such as the need to identify sample origins, relationship with non-human reservoirs and several more (161). In the next sections the different diagnostic modalities will be discussed in more detail.

7.5.1 Parasitology

Parasitological diagnosis involves the demonstration of the parasite in tissue or culture. The amastigote forms of the *Leishmania* parasite are identified intracellularly or extracellularly by size and characteristics morphology, including the nucleus and kinetoplast. The kinetoplast is particularly important to identify, as the parasite without the kinetoplast can be mistaken for other pathogens, like *Histoplasmosis capsulatum*, which also may be found in phagocytic cells (163,164).

For VL, smears from spleen have the highest sensitivity, followed by bone-marrow and lymph node (165). Spleen aspirate is the recommended reference method for VL, especially in studies comparing diagnostic methods (148). Patients with VL often have pancytopenia, meaning they are at higher risk of fatal bleeding when a spleen biopsy is taken. However, Sundar et al. (167) only report two fatal bleedings after 9612 splenic aspirations. Regardless, other techniques that require less invasive diagnostic techniques are important for VL. Microscopy, although in trained and capable hands, may be very specific, sensitivity on the other hand is comparatively low and highly influenced by smear quality, reagents and experience (168).

In CL, a biopsy from the ulcer is sensitive (17). This requires invasiveness often not usable for large-scale screening, but also for follow-up. For CL, some studies have proposed that the use of filter papers able to store DNA (e.g. FTA-cards), could be a useful solution to avoid taking a biopsy. But our study (Paper III) and others have shown that although they might be of value in a research setting, in the clinical setting their role is more dubious (169).

The traditional culture of *Leishmania* from biopsy specimens from patients is both labour intensive, lacks sensitivity and requires considerable specialist infrastructure

(170). Developing of new culturing techniques, like microculture, could make the culture approach cheaper, more sensitive and specific (170). Despite this, processing time did not improve greatly, only reducing the culture time from 5 to 3 days compared to traditional culture, making the technique unattractive for diagnosis, especially VL. Culturing was particularly attractive before the molecular approaches became established because it allowed drug sensibility testing and species identification with multiloci enzyme electrophoresis (MLEE). However, today, all these parameters can be explored with different molecular approaches.

Microscopy has the advantage of being fast, and some argue it is easy to perform for the trained microscopist. One severe limitation is that in chronic lesions, the number of parasites may be difficult to find. The older the lesion, the lower the number of parasites in the lesion. A study from Para in Brazil, found that the parasitism was very low in lesions older than 12 months (171).

Immunofluorescence in general is sensitive but lacks specificity. In Brazil, cross-reactivity with other common infections, such as Chagas disease (*Trypanosoma cruzi)* has been reported (172). It has furthermore been shown to lack sensitivity in patients with few lesions or acute lesions (171).

7.5.2 Serology

Anti-leishmanial antibodies can be detected using several methods, such as enzyme-linked immunosorbent assay (ELISA), direct agglutination test (DAT), indirect immunofluorescence (IFA) and immunochromatographic strip tests (IST). ELISA requires considerable experience and is probably only suitable for well-equipped reference centres. The inherent problems with ELISA could probably be overcome in some field-settings by a collection of material in the field and transport of the material to a reference centre thereafter. Although suitable for research studies, this would be costly, time-consuming and delay the diagnosis. DAT has and is still in use. The applicability of DAT in the field setting has been increased with the development of assays where the antigen is freeze dried, thus reducing the need for a cold chain

(173–175). Although relatively cheap, sensitive and specific, it requires considerable laboratory time (partly due to long incubations), test readings to be standardised and some infrastructure which is not always available in the field (176). In IFA the antigen is adsorbed to a slide, and antibodies present in the patient sample (serum) are detected by a fluorescein labelled secondary antibody using an immunofluorescence antibody. Comparing ELISA and IFA, a study found that for patients with CL IFA, along with parasitological confirmation, could be used in diagnosis. There are also other sporadic reports of the usefulness of IFA in CL (177). A study from Iran, comparing ELISA, DAT and IFA, found the sensitivity and specificity of the latter to be 80.3% and 90.5% respectively (148). Although IFA may have a role in diagnosis, the lack of a standard antigen, proper validation in different endemic settings, and the requirement of considerable infrastructure and expensive equipment mean that the technique has limited value in a diagnostic setting. IST can have a prominent role as point-of-care tests, especially in rural settings.

In endemic settings, the necessary skill, quality of tissue smears and staining, and a high quality microscope is often unavailable (167). HIV is also a major public health issue in many endemic regions for VL, and in half of HIV-*Leishmania* coinfected patients, serology has been found to be negative (178,179). Furthermore, seropositivity has been reported in over 30% of an apparently healthy population, probably due to the high number of asymptomatic individuals in endemic regions (174,180,181). For the leishmaniases, serological tests only have very limited value in CL and MCL but may have a more prominent role in VL. Especially useful is serology for VL patients where other diagnostic tests, such as spleen biopsy or bone marrow aspirate cannot be performed. They also have a role in screening in endemic areas. In elimination programmes especially, active case detection is important, requiring a good point-of-care (POC) test: for VL an IST seems like a very good option. However, currently, the available tests have great limitations in terms of sensitivity and specificity when it comes to different geographical locations. Sensitivity and specificity vary depending on the antigen used, geography and host factors. Furthermore, as antibodies can be detected years after clinical cure, caution

should be taken in the interpretation of the results. A study from Ethiopia, found that 89% of treated and cured VL cases had DAT positive antibodies 50–90 months (7.5 years) post-treatment (182). The same was found in Brazil, where many patients with VL had persisting and detectable antibody levels years after treatment, one even reporting 12 years post-treatment (183). In a study from Sudan, antibody levels could be detected up to 24 months post treatment measured by ELISA and DAT (184). On the other hand, a study from India found that for 55 patients in India, IgG titer reached a maximum after 6 months, declined rapidly and were not detectable after 2–3 years, measured by ELISA (185). The discrepancies between these results are not easily explained but it seems clear that in most cases and for most endemic areas, anti-leishmanial antibodies remain detectable for a long time after treatment.

Several antigens have been developed for serological tests for leishmaniasis. Antigens for serology should, due to standardization purposes and the ability to produce enough antigen, preferably be manufactured as recombinant proteins. rK39, rK28 and rKE16 are examples of this. rK39 is based on a 39 amino acid sequence from the kinesin gene in *L.(L.) infantum* and expressed in *E. coli* (186). rK28 is a polyprotein based on three proteins: *L.(L.) donovani* haspb1, *L. (L.) donovani* haspb2 and *L. (L.) donovani* kinesin (187). rKE16 is a development of the rK39, but the sequence is based on a kinesin gene in *L. (L.) donovani* (188). A study from Kenya, involving 131 VL patients and 88 healthy controls, and comparing a rK39 tests (IT-LEISH, Bio-Rad) with a rKE16 test, found that for rK39 the sensitivity was 89.3% and specificity were 89.8%, while for rKE16 sensitivity was 77.10% and specificity was 95.5% (189). Although the authors strongly stress the need for improved RDTs for East Africa for VL, they suggest that rK39 can be used. In Bihar in India rK28 ELISA was found to be performing as well as rK39, yielding a sensitivity of 99.6% and specificity of 95.45% (190).

In a World Health Organization (WHO) coordinated study, 250 cases and 250 controls were included for each region (191). Patients were recruited from 4 labs from India, Bangladesh and Nepal, 2 labs in Brazil and 3 labs in Kenya and Sudan.

For India, Bangladesh and Nepal the results were uplifting with good sensitivity and specificity, and all the products had good heat-stability. Among the RDTs tested, sensitivity ranged from 92.8–100% and specificity 96–100%. For East Africa and Brazil, the situation was not as promising. In East Africa, the sensitivity ranged from 36.8–87.2% and specificity 90.8%-98%. The same situation was reported for Brazil with a sensitivity of 61.5–92%, and specificity of 95.6–98.8%. For these two regions, the rKE16 performed worse than rK39. Not only antigen diversity might explain the difference in the test, but also differences in parasite and antibody concentration among the study participants, again linked to age, immune status and nutritional status (191).

A Cochrane review compared rK39 ICT, rK26 ICT, rKE16 ICT, KAtex (see section 7.5.3) in urine and FAST agglutination test. The authors conclude that the rK39 is useful in well-defined cases with no previous history of VL in India, Bangladesh and Nepal. However, they consider the test unsuitable in East Africa due to low sensitivity. The other tests, they concluded, lacked both accuracy and validation (192).

Lateral flow techniques are often optimized for serum, although in the field setting, whole blood is the more common material (193). A study from Sudan found that, the rK28 test was positive for two controls (2%) for serum, while none were positive for whole blood (194). Another study from India, compared the use of the rK39 on whole blood and serum and found 40% of the cases to be blood and serum positive, while 4% were blood negative and serum positive (193).

To conclude, serology may be useful in the field for clearly defined cases of VL in India, Bangladesh and Nepal, but should be used with caution for surveillance also here. For other regions, the use of serology remains unclear. Serological tests are also limited for immunosuppressed individuals who may present with lower antibody titers (195). In addition, several serological tests for leishmaniasis have the potential for cross-reactivity with other trypanosomatids and malaria (173).

7.5.3 Antigen detection

Antigen detection has been reported to be more specific than antibody detection (196). It is believed that the levels of antigen correspond to parasite load, thus enabling the test to be used for clinical cure and relapse. Furthermore, as antibody detection is thought to be of limited value in the immunosuppressed, antigen detection could have a more prominent role in this group. One of the most commonly used assays is KAtex (Kalon Biological, UK). KAtex detects a carbohydrate antigen present in both promastigote and amastigote stages. KAtex is fast and easy to use without the need for specialized training. However, the assay procedure involves several steps that must be followed strictly to yield a reliable result. The read-out of the tests is subjective, and the reagents require refrigeration (2-8 °C).

In a study from Bangladesh with 36 patients and 40 healthy controls, KAtex was 75% sensitive and 100% specific (159), while another study from Bangladesh, including 50 patients and 50 healthy controls, found the sensitivity to be 94% and specificity to be 98% (162). A study from India found that out of 392 patients and 185 healthy controls in India that the KAtex was 87% sensitive and 99% specific (197). Furthermore, a study of 155 VL patients and 77 healthy controls in Nepal found the KAtex to perform poorly with 47.7% sensitivity and 98.7% specificity, and with a moderate reproducibility (198).

Another study performed in India compared parasitological diagnosis with DAT-FD, rK39 strip test, rK25 strip test and KAtex in 230 confirmed patients, 52 probable cases, 70 non-cases and 100 healthy endemic controls, and found the sensitivity of KAtext to be 67% (163). This was significantly lower than the DAT-FD (98.9%) and rK39 (98.9%). However, the specificity was above 90% of all the tests. On the other hand, a study in Ethiopia comparing the same tests as the study from India found KAtex to perform poorly compared to both DAT-FD and rK39 (199).

A study from a rural setting in Nepal including 142 patients compared the formol gel test (FGT), KAtex and rK39 (200). The sensitivity of FGT was 52%, for rK39 89%, and KAtex 57%. All tests had a specificity of approximately 90%. It is unclear why the sensitivity is significantly lower in Nepal than Bangladesh and India as the agent should be the same in the region, *L. (L.) donovani*.

To conclude, antigen-based tests still seems to have too unsatisfactory sensitivity and specificity to be useful in a clinical setting.

7.5.4 Detection of DNA

So far, molecular diagnostic tools have had limited to no role in the diagnosis of all the clinical forms of leishmaniasis, but are introduced as a diagnostic tool in severeal diagnostic laboratories. In resource-limited settings, the molecular approaches are limited as they, in general, require a well-equipped laboratory, highly skilled technicians and expensive equipment and chemicals.

Molecular approaches to diagnosis of leishmaniasis have been available since the 1990s (201). By polymerase chain reaction (PCR), sequences of DNA or RNA are amplified by repetitive cycles. It requires isolation of DNA or RNA beforehand. PCR-based methods are sensitive and are therefore prone to contamination if a strict uni-directional work-flow is not established. In addition to both high sensitivity and specificity, PCR based methods may also be able to quantify the parasite in the tissue.

PCR is a specific, sensitive, versatile and fast technique (202). PCR has been used on a range of materials; cell-culture, lesions, blood and sand flies. A range of different PCR-methods have been developed, but only a few are in routine use. One example is the oligochromatography-PCR (OC-PCR), where the amplification of the nucleic acid sequence is visualized on a dipstick. It is a simple technique involving little to no expert knowledge and equipment (203,204)

PCR techniques may not only be used for detection, but also for species differentiation. A range of targets and techniques have been explored. Several are in routine use, but there is no gold standard (145,163). By conventional PCR, the amplified nucleic acid fragment (amplicon) is visualized by gel-electrophoresis where differences in length are used either for detection or for species differentiation. Specificity is dependent on a range of factors, but central are the enzymes used. Taq-polymerases, first isolated from the thermophilic bacterium *Thermus aquaticus* in the 1970s, is still in wide use (205). Taq polymerase, however, lacks 3´-5´exonuclease activity, and is therefore prone to producing mistakes during amplification. Using an enzyme that has exonuclease activity may increase specificity at the cost of sensitivity (205).

PCR products may also be digested by DNA restriction enzymes, that cut a nucleotide sequence at a specific site, leading to different profiles of the digested product. Several assays using restriction enzymes have been developed for *Leishmania* discrimination. Examples of targets are ITS1 (206) and hsp70 (207). A PCR may be improved by running a second PCR on an already amplified product. In this way, both specificity and sensitivity might be improved (208). Several targets may also be amplified in a multiplex PCR. Multiplex PCR are, for example, in routine use for parasites in stool (209).

PCR products may also be used for subsequent sequencing using for example Sanger sequencing (first generation sequencing). This is a technique more commonly used for species identification. It does require expertise both in preparing the product for analysis and in analysing results, can only sequence a limited range of base pairs, and requires advanced technical facilities.

In most laboratories, conventional PCR has been replaced by real-time PCR. In real-time PCR the amplification of the target sequence is detected at each cycle (paper I and II). A further development of real-time PCR is High Resolution Melting analysis - HRM (paper II), that measures the fluorescence intensity of dissociation from

dsDNA to ssDNA. HRM analysis in distinct PCR products can differentiate *Leishmania*-spp. based on their composition, length, GC content and strand complementarity. The method can also be automated (210).

ELISA, a technique widely used in immunology to detect an antigen or an antibody may also be combined with PCR. In a PCR-ELISA, a PCR product is detected after being immobilized (211). The technique may be rapid and sensitive. It is, however, not of widespread use in diagnosis.

Loop-mediated isothermal amplification (LAMP) may be a specific and sensitive technique for diagnosing *Leishmania* in several endemic regions where diagnostic and laboratory facilities are scarce. The amplified sequence may be visualized to the eye. Although LAMP is hailed as sensitive and simple without the need for specialized equipment and reagents (212–214), there are several limitations to the technique, such as very specific requirements for the design of primers that may limit the development of an assay.

Multilocus enzyme electrophoresis (MLEE) has, despite its limitations, been used for species differentiation (215). Other molecular techniques, like multilocus sequence typing (MLST) are not discussed further in this thesis.

7.5.5 Molecular targets

There are a range of molecular targets available, and Akhoundi et al. give a comprehensive review of the most common targets (163). In general, protein coding sequences in *Leishmania* have high specificity, but lower sensitivity compared to non-protein coding targets like kDNA. This is due to the lower level of copy numbers of chromosomal versus non-chromosomal (mitochondrial, maxicircles, minicircles) sequences. An example of a commonly used protein-coding target is the heat-shock protein 70 kDa – Hsp 70 (163,207,216). It is, rather uncommonly, a multicopy gene both used for detection and species identification using a range of methods (163,216).

Akhoundi and colleagues list important factors for validation of molecular methods that must be take into account when choosing a target sequence (size, number of copies in genome, specificity within genus and species), efficacy of the methodology (precision, reproducibility), and clinical sensitivity and specificity (217). In general terms, multi-copy genes are preferred for detection, as they improve sensitivity. However, multi-copy genes may vary in the copy number and may therefore be inappropriate to use in quantitative assays. For that purpose, single-copy genes may be more appropriate.

There is no perfect diagnostic target for leishmaniasis that is perfect in molecular diagnostic assays. In line with Akhoundi and colleagues (217), and others, this thesis argues that for diagnostic purposes, a two-step approach is probably the most appropriate, where multi-copy genes are used for genus detection and a single or few-copy gene is used for species differentiation. There are potentially genes that may work for both purposes: Hsp70 has been shown to be useful for both genus detection and species differentiation with a good specificity and sensitivity, using HRM (210).

7.5.6 Other diagnostic possibilities

Other techniques, which are more commonly used in microbiological diagnosis may have a role in diagnosing leishmaniasis and species identification. One example is MALDI-TOF mass spectroscopy. MALDI-TOF is increasingly being used for bacterial identification. There is a limited amount of evidence on the applicability of this method for leishmaniasis. In a relatively recent paper, MALDI-TOF was shown to be able to discriminate *Leishmania* at a species level (218). However, this technique is far from being useful in most settings as it requires a well-established mass spectral library, requiring considerable expertise and infrastructure. The requirement of a culture means it is of little relevance in most endemic settings in addition to being time-consuming. Although mass spectroscopy today is an integrated part of many routine diagnostic laboratories, it is still far from being applicable to *Leishmania.*

In the Montenegro skin test, 0,1 mL of *Leishmania* antigen is injected in the forearm. The local induration is measured 48-72 h after, and if its > 5 mm it is considered positive. This test measures delayed-type hypersensitivity (59). However, caution has to be taken as not all CL patients yield positive results. Furthermore, species used and how it is prepared for antigen preparation varies, thus comparability is also an important issue with this test (59). In a study from Tunisia, the test remained positive in 20/22 patients post treatment (2 years follow-up) and in 75% of the individuals living in the endemic area without a history of leishmaniasis (219). This test is not included in most guidelines for diagnosing leishmaniasis (145).

8. Aims

Gaps in the literature

The leishmaniases remain, in general, diseases related to poverty and are considered as neglected tropical diseases. There are considerable gaps in our understanding of the parasite biology, as well as its relationship with hosts (insect and mammal). In addition, there is no gold-standard technique for diagnosis, classical or by molecular methods. Therefore, there is a need to explore further diagnostic methods and molecular targets that can be used in both endemic and non-endemic regions (217). To distinguish *Leishmania* at the species level is paramount for several reasons: (i) in several endemic areas several species coexist and the correct identification of them is important to apply the correct therapeutic and follow-up regimes, and (ii) epidemiological and ecological reasons. For this purpose, techniques using genetic material (DNA or RNA) are useful. To be able to explore targets, precise DNA nucleotide sequences are central, as this has been a challenge for several *Leishmania* species. This is important also for the understanding of the physiological role of a gene.

Primary aim

The aim of this thesis was to explore a new potential target, the amino acid permease (AAP3) coding sequence and flanking nucleotide sequences for parasite detection and species identification using molecular methods.

Secondary aims

- To develop an assay to detect *Leishmania* spp., and to quantify the parasite load in mice (Paper I).
- To develop a diagnostic assay for species identification where the amplified product is contained in the tube thereby lowering the risk of laboratory contamination (Paper II).
- To validate *aap3* as a target in clinical samples (Paper II and III).

- To improve the quality of available complete nucleotide sequences of *aap3* coding sequences and flanking regions, to determine the nucleotide sequence in a larger range of *Leishmania* spp. comparing the genomic organization in both subgenera of *Leishmania* pathogenic to humans (Paper IV).

9. Methods and methodological considerations

9.1 Patient recruitment (paper III)

The basis of clinical studies, and sometimes experimental studies, is patient recruitment. In this thesis, patients were recruited when either *Leishmania* infection was established (paper II) or suspected (paper III). In paper II biopsy material from patients was included, however, this was conducted at an earlier time-point and in relation to another study. In paper III, patients were included prospectively, when *Leishmania* infection was suspected at one of the participating hospitals in Norway. Ethical approval was obtained for both studies. For the study in paper III, it was planned to include approximately 50 patients. This study probably fell victim to Lasagna´s law, which states that investigators greatly overestimated the pool of patients (220). One goal of the project was to stimulate the establishment of molecular diagnosis of *Leishmania* in Norway. This was achieved when molecular diagnosis was established in Oslo (Ullevål University hospital) in 2015. Leishmaniasis is not part of the Norwegian Surveillance System for Communicable Diseases (MSIS), and hence the occurrence is not known. The early termination of the study could have been avoided if we had been more cautious when estimating the occurrence of leishmaniasis in Norway.

9.2 Parasites (paper I and II)

Leishmania promastigotes can be cultured in a range of media, from semi-solid to liquid medium. Novy-MacNeal-Nicolle (NNN) blood agar is used for diagnostic purposes in several reference laboratories. Biopsy material from splenic aspirates, bone marrow or lymph node is commonly cultured directly in this medium (221). For continuous propagation of promastigotes, a range of liquid media is commonly used (RPMI, M199, Schneider etc.). In this thesis, we worked in general with two media, M199 and RPMI1640. These two media are well-established and it is unlikely that

any of the results have been affected by the use of these media. In general, they consist of a buffer-system (for example for RPMI1640 a bicarbonate buffer) and are commonly supplemented with heat inactivated serum, hemin, glutamine and sometimes other amino-acids, in addition to antibiotics. Human urine has been shown to stimulate growth of several *Leishmania* spp. *in vitro* and may be added, although it is not clear which substance is causing the increased growth (222). In addition, several *Leishmania* spp. may be converted to amastigotes *in vitro* by increasing incubation temperature and by making the growth medium more acidic (223,224). In this thesis, we worked only with promastigote forms cultured in liquid media. For all the works, we limited the number of passages in liquid culture to a minimum, generally below five passages. Although, this is probably not essential when working with *Leishmania* for diagnostic purposes and the isolation of enough DNA is the purpose, it is indeed the case that the parasite is adaptable to long-term culture and it alters several biological properties (224). Therefore, care has to be taken when experiments are designed and results are analysed. For example, if a particular gene is to be studied for functional properties, the experimental model should strive to reflect the situation *in vivo*. By adapting the experiment too much to the convenience of the researcher, one risks removing oneself too far from the situation *in vivo* and consequently may make false conclusions.

9.3 Mice (paper I and II)

Animal models are central to the study of the leishmaniases. They are, for example, essential when studying the immune response, pathology and functional studies of genes (224–227). Mice, hamsters, domestic dogs and non-human primates have been used, especially in the study of the immunological response to the parasite. Studies using mice have been central in defining cells and molecules (e.g. cytokines) that are important in the immune response against *Leishmania* (228). However, all the models do not necessarily represent the situation in the infection in the human host. For immunological studies, all aspects of the infection, from the amount of inoculum, the saliva of the sand fly, the cells first recruited to the site of infection, the genetic

background of the host, and much more, is essential for the outcome and interpretation of the experiment (225–227). In the papers of this thesis only the mice model was used and will be further discussed. The hamster model, using the Syrian golden hamster (*Mesocricetus auratus*) is considered the best experimental model for visceral leishmaniasis. The model is, however, constrained by the lack of molecular and immunological markers available to study the gene expression and immunological response (225). Dogs are the main reservoir of zoonotic visceral leishmaniasis in Europe, the Middle-East, South-America and parts of Asia. Dogs have been used as experimental models. However they are expensive and also here the available reagents are often lacking. Primates, like the rhesus macaques (*Macaca mulatta*) could be an attractive experimental animal because of their similarity to humans. Of the available animal models, macaques are evolutionarily closest to humans (229). They have, for example, been used in vaccine testing (229). Their main draw-back is that they are expensive, difficult to obtain and handle. Furthermore ethical committees are not encouraging the use of monkeys in animal experiments (225). Mice are the most common animal used, especially the BALB/c and C57BL/6 strains. Where BALB/c mice are considered susceptible and develop a classical Th2 immune response, while C57BL/6 are considered resistant to cutaneous strains and develop a strong Th1-response, although this is dependent on the inoculation site. In general for animal studies, but absolutely central for mice studies, is consideration of the developmental stage of the parasite stage, route of infection (subcutaneous, intraperitoneal, dermal), site of infection, inoculation dose and mouse genotype, as these issues will influence the results (226).

The use of animals in experiments are an integral part of research, especially where studies cannot be undertaken on humans, for ethical reasons (drug testing, vaccine studies and alike). No model is perfect for leishmaniasis, no model can reproduce the infection in humans. Thus, care has to be taken when drawing general conclusions from such experiments. For all the animal experiments (paper I and II), ethical approval was obtained from the appropriate authorities, the animals were taken care of by trained staff, they had food and water *ad libitum*, and the minimum number of

animals, to statistically guarantee the results, were used. To prove that a diagnostic method is able to detect the target in various animals (paper I and II) it is important to test it under controlled conditions. It is arguable, though, that it might not have been necessary to test it in laboratory animals, wild infected animals would have sufficed. However, for paper I, the use of the method for quantitative purposes was essential to prove and mouse experiments were difficult to avoid. For paper II, it was important to prove that the diagnostic method could also be used in an experimental setting.

9.4 DNA (paper I-III) and RNA extraction (paper IV)

Extraction of nucleic acids from cultured cells or tissue is a central technique in any modern research and diagnostic laboratory. The extraction techniques are often overlooked as simple and just a pre-step before the real experiments. However, care has to be taken when nucleic acids are separated from proteins, cell membranes and other material as contamination has to be avoided, and one should strive for as pure a sample as possible to avoid inhibitory molecules for later applications. Furthermore, the nucleic acid extraction itself may be one of the most labour-intense parts of an experiment. In general, any nucleic acid extraction protocol consists of some basic steps: (i) lysis of the cells, (ii) purification or isolation of nucleic acid from other cell components.

The DNA extraction procedure is generally divided into organic purification, using phenol/chloroform or inorganic purification that uses, for example, silica beads to bind the DNA. In papers I and III commercial kits were used to isolate DNA, based on DNA binding to silica beads. This method is prone to contamination, as it does involve multiple tube changes. However, the method may be automated which allows processing of a large number of samples. In paper II an organic method, using a salting out technique, was applied to extract DNA from trypanosomatids. The method was based on a protocol already described by Miller et al. (230). The protocol was laborious, but relatively simple, cheap and a high quality and pure DNA was isolated.

In paper III, FTA filer paper cards was also used for DNA isolation. The cellulose-based matrix contains several reagents to stabilise nucleic acids for long-term storage.

Compared to DNA extraction, RNA extraction is inherently more difficult as there are several enzymes in cells and tissues that rapidly degrade RNA. Thus, care has to be taken to ensure high-quality RNA is isolated necessary for several downstream applications, such as reverse- transcription real time PCR and RNA sequencing. Several applications exist, generally divided into: (a) organic extraction methods, (b) filter-based extraction methods, and (c) magnetic particle methods. In paper IV, TRIzol was used for isolation, a liquid extraction technique using guanidinium thiocyanate-phenol-chloroform for extraction. TRIzol maintains RNA integrity while at the same time contributing to the digestion of cell membranes and components. The advantage of TRIzol is that it enables high purity extraction and has a high recovery. However, the technique is relatively time-consuming compared to filter- and bead-based techniques.

9.5 Polymerase chain reaction (PCR) (paper I-III) and high-resolution melting *analysis (HRM) (paper II)*

> "upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme"(231).

The quote above is from a seminal paper by Professor Kjell Kleppe from Bergen where he describes one of the first polymerase chain reactions (PCR) ever conducted. PCR is a standard method used in molecular biology to amplify a specific DNA or RNA sequence.

Conventional (or sometimes also referred to as standard or simple) PCR is relatively simple and highly sensitive. In reverse transcription PCR (RT-PCR), RNA can be used as template. RNA is reversely transcribed to complementary DNA (cDNA). RT-

PCR can be the first step in real-time PCR (sometimes also referred to as qPCR) to quantify RNA transcripts in a sample.

Conventional PCR is widely used in a research setting. It is laborious and generally needs the visualisation of the product by gel electrophoresis to estimate quantity and size. It has, therefore, in a diagnostic setting, largely been replaced by real-time PCR. Real–time PCR can be broadly divided into dye-based (paper II) and probe-based (paper I). In dye-based real-time PCR, a fluorescent label binds to dsDNA, the fluorescent signal increases proportionally to the amount of DNA and can therefore be measured in real time. Dye-based methods are relatively cheap but can only be used for one target at a time and the dye does not discriminate between target DNA or any other DNA that might be present in the reaction. Probe-based real-time PCR commonly consists of a hydrolysis probe, including a fluorophore and a quencher. When the target sequence is amplified, the fluorophore is separated from the quencher, and the fluorescence can be measured real-time. Probe-based technology is more specific and more commonly used in diagnostic assays.

Real-time PCR may be used to quantify a target, such as a parasite, in tissue. Absolute or relative quantification may be applied. Absolute quantification relies on a standard curve. This method requires information about the copy number per cell of the target sequence. Furthermore, for *Leishmania*, the copy number should not vary between the species. Kinetoplastid DNA (kDNA), a common target in *Leishmania*, may indeed vary both between and within species (232–234). Single or less copy genes can have a more prominent role for quantification but may fall short in sensitivity, as can be argued for paper I and III.

Real-time PCR is a sensitive, specific, robust and efficient technique for detecting *Leishmania*-spp. However, there has not been one sensitive method that is able to detect the parasite, quantify and also discriminate at the species level until high resolution melting analysis (HRM) was introduced. High resolution melting analysis (HRM) was first described in 2003 (235) and has been described as a method for

detection and genotyping for several infectious agents (236–239). The method relies on the accurate detection of denaturation of a DNA fragment with increasing temperatures. The melting temperature (Tm) is related to sequence length and GC-content. The limit of measuring differences in Tm is approximately 0.2°C, depending on the instrument and chemical used. There are also limits to the length of DNA fragments that can be applied to yield a robust HRM analysis (240). Besides the Tm, the shape of the melting curve is central, which is related to the DNA sequence and especially the GC-content. HRM has led to development of new DNA binding dyes that do not have an inhibitory effect on the PCR at saturating conditions. One limitation of HRM is that it does not reveal all sequence variations and base differences which, however, do not change the GC-content in a DNA-fragment and thus will not be detected. In a diagnostic setting it would require a trained technician to perform the experiments and to analyse the results, it would also require standard strains. On the other hand, the speed, cost and the potential to process large samples makes this technique attractive.

In microbiology, HRM has been used for identification of infectious agents (236–239), genotyping (210,238) and detection of resistance genes (241,242). For leishmaniasis, there are relatively few studies using HRM. With a few exceptions, most of them are using the technique to detect and differentiate a few species in a certain geographical region. The earliest report is by Nicolas et al. who used the technique to differentiate *L. (L.) major*, *L. (L.) donovani*, from *L. (L.) tropica* and *L. (L.) infantum* (243). Talmi-Frank et al. used the technique to differentiate the same species, with the addition of *L. (L.) aethiopica*, by targeting polymorphisms in the Internal Transcribed Spacer (ITS1) gene (244). Both Nicolas et al. and Talmi-Frank et al. only target species from Eurasia and Africa, thus making their approaches of limited value in the Americas and in a non-endemic setting where infections from all endemic regions can be expected. Studies by Pita-Pereira et al. (245) and Ceccarelli et al. (246) were both able to differentiate some species from the subgenus *L. (Leishmania)* from the subgenus *L. (Viannia),* thus making their targets more attractive for the Americas. Zampieri et al. (210), targeting several polymorphic sites

on the *hsp70* gene, was able to differentiate several species from both subgenera, making it a very attractive target. As we had before shown that the *aap3* coding sequence could be an attractive target for detection of *Leishmania* (paper I), we also wanted to investigate if the target could be used for species identification (paper III).

qPCR may have a role, as proposed in paper I, in monitoring treatment response. An argument against this is that parasite DNA is potentially detectable a long time after the parasite is killed, mainly if the DNA target used is stable. However, Pessoa-e-Silva and colleagues (247) have demonstrated that parasite DNA was only detectable in urine before treatment for VL with *L. (L.) infantum*, not after. Although recent guidelines for diagnosis, treatment and follow-up do not recommend testing the patients for parasites after treatment (145), qPCR may be important in vaccine development and research settings. Host DNA can affect the reliability of qPCR results. The quantitative part of a real-time assay should therefore always be tested with host DNA (paper II).

9.6   Nucleic acid sequencing (paper IV)

Nucleic acid sequencing can be performed in a range of ways, all with their inherent benefits and drawbacks. The technology can be used for sequencing small parts of a genome, chromosomes, or entire genomes. By DNA sequencing, the order of the four bases; thymine (T), adenine (A), cytosin (C) and guanine (G), is determined. The technology of DNA sequencing has developed greatly from the initial steps in the 1970s (248,249). Sequencing technologies are, by convention, divided into generations. In first generation sequencing technology, such as Sanger sequencing, dideoxynucleotides (dNTPs), corresponding to each of the DNA bases, are separated according to their size and visualised by an imaging system. This technology is still widely used but has limitations in output and cost. Second generation technologies improved output exponentially and reduced cost. Several second-generation technologies exist, where Illumina (originally Solexa) is the most common. Illumina uses sequencing by synthesis technology (SBS). The technology works by adding a

single base to a nucleotide strand by a polymerase enzyme. It uses a modified dNTP which contains a fluorescent terminator blocking further polymerisation. After the addition of the dNTPs, an image of the fluorescent label is taken, the terminators removed, and the process repeated. The process is conducted on a million templates simultaneously. The second-generation technology comes with their inherent drawbacks of short reads and problems of underrepresentation of GC-rich regions. On the other hand, the technology can be used to sequence multiple strands simultaneously, thereby facilitating high output.

Third generation technology, such as single molecule real time sequencing (SMRT), is relatively cheap, fast and does, in general, not require PCR amplification. In SMRT, by fluorescent labelling, the nucleotide is detected in real time. This technology can provide very long reads. The drawback is a higher error rate however, the errors are randomly spread. Another third-generation technology is the Oxford Nanopore, where the MinION device is small and can be connected to a USB-port of a computer. In this technology, the DNA is passed through a protein pore, the ionic current is then detected and translated to a nucleotide sequence. The error rate is relatively high. This type of sequencing is relatively low cost, data are available in real time, and it can provide very long reads.

In paper IV we used third generation sequencing technology through SMRT sequencing. This technology produces relatively long read lengths (>20 kb) but, the error rate is slightly higher with SMRT compared to other techniques, but as the errors are randomly spread, the sequences remain highly accurate as the same molecule are sequenced multiple times. SMRT sequencing has also been used in whole-genome sequencing of *Leishmania* (250,251). In this work, we show that SMRT can be a very useful technology also for studying target sequence fragments in multiple strains of *Leishmania* spp. with a high accuracy, generating data that could be important for the understanding of gene expression regulation as well as for diagnostic purposes.

In paper IV RNA sequencing was used to establish where the untranslated regions (UTRs) were in relation to the coding sequences of the two copies of *aap3*. This was done using next-generation sequencing technology (Illumina). RNA sequencing relies on some initial steps to prepare cDNA before sequencing. RNA has to be isolated, a crucial and difficult step, then different types of RNA can be selected for further analysis (such as mRNA only), and then cDNA is synthesised. The resulting transcriptomic analysis often requires specialist knowledge. RNA sequencing can be used for a range of applications, such as differential expression (DE) studies, variant discovery, fusion gene detection and more. The challenge to RNA seq is potentially the technology itself, as it does require expertise to prepare the samples and bioinformatical expertise to analyse the result.

9.7   Ethics

For the study in paper II, ethical approval was obtained from the Committee of Ethics of Irmandade de Santa Casa de Misericórdia de São Paulo. For paper III, ethical approval was obtained from the Regional Committee for Medical and Health Research Ethics.

The animal study in paper I was approved by the Laboratory animal facility at the University of Bergen and conducted according to local guidelines and procedures. Ethical approval for the experimental infection of mice in paper II was obtained from Instituto de Biociências at the University of São Paulo. The animals in paper I and II received food and water *ad libitum*.

9.8   Statistics (paper I–II)

In general, for this thesis, all experiments were performed at least three times and all samples were tested in duplicate or more. In paper II, statistical melting temperatures were analysed using a one-way ANOVA. Results were presented with 95%

confidence intervals and statistical significance set to $p < 0.05$. Analysis was performed in Excel and GraphPad Prism version 7.

10. Summary of papers

10.1 Paper I

For *Leishmania*, several publications exist on different molecular targets and methods for identification and quantification with molecular methods (217). The aim of this study was to develop an assay to detect *Leishmania* and to quantify the parasite in mice. Therefore, a primer targeting a 74 bp region of the *aap3* coding sequence in *L. (L.) major* was coupled with a hydrolysis probe (TaqMan). The assay was able to detect 9 different *Leishmania* species; six from subgenus *L. (Leishmania)* and three from subgenus *L. (Viannia)*. The assay did not react with other trypanosomatids tested (*T. cruzi* and *T. brucei*), nor human or mice DNA. The assay had a limit of detection ranging from 10 fg DNA for *L. (L.) major* to 100 fg DNA for *L. (L.) braziliensis*. When coupled with an assay targeting the transferrin receptor (*tfrc*) in mice, it was also able to quantify parasites in mice. The limit of detection (LOD) of the assay was equal to other assays. The assay showed little intra-species variation in terms of Cq-values, but some inter-species variation for *L. (V.) braziliensis* and *L. (V.) guyanensis*. Coupled with *tfrc*, the assay was able to quantify *Leishmania*-DNA in mouse tissue independent of the amount of DNA harvested. Thus, this is one of the first reports of a real-time quantitative PCR assay with a low copy-number gene able to detect parasites with relatively good sensitivity, specificity, and replicability. Being an only two-copy gene, it is very useful for quantitative purposes as it is not prone to mis-quantification as multicopy genes are.

10.2 Paper II

This project was largely based in Brazil. The background were the promising findings in paper I, and the aim was to develop a diagnostic assay for species identification. In addition, to avoid over-complicated laboratory procedures, and the potential of

laboratory contamination, we wanted to develop a closed-tube molecular diagnostic method that could discriminate *Leishmania* species.

Real-time PCR coupled with HRM is a relatively new technique that could offer the possibility that this project aimed for. Using three amplicons we managed to distinguish eleven *Leishmania* species, six from *L. (Leishmania)* subgenus and five from *L. (Viannia)* subgenus. The sensitivity ranged from 1 parasite per reaction (amplicon 1 and 2), to 5 parasites per reaction (amplicon 3). The protocol was validated with samples from humans, cats, sand flies and experimentally infected mice.

The method, using advanced real-time PCR technology coupled to HRM, showed that *aap3* could be a valuable target for *Leishmania* species discrimination and therefore also has potential as a diagnostic target. Furthermore, the methodology is relatively cheap, fast and robust, and offers the possibility for automation. It was able to detect *Leishmania* in clinical, field and experimental samples, indicating its usefulness in an ecological and epidemiological setting. The method was, however, validated on relatively few standard strains and few clinical, field and experimental samples, and would need further testing to reliably determine its usefulness.

10.3 Paper III

This project was initiated by the National Advisory Unit on Tropical Infectious Diseases at Haukeland University Hospital in Bergen. The aim of the study was two-fold: (i) to gather information on occurrence, diagnosis, treatment and outcome of leishmaniasis at five university hospitals in Norway, and of particular relevance for this thesis (ii) to evaluate molecular diagnosis by comparing *aap3* with *SSU rDNA* as a target and test the usability of various sample material. Patients were prospectively recruited from all health regions in Norway (Bergen, Oslo, Stavanger, Trondheim and Tromsø) from March 2014-September 2017.

Thirteen patients were included, of which 2 had VL and 11 CL. Both VL patients presented with classical symptoms and findings (fever, pancytopenia, splenomegaly, hypergammaglobulinemia and hypoalbuminemia). One patient was initially diagnosed with serology only, and both received treatment with liposomal amphotericin B. Eleven patients were diagnosed with CL, 10 with molecular methods and one was diagnosed clinically. Seven received treatment with liposomal amphotericin B, of which one did not respond, but responded well on subsequent treatment with sodium stibogluconate. Three patients with CL healed without medical treatment. SSU rDNA and *aap3* was tested with PCR, samples from 11 patients were included. Using SSU rDNA as target, all patients were identified as positive, while only 7 were identified as positive with *aap3*. The material was not sufficient for a meaningful statistical analysis and comparison, but clearly indicated that diagnostic target is essential, and that SSU rDNA is more reliable than *aap3* when conventional PCR was used. Furthermore, punch biopsy is the best sample for diagnosis with molecular techniques. This study shows that *Leishmania* is a rare disease in Norway, but important to know about, since VL may be lethal. In Norway, PCR and sequencing is recommended for species identification, to ensure the right treatment and follow-up regime.

10.4   Paper IV

This project was based in Brazil and Norway. The background was the promising findings in paper I and II into the diagnostic potential of *aap3* as a target. During the work with optimisation of oligonucleotides we quickly realised that although there were relatively good sequences for some species, there were ambiguities in others. To explore *aap3* further, we needed better quality sequences. After carefully exploring different technologies, it became clear that third generation sequencing technology was the most appropriate, considering speed, cost and accuracy.

The aim of the study was to improve the quality of the available nucleotide sequences of *aap3* coding and flanking regions in both subgenera pathogenic to humans. We

also wanted to investigate the presence of both copies of the gene in all species, explore the similarity in the coding and flanking regions, describe sequences that could be regulatory in transcription and/or translation, and explore whether there was any marked difference at the protein level we could predict.

We managed to sequence long amplicons including both copies of *aap3* in both subgenera including nine species in subgenus *L. (Leishmania)* and six species in *L. (Viannia)*. We confirmed that *aap3* is coded for by two coding sequences (CDS) and that they are organised *in tandem*. We found a high similarity between the copies in a strain, but also a high similarity between species, but a slightly lower when comparing the copies between species at the subgenus level. Using transcriptomic data from *L. (L.) major* Friedlin we analysed the flanking regions, being able to reliably predict the untranslated regions (UTRs) for subgenus *L. (Leishmania)*. For this subgenus we found relatively long 3´UTRs compared to 5´UTRs. We could also find large similarities in the UTRs comparing at the strains and species level, but there was a marked reduction of similarity between *L. (L.) major* and *L. (L.) mexicana* on the one hand, and *L. (L.) donovani* and *L. (L.) tropica* on the other. At the protein level, all copies of AAP3 presented with 11 predicted transmembrane domains, while subgenus *L. (Viannia)* presented with CDSs with three nucleotides less, leading to a predicted shorter protein. We did not find any differences in protein conformation or transmembrane domains.

Our findings indicate that the general organisation of the CDS is conserved in the *Leishmania* genus despite having undergone evolutionary processes leading to differences in the flanking regions. Altogether, we managed to sequence and thereby improve the quality of available nucleotide sequences for *aap3*. This could be important for further studies into gene and protein function and regulation.

11. Results and discussion

11.1 Paper I

The real-time PCR assay, in this paper, was designed on the *L. (L.) major* gene encoding AAP3. To investigate if the assay could be used for quantitative purposes in an animal model, it was also coupled to a coding sequence from the transferrin receptor gene (*tfrc*) in mice. To ensure the assay would be useful for the identification of other *Leishmania* species, the test was applied on nine different species, and several strains of the species. The assay controls were: DNA from *T. cruzi*, *T. brucei*, human and mice. The controls were all negative in the amplification with *aap3* primers, the *tfrc* was positive only for mice DNA. Furthermore, *in silico* analysis revealed no unspecific binding to bacterial genomic sequences. Three strains were chosen for sensitivity analysis, representing each clinical form: *L. (V.) braziliensis* (MCL), *L. (L.) donovani* (VL) and *L. (L.) major* (CL). The limit of detection ranged from ≥ 10 fg for *L. (L.) donovani* and *L. (L.) major*) and ≥ 100 fg for *L. (V.) braziliensis*. While the limit of quantification ranged from ≥ 10 fg for *L. (L.) donovani*) to ≥ 1 pg for *L. (V.) braziliensis*. The assay presented a good reproducibility. Furthermore, one aim of this project was to find a method for quantification of parasites in mice. To enable this, the *aap3* assay detecting *Leishmania* DNA was run in duplex with a *tfrc*-assay detection mice DNA. The assay was able to quantify *Leishmania* DNA present in mouse tissue, independently of the DNA loaded.

Traditionally, *Leishmania* parasites have, and are to a great extent still, been quantified using indirect techniques, such as the measurement of the swelling of the footpad of mice and limiting-dilution assay. Although these forms of measurements do have some value, they also have severe limitations (227). Footpad swelling in mice measures the extent of tissue edema in the foot, which in turn is related to inflammation. It is common to measure the footpad swelling as a correlate to the

amount of infection with parasites. This is problematic as (a) parasites may be killed long before the reduction in footpad size, (b) swelling in foot-pads has been reported without an increase in parasite numbers, (c) low numbers of parasites can be found in resolved lesions (227,252,253). Limiting-dilution assay may be used for all types of infected tissue. It is, however, a laborious and time-consuming technique where the infected tissue is incubated in culture medium over several days, where the amastigotes differentiate to promastigotes and then, the replication of this form can be followed in the limiting dilution. The incubation time increases the risk of contamination (254). Real-time PCR offers the advantage of great sensitivity, specificity, capacity to analyse many samples at once, and reproducibility. As long as DNA can be isolated, the technique can be applied. One immediate drawback is also its strength. It measures the presence of nucleic acids, not live parasites. That is one reason why some researchers have been hesitant in introducing the technique. Some have argued that when comparing limiting dilution assay with real-time PCR, that the lack of correlation between the techniques could be because limiting dilution assay measures only live parasites (255). For sure, the detection of DNA depends on the origin of the target molecule, since chromosomal DNA is more sensitive to DNase than the circular form of kDNA.

A real-time PCR assay for quantifying *Leishmania spp*. has to take into account the copy number of the target gene. Many real-time PCR assays for quantification of *Leishmania* use multicopy genes which increases sensitivity greatly. But as the copy number among different *Leishmania* strains varies, and sometimes, the variation also occurs during the life cycle of the same strain, they are often unreliable targets for quantification (25,254–257).

11.2 Paper II

In this study, we wanted to investigate if *aap3* could have potential as a diagnostic target and, more specifically, be able to identify *Leishmania* species. We designed three primer pairs for several amplicons, where three primer pairs were found to

generate good amplicons for an HRM assay. The *in silico* study indicated that the primer pairs used did not produce an unspecific amplification product. Theoretical melting temperatures were calculated *in silico* based on available sequences in GenBank and TriTryp Database. The amplicons were then tested for specificity in a conventional PCR. All amplicons were specific without any primer dimers formation that could potentially inhibit the performance of the assays. We also included, as biological controls, DNA from *T. brucei*, *T. cruzi*, *Crithidia fasciculata, Endotrypanum schaudinni*, rat, mice and human. Only *C. fasciculata* DNA was positive for amplicon 3, producing a fragment of approximately the same size of *Leishmania* DNA, in the conventional PCR. After the conventional PCR, the amplicons were tested in a real-time PCR followed by HRM. Although the essential parameter for the assay is the melting temperature (Tm), Cq was also considered. The limit of the equipment was 0.2 °C, but we considered a Tm value exceeding ±0.25 °C as discriminatory between the species.

By using amplicon 1, the assay was able to distinguish the species into three clusters: (i) visceral - *L. (L.) donovani* and *L. (L.) infantum*, (ii) cutaneous from Eurasia and Africa - *L. (Leishmania)* strains, and (iii) cutaneous and mucocutaneous strains from South America. Within this last cluster, the assay was able to differentiate *L. (L.) amazonensis, L. (L.) mexicana* from *L. (Viannia)* strains, and the last cluster *L. (V.) guyanensis*, *L. (V.) naiffi* and *L. (V.) shawi*.

Amplicon 2 was designed specifically to differentiate between the *L. (Leishmania)* subgenus. By using amplicon 2, the assay was able to differentiate *L. (L.) donovani* from *L. (L.) infantum* and *L. (L.) major* from *L. (L.) tropica*. It was, however, not able to differentiate *L. (L.) amazonensis* from *L. (L.) mexicana*, a common problem as the species are phylogenetically close. Therefore, amplicon 3 was designed. By using amplicon 3, the assay was able to amplify *L. (Viannia)* subgenus, in addition to being able to differentiate *L. (L.) amazonensis* from *L. (L.) mexicana*.

Although we did not propose the use of Cq-values for diagnostic reasons, the parameter is important to assess the specificity and efficiency of a real-time PCR assay. Using 25 ng as the standard DNA amount in our assay, amplicon 1 showed roughly similar Cq-values for all species. For the controls, we found unspecific amplification of human DNA at Cq >30, a different Cq and Tm profile than the ones obtained for *Leishmania* spp. Amplicon 2, designed for *L. (Leishmania)* subgenus proved to be the most effective for *L. (L.) donovani, L. (L.) infantum* and *L. (L.) tropica*, while the Cq value for *L. (L.) major* was around 30 for *L. (L.) amazonensis* and around 26 for *L. (L.) mexicana*. This finding was also clearly visualised in the conventional PCR, where there was no band detectable for *L. (L.) major*. For amplicon 3, the Cq values were similar for all species, indicating a similar specificity and efficiency of the assay for all species.

The inter-species variability of Tm was also investigated for those species where additional strains were available. For amplicon 1, the variability was little except for *L. (L.) major* (average Tm 82.20 °C, SD 0.24) and *L. (V.) naiffi* (average Tm 83.23°C, SD 0.26). For amplicon 2, the variability was significant for *L. (L.) donovani* (average Tm 85.23°C, SD 0.3), while for amplicon 3, the profiles were consistent for all strains.

Sensitivity was estimated for all amplicons by diluting DNA from standard strains. For amplicon 1 and 2, the LOD was 100 fg for all species, except *L. (L.) mexicana* (LOD 250 fg for amplicon 1, and 50 pg for amplicon 2). The reason for this discrepancy is discussed later. For amplicon 3, the LOD was higher, 500 fg, except for *L. (V.) braziliensis*, which has a LOD of approximately 50 pg. The efficiency for all amplicons was investigated using 25 ng, 5 ng and 50 pg of DNA from selected species. The efficiency calculations for all amplicons showed Cq-values between 20 and 30, slopes between -3.58 and -3.1 (except *L. (L.) donovani* (-4.255) and *L. (L.) mexicana* (-3.975) in amplicon 3) and R^2-values >0.99 except for *L. (L.) donovani* in amplicon 1 ($R^2 = 0.985$).

To finalise the analysis of the assays, we validated the assays on biological samples. Samples from humans, cats, mice and sand flies were tested. The results showed a good correlation with earlier diagnostic tests that had been performed on the same samples. As expected, using amplicon 2, the assay did not detect samples from *L. (Viannia)* subgenus. Using amplicon 2 or 3, the assay was not able to detect *L. (L.) amazonensis* in the human sample.

Molecular methods are increasingly being used for the detection and identification of *Leishmania* spp. (258,259). In Europe, PCR followed by sequencing is increasingly being used, although it is relatively laborious, time-consuming, and requires expert knowledge and product manipulation, thereby increasing the risk of laboratory contamination (259). Therefore, we wanted to test if the PCR coupled HRM assay would be a technique where *aap3* could be used as a target for species discrimination. The HRM methodology is attractive as the whole process is performed in a closed tube system, avoiding contamination. Apart from the work by Zampieri et al. (210) that targets hsp70, all HRM studies published so far target a limited number of species, or species from one or few endemic areas (243,244). Assays that target only a limited number of species may be of value in a study setting or for research purposes, but they are of limited value in a diagnostic setting. With increasing migration and travel to and from several endemic areas, the ability of an assay to target species outside the endemic region is important.

The *aap3*-assay, by using 3 amplicons, was able to differentiate all *L. (Leishmania)* subgenus tested, and several of the species from *L. (Viannia)* subgenus. Any medical diagnosis does not rely solely on laboratory diagnosis alone. Diagnosing leishmaniasis relies on a rigorous patient and travel history, clinical symptoms and findings, and results from laboratory tests. Thus, the inability of amplicon 1 to reliably distinguish *L. (L.) donovani* from *L. (L.) infantum*, although compensated by both amplicon 2 and 3, is of limited importance. A patient diagnosed with VL would receive the same treatment and follow-up regime regardless (258). However, to

reliably distinguish the species may be central in a public health perspective, epidemiology and research setting.

Although *Crithidia fasciculata* was amplified in the conventional PCR producing a fragment of the same size as for *Leishmania* spp., *Crithidia* is a non-pathogenic parasite for humans that only parasitizes insects and is regarded as a model organism in trypanosomatid research (260). This way, for a diagnostic assay for human, unspecific amplification of *Crithidia* is less of a problem. It could, however, be a problem for an assay in an ecological setting. However, the HRM assay for amplicon 3 showed that the Cq and Tm values for *C. fasciculata* DNA were distinct from the ones observed for *Leishmania* spp.. In addition, as the assay includes 3 amplicons, where the other two amplicons do not amplify *Crithidia*, the issue is not a problem at all.

The sensitivity of a diagnostic assay is key to evaluating its usefulness. For *Leishmania*, multi-copy genes are known to be sensitive targets (217), while single-copy genes are commonly used for species differentiation. Ideally, a diagnostic test for *Leishmania* should be able to detect the parasite in different material (skin, spleen, bone-marrow, blood). This is then also one of the attractions of a molecular approach to diagnosis. As long as *Leishmania* DNA can be isolated, it has also the potential to be detected. There are several variables affecting the sensitivity of a molecular method, such as the quality of the target, quality of the DNA, and the modality used. Conventional PCR commonly uses agarose gel electrophoresis to visualise an amplified product. It is thought of as a fairly easy technique, but the resolution is comparatively poor, it lacks precision, and has considerable limitations for quantitative purposes. Real-time PCR does not measure the end-point amplification, but the amplification of a nucleotide sequence in real-time in the exponential phase of an amplification. The fluorescent signal is proportional to the number of amplicons generated. Real-time PCR does not require any post-PCR processing and has a greater sensitivity than conventional PCR. For example, Talmi-Frank et al., Hernández et al. and Zampieri et al. all report LODs of less than one parasite for their

assays (210,244,261). We determined a LOD of 100 fg (approximately 1 parasite) for amplicon 1 and 2, and 500 fg for amplicon 3. The assay was able to detect *Leishmania* in all samples tested. In the publication of this work, we suggest that the sensitivity could be improved with a pre-amplification step (262). Although this could be a solution, and indeed would be likely to improve the sensitivity, a pre-amplification step would involve removing one of the main attractions of the method, i.e. the closed-tube system, thereby increasing the possibility of contamination. The likelihood of contamination could, of course, be greatly reduced by strict adherence to good laboratory practices (such as unidirectional workflow), but the risk would remain.

We found it very encouraging that the assay, although tested on a limited number of biological samples, did indeed detect *Leishmania* and correctly identified the species. The assay itself includes three amplicons, and thereby species might be confirmed in all amplicons being controls in themselves. We did not have samples giving ambiguous answers. If so, this would have to be reported as undetermined.

For the potential of the assay and the target to be shown reliably, it would have to be tested on more samples, on samples using different DNA extraction techniques, using different PCR machines and, preferably, also tested by independent laboratories. We did not test the assay using different reagents and equipment. Thus, this is very likely to affect the result. However, the standardisation of the test in different labs, using DNA obtained from standard reference *Leishmania* DNAs can solve this problem.

11.3 Paper III

This was a clinical study of patients with leishmaniasis in Norway where *aap3* was also tested as a diagnostic marker. For this thesis, the latter results are of particular relevance and the results from the clinical part of the study are only briefly presented and discussed.

Twenty-one patients were included from the participating university hospitals; 13 patients were diagnosed with leishmaniasis. Of the 13, two had VL and 11 had CL. The patients had been infected in South America, Europe and the Middle East – as can be expected in a non-endemic country. The VL patients were diagnosed with serology and PCR on peripheral blood and spleen tissue. For one patient, the strain was also determined by sequencing. Ten of the CL patients were diagnosed by PCR, while one was diagnosed clinically. Both VL patients were treated with liposomal amphotericin B and recovered fully. Seven of the 11 CL patients were treated successfully with liposomal amphotericin B, one patient did not respond to liposomal amphotericin B, but responded well to sodium stibogluconate. Three patients healed spontaneously without medical treatment. *Aap3* as a diagnostic target was tested and compared with SSU rDNA, using conventional PCR. Depending on the clinical manifestation, samples from diverse sites were collected from the patients. One of the patients with VL had blood and spleen samples collected, all the CL patients had punch biopsies taken, and from several patients, filter paper had been pressed into the wound for DNA conservation. Of the 11 known *Leishmania* positive patients from other diagnostic tests, ten were detected by either *aap3* and/or SSU rDNA. All were detected by SSU rDNA, while only seven of ten were detected by *aap3*. For one CL patient, only the filter paper was positive, while for another the biopsy was positive. None of the negative patients from earlier diagnosis or negative controls came out positive in either *SSU rDNA* or the *aap3* assay.

Leishmaniasis, and VL in particular, is a severe parasitic disease that should be suspected in travellers with fever from endemic areas. Delayed diagnosis and treatment can be life-threatening. One of the VL patients was only diagnosed with serology, while the other patient was diagnosed with microscopy, serology and real-time PCR and sequencing. The aim should be to diagnose using several different modalities. This is especially the case for VL where false negative results or delayed diagnosis may have fatal consequences. All patients with CL were diagnosed with real-time PCR and subsequent sequencing for species determination, except one patient that was diagnosed clinically. Modality and molecular target differed between

the samples. Determining the species is of value in CL, especially when the patient has a travel history from an area endemic for MCL. All punch biopsies were positive except for one when comparing *aap3* and *SSU rDNA*. Punch biopsies are usually taken from the border of an ulcer and will involve several layers of the skin. In general, parasites are scarce in an ulcer so involving several layers will increase the likelihood of including a parasite in the specimen. However, punch biopsies are invasive. Membranes that can store nucleic acids, such as the FTA-technology (Whatman), can be extremely useful. Nucleic acids, from for example pathogens like *Leishmania*, can be isolated and stored on the membrane without requiring specific expertise, toxic chemicals or special equipment. For *Leishmania*, it seems that using the FTA-technology on cutaneous lesions can be useful in ecological settings, but lacks the required sensitivity for diagnostic purposes (169) In paper III, however, filter papers were negative in two out of five samples, most likely because of few parasites in the ulcer. This shows that not only the target, but also the sampling technique, is central when *Leishmania* is diagnosed.

We wanted to investigate if *aap3* could be used in a diagnostic setting for humans and if its sensitivity was comparable to commonly used targets, such as SSU rDNA. The results in this study were limited and not sufficient to make any meaningful statistical analysis. However, there was a clear trend that SSU rDNA was a more sensitive target. Several of the samples were negative when using *aap3*, a result that could have detrimental results for the patient. This indicates that *aap3* may be a more suitable target for species differentiation than for genus detection (see paper II).

This was a prospective study where medical doctors at five Norwegian university hospitals sent patient data, clinical, biochemical and diagnostic information, and samples from each patient were sent to the coordination centre in Bergen. When planning the study, we had very limited information about the occurrence of leishmaniasis in Norway. Therefore, the prospect of including 50 patients was somewhat arbitrarily set. In hindsight, this was too high. Furthermore, the study protocol did not include any way of determining if we had included all patients

diagnosed with leishmaniasis at the respective hospitals. We determine it as likely that all patients were included, but resources could have been spent on regular follow-up visits to the participating study centres. Furthermore, in Norway, all patients and their diagnosis are registered in the Norwegian Patient Registry. The reliability of the study could have been improved by cross-checking if all patients diagnosed with leishmaniasis in the study period were included. This should have been included in the initial study protocol.

11.4 Paper IV

In this study, we wanted to improve the quality of the available sequences of *aap3* in several *Leishmania* species from both subgenera pathogenic to humans. Furthermore, we wanted to determine the similarity of the copies of the coding sequences, and their flanking regions, within a strain, between species and between subgenera. In addition, we wanted to explore whether there were any marked differences that we could predict at the protein level.

DNA was extracted from nine species belonging to subgenus *L. (Leishmania)* and six belonging to subgenus *L. (Viannia)*. We extracted DNA from all species and RNA from one species, *L. (L.) major*. Oligonucleotides were designed to amplify a long fragment including the CDSs and flanking regions of both subgenera. However, because of differences in the nucleotide sequences, it was necessary to design one set of oligonucleotides per subgenera.

The average sequence length was 10 799 bp for *L. (Leishmania)* spp. and 11 083 for *L. (Viannia)* spp. In all species we sequenced, we identified two CDSs organised *in tandem* in the genome. Both of the copies in *L. (Leishmania)* spp. were 1443 nucleotides long, coding for a predicted 480 amino acid protein, while the copies in *L. (Viannia)* spp. were 1440 long, coding for a 479 amino acid protein. Comparing to the *L. (Leishmania)* species, there was no clear pattern in which amino acid was deleted.

Comparing the similarity of the CDSs (named *aap3-1* and *aap3-2*), we found the average similarity for *aap3-1* for *L. (Leishmania)* to be 97.6% and for *L. (Viannia)* to be 99%. Comparing the subgenera to each other, there was a marked reduction in similarity to 83.5%. The same pattern was found for *aap3-2* as similarity for *L. (Leishmania)* was 97.6% and for *L. (Viannia)* at 99.4%. Comparing *aap3-1* to *aap3-2*, there was a high similarity within the subgenus, at average 97.7% for *L. (Leishmania)* and 98.7% for *L. (Viannia)*. Comparing the subgenera to one another, the similarity was reduced to 83.4%. Codon usage was, in general, identical for both CDSs, with some peculiar exceptions, where the most notable was the consistent use of TAA as stop-codon for *L. (Viannia)* spp., while *L. (Leishmania)* used TGA or TAG, depending on the CDS. Codons ending with A and T seemed to be avoided.

By using transcriptomic data for *L. (L.) major* Friedlin, the untranslated regions (UTRs) for both *aap3-1* and *aap3–2* CDSs were mapped. For species in *L. (Viannia)* it was not possible to map the UTRs as the alignment of these regions were weakly similar. We found 5´UTR of *aap3–2* to be 859 nucleotides, while the size of *aap3-1* was 296 nucleotides. The size of 3´UTR of *aap3–2* was 2017 nucleotides, while for *aap3-1* it was 4001 nucleotides, of which our sequences only covered 1486 nucleotides. The similarity was also compared and was relatively high for all UTRs when comparing within species 98.4% for 5´UTR *aap3-2* and 99.7% for *aap3-1,* and for 3´UTR *aap3-2* it was 98.3% and for *aap3–1* 99.1%. However, the similarity was greatly reduced when comparing *L. (L.) major* and *L. (L.) mexicana* on the one hand, to *L. (L.) tropica* and *L. (L.) donovani* on the other, with average 74.2% similarity for 5´UTR *aap3-2*, 89.3% for 5´UTR *aap3-1*, and 80.7% for 3´UTR *aap3-2* and 83.7% for *aap3-1.* In all species, the gene products from both *aap3-1* CDS and *aap3-2* CDS presented with 11 trans-membrane domains and had similar structure, according to *in silico* analyses.

Amino acid permease (AAP3) was first described in *L. (L.) donovani* (86,87) and later in *L. (L.) amazonensis* (90). Both works found two copies coding for the amino

acid permease, organised *in tandem*, and found that there was a relatively high similarity of the CDSs. We confirmed these findings and increased the analysis, being the first to describe *aap3* in several species of subgenus *L. (Viannia)*. We found that in *L. (Viannia)* a deletion has led to those species lacking an amino acid in both CDSs, but how this affects the protein needs further studies. Furthermore, why the gene exists in several copies can only be speculated on, but is likely to be related to rapid expression, ability to resist drugs and antigen variation.

Gene expression in *Leishmania* is regulated at the post-transcriptional level, where the 3´UTR play a significant role. This can, for example, be seen in the relatively long 3´UTR domains we found for *aap3* in subgenus *L. (Leishmania)*[6]. As for the CDSs, there was a relatively high similarity within species, while there was a marked loss of similarity between them. It is therefore likely, as has been repeatedly suggested, that gene expression in *Leishmania* spp. is regulated in the 3´UTRs. We also found the presence of a region in the 3´UTR, that has been described as an element regulating mRNA translation in response to intracellular stress (263). Furthermore, studies from our group have earlier found that transcripts of *aap3-1* in *L. (L.) amazonensis* are more abundant than *aap3-2*, indicating that AAP3 regulation can occur at two levels: RNA-stability and translational control.

The study could have been improved through ensuring that all regions, especially including all of 3´UTR of *aap3-1,* were sequenced. This could have been done by dividing the sequencing into two overlapping regions. Furthermore, the sequences described can now be used in the construction of mutants, by deletion or overexpression, of all sequence components, enabling us to explore their physiological role and the description of a possible chemotherapeutic target.

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[6] Recall we did not have enough data to support an analysis of subgenus *L. (Viannia)*.

12. Conclusions

Paper I –To develop an assay to detect **Leishmania spp.** *and to quantify the parasite load in mice*

- The real-time PCR assay targeting the *aap3* coding sequence was able to detect *Leishmania* species from all endemic regions and from both *L. (Leishmania)* and *L. (Viannia)* subgenera.

- The assay had a high sensitivity of 10 fg DNA for *L. (L.) major* and *L. (L.) donovani*, and 100 fg DNA for *L. (V.) braziliensis*.

- The duplex running with a host-gene assay, such as *tfrc* for mice, can be used for quantitative purposes. It may therefore be a valuable tool in monitoring the effects of drugs, testing vaccine candidates and for diagnosis.

Paper II – **T***o develop a diagnostic assay for species identification where the amplified product is contained in the tube and thereby lowering the risk of laboratory contamination. And to validate* **aap3** *in clinical samples.*

- The *aap3* coding sequence could be used to distinguish *Leishmania* at the species level.

- The target and method were able to detect *Leishmania* in clinical, field and experimental samples.

- The assay may be useful for diagnostic purposes, as well as ecological and epidemiological studies.

Paper III – Validate aap3 as a target in clinical samples.

- Leishmaniasis is a rare imported infectious disease in Norway. The patients with VL included in the study were diagnosed late and were infected in countries where clinicians rarely suspect *Leishmania*.

- VL and MCL were diagnosed through PCR and effectively treated with liposomal amphotericin B, according to guidelines. Although not according to guidelines, those CL patients given medical treatment received systemic treatment with liposomal amphotericin B, and one patient received subcutaneous injections with sodium stibogluconate.

- *SSU rDNA* detected all positive samples (11/11), while *aap3* only detected 7/11, indicating that *SSU rDNA* is the best target for genus detection. Punch biopsy was the best material for genus detection.

Paper IV – To improve the quality of available complete nucleotide sequences of aap3 coding sequences and flanking regions, to determine the nucleotide sequence in a larger range of* Leishmania *species comparing the genomic organisation in both subgenera of* Leishmania *pathogenic to humans

- In all species studied, nine from subgenus *L. (Leishmania)* and six from subgenus *L. (Viannia)*, *aap3* was organised *in tandem* and the two coding sequences (CDS) presented a high average similarity at species and strain levels, but lower similarity at the subgenus level. The size of the CDSs differed on the subgenus level, where copies in *L. (Leishmania)* presented the same size (1443 bp), while *L. (Viannia)* presented copies with a three nucleotide smaller size (1440 bp). Codon usage was in general conserved.

- For *L. (Leishmania)* the 3´ UTRs were relatively long for both copies, and the similarity in the UTRs was relatively high at the strain and species levels.

- Long amplicon sequencing using single molecule real-time sequencing can be a useful technology for target sequencing of multiple strains in *Leishmania* with high accuracy.

- The general organisation of *aap3* is conserved in *Leishmania* genus, with some marked differences at the subgenus level and also in the flanking regions, which could indicate some regulatory mechanism of gene expression.

13. Proposal for future studies

Working with *Leishmania* and the leishmaniases is incredibly exciting but also exhausting– as you open one door, numerous other doors also open simultaneously. *Leishmania* is a diverse pathogen with transmission in rainforest, rural and urban areas, as well as dry climates. And the leishmaniases present with a variety of clinical syndromes. Working within the framework of a PhD, this gives you a lot of freedom and opportunity, but also a sense of "angst" – time and resources are limited and maybe the path you are taking is wrong or not as exciting as the other path you could have chosen? Thus, there are a range of different paths I would have liked to have taken. In particular, I would have liked to:

- Investigate the *aap3*-HRM approach with more biological samples from different mammalian and non-mammalian backgrounds to evaluate its true diagnostic and epidemiological potential.

- According to Peeling and Mabey, increasing access to treatment for infectious diseases can have a major impact on disease burden (161). Key to improving this access is diagnosis. In developing countries, due to limited laboratory facilities, increasing the availability of point-of-care tests is likely to be important. To develop a molecular based point-of-care test would therefore be of particular interest and importance in future studies. A likely approach would be an isothermal method using enzymes like recombinase, or similar.

- A multicentre study to evaluate the best sampling method and DNA extraction method from all the leishmaniases.

- Continue studies into *aap3* and flanking regions on more strains and species, include the complete sequences of flanking regions by optimising oligonucleotides further, and to study the gene expression further, combine with RNA-sequencing data from more species.

- Use gene-editing technology, like CRISPR-Cas9 to edit *aap3* CDSs and target sequences in the UTRs to study function and regulation.

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"All things must pass"
-   George Harrison

I

Detection of a broad range of *Leishmania* species and determination of parasite load of infected mouse by real-time PCR targeting the arginine permease gene *AAP3*

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ABSTRACT

Leishmaniasis is one of the world's most neglected infectious diseases, affecting around 12 million people and more than 350 million at risk of infection. The clinical picture varies from self-healing cutaneous lesions to severe visceral infections, but still no commercial vaccines for humans are available and the currently used drugs have unpleasant side effects. Here we report a real-time PCR assay targeting the arginine permease gene *AAP3* that can be applied for all the nine different species of the *Leishmania* genus tested; 4 Old World species and 5 New World species, from both *L.* (*Leishmania*) and *L.* (*Viannia*) subgenera. No cross-reaction was seen with *Trypanosoma cruzi*, *Trypanosoma brucei*, human or mouse genomic DNA. The assay has a high sensitivity, with a limit of detection of 10 fg DNA for *L.* (*L.*) *major* and *L.* (*L.*) *donovani*, and 100 fg DNA for *L.* (*V.*) *braziliensis*, and can be used for both qualitative and quantitative purposes. This AAP3-Assay, run in duplex with a host specific gene-assay, was also successfully used for quantification of parasite load of footpads from *L.* (*L.*) *major*-infected mice. It can therefore be a valuable tool in applications like monitoring effects of drugs, the selection of vaccine candidates and in screening patients, including asymptomatic carriers.

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1. Introduction

Leishmaniasis is a vector-borne disease with different clinical pictures caused by protozoa of the genus *Leishmania*. It is one of the world's most neglected infectious diseases and the second cause of parasite related deaths after malaria (Mathers et al., 2007). Spread of the *Leishmania* parasite is caused by the bite of infected sand flies. Worldwide more than 350 million people in 98 countries or territories are at risk (World Health Organization, 2010). The estimated incidence of new cases each year is 0.2–0.4 million for visceral leishmaniasis and 0.7–1.2 million for cutaneous leishmaniasis, causing 20 000 to 40 000 deaths annually, and these data are probably underestimates (Alvar et al., 2012). In some affected areas both domestic and sylvatic animals seem to be important reservoirs of the parasite, contributing to promote human infections (Quinnell and Courtenay, 2009). Available drugs for treating the disease can be characterized as limited, expensive and often with unpleasant side effects. Moreover, there has been an emergence of drug resistance (Sundar and Chakravarty, 2013). No commercial vaccines are currently available for preventing leishmaniasis in humans. Therefore, in order to control the disease, there is a need for development of new drugs, vaccines and more specific and sensitive diagnostic methods. Assays for quantification of the parasite in the host tissues are essential for development and testing of prophylactic and therapeutic regimes.

Polymerase chain reaction (PCR) and its variations represent highly sensitive and specific methods for *Leishmania* DNA detection. PCR has shown to be superior to other methods like microscopy and various immunologic tests, reducing time from sampling to test result, optimizing sensitivity and specificity and reducing subjective evaluation (Aviles et al., 1999; Bensoussan et al., 2006; Srivastava et al., 2011a; Wall et al., 2012). Real-time PCR is advantageous over conventional PCR because it is faster, less labor-intensive, reduces risk of contamination, and by using probes the sensitivity and specificity can be increased (Dymond, 2013; Mohammadiha et al., 2013; Yang and Rothman, 2004)

Furthermore, by using standard curves real-time PCR can be used for quantification. Exploring the *Leishmania* parasites and the clinical manifestations they cause, quantitative real-time PCR (qPCR) can be useful for detection and species identification, but it also has a wider potential, like monitoring the effect of drug activity and measuring the protection as part of vaccine development. Due to the high sensitivity, qPCR can be used for screening of patients and detection of asymptomatic carriers, and thereby addressing gaps in the understanding of infection with *Leishmania* (Francino et al., 2006; Mary et al., 2006; Pourabbas et al., 2013).

There are many publications of different variants of PCRs for *Leishmania*, using different molecular targets, but most protocols target a single species (Francino et al., 2006; Srivastava et al., 2011b), a group of closely related species (Harris et al., 1998; Odiwuor et al., 2011), or they are not quantitative (Berzunza-Cruz et al., 2009; de Almeida et al., 2011; Harris et al., 1998; Odiwuor et al., 2011; Srivastava et al., 2011b), and some also show low sensitivity (Wortmann et al., 2005). There are only few publications of real-time PCR assays that target all or nearly all of the approximately 20 different *Leishmania* species found in humans (Castilho et al., 2008; Tupperwar et al., 2008; Wortmann et al., 2001). Many protocols target multicopy genes (Bossolasco et al., 2003; Francino et al., 2006; Talmi-Frank et al., 2010) and some protocols for multicopy genes also use SYBRGreen (de Monbrison et al., 2007). Multicopy genes are often preferred to enhance sensitivity, and thus are advantageous for detection, but due to potential variations and instability in copy number of the same gene both between and within species (Weirather et al., 2011) they can be challenging and confounding for quantification using standard curves. SYBR-Green has the disadvantages of more unspecific binding, hence a probe, which is more specific, is to be preferred. However, it can be challenging to design a qPCR with a specific probe that target a DNA sequence universal to all species of the *Leishmania* genus.

L-Arginine is an essential amino acid for *Leishmania*, for which metabolism depends on arginine supply from external sources, as no evidence for endogenous synthesis has been reported. The arginine transporter *Leishmania* arginine permease AAP3 is encoded on chromosome 31 in *Leishmania* (*L.*) *major* and other *Leishmania* species, and on chromosome 30 in *Leishmania* (*L.*) *mexicana* (which

due to chromosome fusion events is the equivalent of chromosome 31 in *L.* (*L.*) *major* (Britto et al., 1998)). The AAP3 gene is identified in several different *Leishmania* species (Shaked-Mishan et al., 2006). Likely more *AAP3* sequences will be published along with the increasing number of sequencing projects.

The aim of the study was to develop a quantitative PCR method that could be applied for infectivity studies in mice, focusing on *L.* (*L.*) *major*, as murine models are widely used in *Leishmania* research, with the benefit that it could also be used for other *Leishmania* species from both the Old- and New World groups. We developed a PCR-Assay targeting the arginine transporter gene *AAP3* and included DNA from cell pellets or cultured promastigotes from nine different *Leishmania* species, as well as different negative controls, to validate the assay. This AAP3-Assay, run in duplex with a host specific gene-assay, was then used for quantification of parasite load of footpads from *L.* (*L.*) *major*-infected mice.

2. Materials and methods

2.1. Strains used in this study

Leishmania strains used in this study are given in Table 1. *Leishmania* promastigotes were grown at 26 °C in RPMI 1640 medium (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco®, Life Technologies – Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin, or in Schneider's Insect Medium (Sigma–Aldrich) supplemented with 20% heat inactivated fetal calf serum and 1% sterile-filtered human urine. Parasites in culture were washed and counted by flow cytometry using reference beads (Flow-Count Fluorospheres®, Beckman Coulter, Brea, CA, USA) after staining with Vybrant® DyeCycle™ Green Stain (Molecular probes®, Eugene, OR, USA).

L. (*L.*) *mexicana* MHOM/BZ/82/BEL21 was a kind gift from Centro de Investigaciones Regionales 'Dr. Hideyo Noguchi', Universidad Autónoma de Yucatán, Mérida, Yucatán, México. Cell pellets from *Leishmania* strains as indicated in Table 1, as well as from *Trypanosoma brucei* and *T. cruzi*, were kindly donated from Dr. Silvia

Table 1
Leishmania strains used in this study, and their C_q-value when 100 pg of DNA was used as template in the AAP3-Assay.

Species[a]	International code or other reference	Origin	Source[b]	C_q[c]
L. (*L.*) *aethiopica*	MHOM/ET/91/Kassaye	Ethiopia	SMI	23.4
L. (*V.*) *amazonensis*	MHOM/BR/73/M2269, LEM 0690	Brazil	CNRL	23.7
L. (*V.*) *braziliensis*	MHOM/BR/87/LTB12MAR87, LEM 2839	Brazil	CNRL	25.0
L. (*V.*) *braziliensis*	MHOM/BR/75/M2904, LEM 2249	Brazil	CNRL	25.1
L. (*V.*) *braziliensis*	SMI 2094	Unknown	SMI	25.3
L. (*L.*) *donovani*	MHOM/CY/2006/CH33, LEM 5298	Cyprus	CNRL	23.8
L. (*L.*) *donovani*	MHOM/IN/80/DD8, LEM 0703	India	CNRL	23.7
L. (*L.*) *donovani*	MHOM/ET/67/HU3	Ethiopia	SMI	23.7
L. (*V.*) *guyanensis*	MHOM/GF/94/22319, LEM 2763	French Guiana	CNRL	27.6
L. (*L.*) *major*	MHOM/AF/2006/LEM5344, LEM 5344	Afghanistan	CNRL	23.9
L. (*L.*) *major*	MHOM/MA/2004/LEM4905, LEM 4905	Morocco	CNRL	24.2
L. (*L.*) *major*	MHOM/TN/2006/LPN296, LEM 5373	Tunisia	CNRL	23.9
L. (*L.*) *major*	MHOM/IL/80/FRIEDLIN, LEM 3150	Israel	CNRL	23.4
L. (*L.*) *major*-np	MRHO/SU/59/LV39	USSR	CNRL	23.6
L. (*L.*) *major*-p	MRHO/SU/59/LV39	USSR	CNRL	24.0
L. (*L.*) *mexicana*	MHOM/MX/96/NAN01, LEM 3816	Mexico	CNRL	23.8
L. (*L.*) *mexicana*	MHOM/MX/93/CRE47, LEM 2695	Mexico	CNRL	23.5
L. (*L.*) *mexicana*-np	MHOM/BZ/82/BEL21	Belize	CIR	24.3
L. (*L.*) *mexicana*-p	MHOM/BZ/82/BEL21	Belize	CIR	24.4
L. (*V.*) *naiffi*	MHOM/GF/97/CRE88, LEM 3426	French Guiana	CNRL	24.0
L. (*L.*) *tropica*	MHOM/SU/74/K27, LEM 0419	USSR	CNRL	24.2
L. (*L.*) *tropica*	MHOM/MA/2000/INHW10, LEM 5277	Morocco	CNRL	24.1

[a] np: not been propagated in mouse; p: propagated in mouse.

[b] SMI: Public Health Agency of Sweden, Sweden; CNRL: Centre National de Réfèrence des Leishmanioses, Montpellier, France; CIR: Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán, Mérida, Yucatán.

[c] C_q-value from the AAP3-assay using 100 pg DNA as template.

Botero-Kleiven and Dr. Leigh Davidsson at the Public Health Agency of Sweden, Sweden, and were kept in ethanol during transport to our laboratory and until DNA extraction.

2.2. Detection of the AAP3 gene in Leishmania species

2.2.1. DNA extraction

Cultured promastigotes from the stationary phase and cell pellets in ethanol were washed with Dulbecco's phosphate-buffered saline (10 mM Phosphate, 137 mM Sodium chloride, 2.7 mM Potassium chloride, pH 7.4) (DPBS) before subjected to DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that Proteinase K was used instead of protease. DNA was eluted in 100 µl of Buffer AE (Qiagen). In all DNA extractions a negative control of DPBS was included to monitor for contamination. DNA concentration and quality was determined by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All DNA samples were stored at −20 °C.

2.2.2. Real-time PCR

Primers and hydrolysis probe (TaqMan® MGB probe) targeting a 74 bp region of the *L. (L.) major* gene encoding the arginine transporter AAP3 were designed by the Custom TaqMan® Assay Design Tool from Applied Biosystems (Applied Biosystems, Warrington, UK). The sequences of the primers and probe were 5′-GGCGGC-GGTATTATCTCGAT-3′ (Forward), 5′-ACCACGAGGTAGATGACAGA-CA-3′ (Reverse) and FAM 5′-ATGTCGGGCATCATC-3′ NFQ (probe). In silico analysis of specificity of the assay was performed using the Vector NTI software (Life Technologies). A global BLAST search was undertaken by the 74 bp region wherein the primers and probe bind. In addition, complete genome sequences of some relevant bacteria (like *Mycobacteria*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and investigated for the extent of binding (BLAST) of the primers and probe.

Each PCR test was performed in triplicate in a 20 µl reaction mixture. The reaction mixture included: 1× TaqMan Universal Master Mix II with UNG (Applied Biosystems), 1× Custom TaqMan® Gene Expression Assay Mix with primers and probe targeting the *Leishmania AAP3* gene, 1× TaqMan® Copy Number Reference Assay, Mouse, Tfrc, water and 100 pg of DNA sample. The PCR was run as a duplex assay after comparing the results of singleplex AAP3-Assay and the duplex with the Tfrc-Assay (results not shown).

The qPCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), with cycling conditions as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min each. All samples were run on MicroAmp® Optical 96-well Reaction Plates (Applied Biosystems) sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems). Each run included multiple no-template controls. Human DNA, *T. brucei* and *T. cruzi* was used as negative controls to check for cross-reactivity. The human DNA for control was provided by Christel G. Haanshuus, National Centre for Tropical Infectious Diseases, Department of Medicine, Haukeland University Hospital, Bergen, Norway.

Tenfold dilution series of *L. (L.) major* DNA (range 12 ng–1.2 × 10^{-7} ng) were prepared for creating standard curves and estimating assay performance for the AAP3-Assay. Besides quantification, dilution series and standard curves were used for estimation of PCR efficiency, limit of quantification (LOQ) and limit of detection (LOD). LOQ and LOD were also estimated for *L. (L.) donovani* and *L. (V.) braziliensis*. The dilution corresponding to the LOQ was the highest dilution used for the standard curve. For estimating repeatability and reproducibility, replicates of a DNA sample from the footpads of BALB/c mice infected with *L. (L.)*

major parasites were used. DNA was quantified using the Absolute Quantification Assay. Thresholds were set automatically. Amplicons were run on a 2% agarose gel (SeaKem™, Lonza, Rockland, ME, USA) with 1X GelRed™ (Biotium, Hayward, CA, USA) to check for the correct size. Replicates with quantification cycle (C_q)-value differing by more than 0.3 were omitted.

A unidirectional workflow pre- to post-qPCR was enforced, and preparation of qPCR reaction mixture, DNA preparations and qPCR were carried out in facilities physically separate from each other.

2.3. Detection of the AAP3 gene in L. (L.) major-infected mice

2.3.1. Mouse tissue samples

The left footpads of female BALB/c mice were inoculated with 10 µl of 10^6 ml^{-1} *L. (L.) major* parasites in stationary phase. After swelling and lesions had developed, 56 days post inoculation, mice were euthanized after first using Isoba vet. 100% (Intervet/Schering- Plough Animal Health, Intervet Denmark A/S, Denmark) for anesthesia. Footpads of control mice (2 animals) and infected mice (8 animals), and liver for providing control DNA, were harvested and stored at −80 °C.

2.3.2. Ethical clearance

The animal experiments were approved by the National Animal Research Authority in Norway and carried out at the Laboratory Animal Facility (AAALAC-accredited) at the University of Bergen, Bergen, Norway.

2.3.3. DNA extraction from mice tissue

Mouse footpads were subjected for DNA extraction by a phenol-chloroform based protocol from Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, available at http://clioc.fiocruz.br/documents/mmp.pdf (Leishmaniasis Epidemiology Network South America, 2009). DNA pellet was dissolved in 200 µl TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and incubated at room temperature overnight. Mouse livers were incubated over night at 56 °C in Buffer ATL (Qiagen) with 2 mg/ml Proteinase K (Qiagen), before subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 100 µl of Buffer AE (Qiagen).

In all DNA extractions a negative control of DPBS was included to monitor for contamination. DNA concentration and quality was determined by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All DNA samples were stored at −20 °C.

2.3.4. Quantitative real-time PCR

In order to normalize the parasite load for amount of mouse tissue DNA, and hence overcome the possible quantification errors due to different cutting points when harvesting the footpads, the qPCR was run as a duplex-assay with the AAP3-Assay and the TaqMan® Copy Number Reference Assay, Mouse, Tfrc (Applied Biosystems, Foster City, CA, USA), as a reference assay. This premade reaction mixture consists of primers and a VIC® dye-labeled TAMRA™ probe which detects the single-copy transferrin receptor gene (*Tfrc*) in the mouse genome.

Each qPCR test was performed in triplicate in a 20 µl reaction mixture. The reaction mixture was as in Section 2.2.2, except that 100 ng of DNA sample was used as template. Initial dilution experiments showed that with 100 ng of DNA the background level, or inhibition, was negligible.

Fivefold dilution series of BALB/c mouse DNA (range 1050 ng–0.33 ng) were prepared for creating standard curve and estimating assay performance for the Tfrc-Assay. For estimating repeatability and reproducibility, replicates of a DNA sample from the footpads of BALB/c mice infected with *L. (L.)*

major parasites were used. DNA was quantified using the Absolute Quantification Assay. Thresholds were set automatically. The parasite load in tissue samples was given as the ratio between *Leishmania* DNA and genomic mouse DNA in the 100 ng DNA applied to each PCR reaction. Since the amount of DNA cannot be reliably estimated outside the linear area of the standard curve, the DNA quantity for samples with *Leishmania* DNA less than the LOQ was set to be equal to or less than the LOQ. Parallels with C_q-value differing by more than 0.3 were omitted.

3. Results

3.1. Specificity of the AAP3-Assay and the Tfrc-Assay

The primers and probe for the AAP3-Assay were designed for *L. (L.) major*. We also experimentally tested the ability of these oligonucleotides to detect other different *Leishmania* species, representing nine different species, either isolated from cell pellets or cultured promastigotes. *L. (L.) aethiopica*, *L. (V.) amazonensis*, *L. (V.) braziliensis*, *L. (L.) donovani*, *L. (V.) guyanensis*, *L. (L.) major*, *L. (L.) mexicana*, *L. (V.) naiffi* and *L. (L.) tropica* could all be amplified with the AAP3-Assay, as seen from Table 1. The PCR resulted in amplicons of the correct size, 74 bp, as visualized by gel electrophoresis (results not shown). Neither the AAP3-Assay nor the Tfrc-Assay could amplify *T. brucei* and *T. cruzi*, or genomic DNA from humans or mice (AAP3-Assay only).

The global BLAST search with the 74 bp region (wherein the primers and the probes bind) gave only hits indicating generation of positive signals of the assay when targeting DNA from *Leishmania* species. Likewise, investigation of the bacterial genomic sequences did not reveal strong binding of any combinations of applied primers in a way that would generate amplification of any segment to which the probe would bind, and thereby giving rise to a positive signal.

3.2. Sensitivity of the AAP3-Assay

LOD and LOQ were estimated for *L. (V.) braziliensis*, *L. (L.) donovani* and *L. (L.) major* using serial dilutions of DNA purified from in vitro cultivated parasites. With the applied method used for DNA extraction, 100 fg corresponded to 2 parasites (p). For *L. (V.) braziliensis*, *L. (L.) donovani* and *L. (L.) major* the LODs, given in fg with number of parasites with our extraction method in brackets, were: ≥100 fg (2 p), ≥10 fg (0.2 p) and ≥10 fg (0.2 p), respectively. The LOQs for *L. (V.) braziliensis*, *L. (L.) donovani* and *L. (L.) major* were ≥1000 fg (20 p), ≥10 fg (0.2 p) and ≥100 fg (2 p), respectively.

3.3. Assay performance

From the DNA dilution series parameters of assay performance other than sensitivity was calculated. From repeated runs the mean slopes of the AAP3-Assay and Tfrc-Assay were −3.205 (range −2.994 to −3.305) and -3.193 (range −3.083 to −3.313), respectively. Efficiency, as determined from the slope and using the formula $E = 10^{-1/\text{slope}} - 1$, was 105.1% for the AAP3-Assay and 105.7% for the Tfrc-Assay. The correlation coefficient, R^2, was 0.999 (range 0.996–0.999) for the AAP3-Assay and 0.997 (range 0.996–0.999) for the Tfrc-Assay, and the Y-intercept was 19.48 (range 18.26–20.85) for the AAP3-Assay and 31.83 (range 31.43–32.11) for the Tfrc-Assay. A DNA sample from the footpads of BALB/c mice infected with *L. (L.) major* parasites was used for estimating repeatability and reproducibility. The intra-assay coefficient of variation (CV) for the AAP3-Assay, calculated from DNA quantity of replicates, was for each of three separate runs 0.0908, 0.0901 and 0.0882, respectively. For the Tfrc-Assay the intra-assay CV was for each of two separate runs 0.0179 and 0.021. Inter-assay
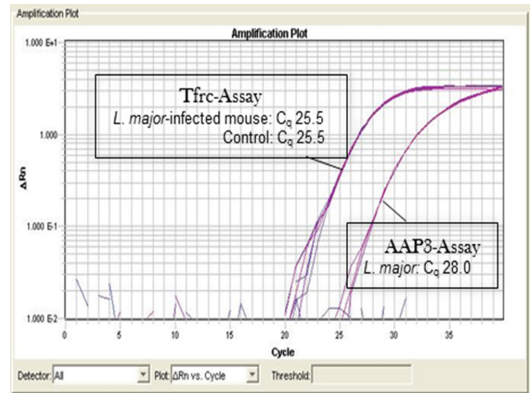

Fig. 1. Amplification plot for the AAP3-Assay and the Tfrc-Assay. Amplification curves for DNA isolated from footpad of uninfected Control mouse (Control) and mouse infected with *L. (L.) major*.

CV, calculated from DNA quantity of separate runs, was 0.052 for the AAP3-Assay and 0.059 for the Tfrc-Assay. The ratio in 100 ng DNA between quantity of *L. (L.) major* DNA, as measured by the AAP3-Assay, and mouse DNA, as measured by the Tfrc-Assay, was 3.22×10^{-4} and 3.25×10^{-4} for the same sample on two separate runs.

No amplification of any of the no-template controls was detected.

3.4. Estimation of L. (L.) major DNA in mice footpads

For analyzing and quantification of parasite load of footpads from *L. (L.) major*-infected mice, the AAP3-Assay was run in duplex with a host specific gene-assay; the Tfrc-Assay, which detects mouse DNA. *Leishmania* DNA was successfully amplified from all the infected mice, but not from the control mice. Fig. 1 shows amplification plots for the AAP3-Assay and the Tfrc-Assay when analyzing a sample of *Leishmania*-infected tissue, together with plots for the Tfrc-Assay when analyzing a control sample of uninfected tissue. As the mice DNA constitute the majority of the DNA in the infected tissue, all plots from the Tfrc-Assay make up a concurrent curve. Values for the AAP3-Assay triplicates and for the triplicates of the Tfrc-Assay are given in Table 2. In addition, DNA isolated from the footpad of one of the other *L. (L.) major*-infected mice was analyzed, where we made two different dilutions of the DNA to confirm that the same ratio between *Leishmania* DNA and mouse DNA would still be obtained. For 148.5 ng sample the ratio was 1.99×10^{-4} and using 74.3 ng of the same sample the ratio was 1.93×10^{-4}.

4. Discussion

Microscopy has traditionally been the cheapest and easiest method for detecting and counting, *Leishmania* parasites. However, real-time PCR is superior regarding sensitivity, specificity, capacity and has in addition short time of analysis and minimized subjectivity of laboratory staff. We have developed a qPCR assay able to detect as little as 10 fg of DNA, and to quantify down to the limit of 10 fg (*L. (L.) donovani*). The AAP3-Assay is effective in detecting several different species, and possibly the species range for our assay is broader than we have tested for. Regarding specificity, both experimental and bioinformatic analyzes show no homology of the target sequence to non-*Leishmania* sequences.

Table 2
Quantification of *L.* (*L.*) *major* DNA in footpad from experimental infected mouse.

Sample[a]	$C_{qAAP3\text{-}Assay}$[b,c]	Qty LeishDNA[d]	$C_{qTfrc\text{-}Assay}$[b]	Qty mouseDNA[e]	Qty LeishDNA/Qty mouseDNA
L. (*L.*) *major*-1	28.08	2.3×10^{-3}	25.55	90.03	2.55×10^{-5}
L. (*L.*) *major*-2	27.96	2.5×10^{-3}	25.46	96.18	2.60×10^{-5}
L. (*L.*) *major*-3	28.03	2.4×10^{-3}	25.46	95.99	2.50×10^{-5}
Control 1-1	Undet.	–	25.46	95.79	–
Control 1-2	Undet.	–	25.51	92.54	–
Control 1-3	Undet.	–	25.49	93.84	–

[a] Control 1, uninfected mice; results are shown for all triplicates for each sample.
[b] C_q, quantification cycle.
[c] Undet., Undetermined.
[d] Qty LeishDNA, Quantity *Leishmania* DNA in ng detected by the AAP3-Assay per 100 ng DNA.
[e] Qty mouseDNA, Quantity mouse DNA in ng detected by the Tfrc-Assay per 100 ng DNA.

With the applied method used for DNA extraction, 100 fg corresponded to 2 parasites. Assuming 80 fg of *Leishmania* DNA is equivalent to one parasite, then 100 fg corresponds to 1.2 parasites, indicating that the sensitivity of the AAP3-Assay actually is better than experienced, and can be further improved by optimizing DNA extraction.

Regarding sensitivity in terms of DNA quantity, the LOD of our assay equals that of some of the publications targeting both rRNA- and kDNA minicircle genes (Berzunza-Cruz et al., 2009; Nicolas et al., 2002; Prina et al., 2007; Gomes et al., 2012; Talmi-Frank et al., 2010; Wortmann et al., 2001), which are genes with high copy numbers, but there are also publications targeting multicopy genes showing higher sensitivity (Francino et al., 2006). However, when using multicopy genes for quantification there might be a need for a species specific standard curve as number of gene copies vary between species (Weirather et al., 2011), thus making those assays little suitable for a universal quantitative *Leishmania*-assay. Like the AAP3-Assay, the assay of (Wortmann et al., 2001), targeting the *Leishmania 16S rRNA*, was also able to amplify a wide range of *Leishmania* strains at the genus level. However, the sensitivity was only determined for *L.* (*L.*) *mexicana*, the C_q-values both within and between species had a great range, though they not clearly specify if the same amount of DNA was used, and parameters for assay performance, like efficiency, LOQ, reproducibility and repeatability was not estimated, or at least not given in the text.

The chromosome that hosts the *AAP3* gene is supernumerary; tetrasomic for some species, like *L.* (*L.*) *major*, *L.* (*L.*) *infantum* and *L.* (*L.*) *donovani*, and hexasomic for *L.* (*V.*) *braziliensis* (Rogers et al., 2011). In *L.* (*L.*) *major*, *L.* (*L.*) *infantum*, *L.* (*V.*) *amazonensis* and *L.* (*L.*) *donovani* there are two identical copies of *AAP3* (Castilho-Martins et al., 2011; Shaked-Mishan et al., 2006), whereas in *L.* (*L.*) *mexicana* there is no reported equivalent gene duplication. We have included 22 *Leishmania* strains, representing 9 different species. The C_q-values for these strains using 100 pg DNA as template in the AAP3-Assay are shown in Table 1. There is very little intra-species variation in C_q-value, but some inter-species variation, mainly between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* against the others species included in our study. This might indicate that ploidy between strains and species does not largely affect the results of the assay, but this should be investigated further. Tupperwar et al. (2008) describes a PCR assay targeting the *Leishmania GP63* able to amplify several different species, but compared to the AAP3-Assay there is a markedly difference in the C_q-valuable between species when the same amount of template was used. Whether there is a need for a species specific, or even strain specific, standard curve for the *AAP3*-assay depends on the specific application and the required accuracy, and has yet to be more thoroughly evaluated. In contrast to other multicopy genes like the kDNA minicircle and ITS1, with copy numbers around 10 000 and 200 respectively, and as seen from Table 1, it is less likely, and for several species probably species group-specific standard curve will be sufficient.

A way of avoiding a species specific standard curve could be to use the DNA concentrations for the dilutions of the standard curve instead of the number of parasites, and then using species related conversion factors.

In this study we developed a qPCR assay that can be applied on a broad range of species of the *Leishmania* genus, from both the Old- and New World groups, including both *L.* (*Leishmania*) spp. and *L.* (*Viannia*) spp., and which in addition can be used to analyze DNA from both cell cultures and mouse tissue, and most likely DNA from several other kinds of sample materials. By using a standard curve one can determine the amount of target DNA in a sample, and from that also calculate the number of parasites. However, when isolating DNA from infected tissues the amount of DNA will be dependent on the size of the tissue sample and the recovery of DNA at the different steps of the isolation protocol. The use of host reference gene can circumvent this problem. When applied to *Leishmania* infected mouse, the AAP3- and Tfrc-Assays give a ratio between *Leishmania* DNA and mouse DNA. This ratio, which is independent of the amount of total DNA applied for the analysis, is reflecting parasite load in the tissue and hence differences can be detected. In a study by Tupperwar et al. (2008), they normalized the parasite number to the total DNA isolated from the mice tissue samples, and finally reported the parasite number per milligram of original tissue, not using a host reference gene. This reported result could then be prone to differences in recovery of DNA during the extraction process. Nicolas et al. (2002) describe a real-time PCR assay which successfully detects four different *Leishmania* species from a wide range of mouse infected tissues. However, the study does not include a method, like using a host reference gene, for estimating and normalization of the parasite burden of the tissue. They also target the high copy number minicircle kDNA, which might have drawbacks, as mentioned above. A limitation of our method is that it not necessarily discriminates between live and dead parasites. However, the levels of *Leishmania* DNA detected will still indicate the parasite load at the time of sampling.

In summary, we have developed a very sensitive method for detection of *Leishmania* that can be applied for several different species of the parasite. In combination with an assay for quantification of host DNA, it is possible to measure the load of *Leishmania* in infected tissues. With its high sensitivity the method has potential as a tool for diagnostic purposes, including detecting asymptomatic infections. Due to the quantification possibility, the method can be used to monitor the progress of infection, which will be a valuable tool in testing new drugs and in vaccine development.

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Amino acid permease 3 (*aap3*) coding sequence as a target for *Leishmania* identification and diagnosis of leishmaniases using high resolution melting analysis

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Abstract

Background: The leishmaniases comprise a spectrum of clinical manifestations caused by different species of *Leishmania*. Identification of species is important for diagnosis, treatment and follow-up management. However, there is no gold standard for species identification. High resolution melting analysis (HRM) offers a possibility to differentiate *Leishmania* species without the need for processing of the PCR-product. The amino acid permease 3 (*aap3*) gene is an exclusive target for trypanosomatids and is conserved among *Leishmania* spp., thus it can be a valuable target for an HRM assay for diagnosis of the leishmaniases.

Results: The HRM dissociation profiles of three amplicons targeting the *aap3*-coding region allowed the discrimination of *L. (Leishmania) donovani*, *L. (L.) infantum*, *L. (L.) major*, *L. (L.) tropica*, *L. (L.) mexicana*, *L. (L.) amazonensis*, *L. (Viannia) braziliensis*, *L. (V.) guyanensis*, *L. (V.) lainsoni*, *L. (V.) naiffi* and *L. (V.) shawi* using DNA from promastigote cultures. The protocol was validated with DNA samples from clinical infection in humans and a cat, naturally infected sand flies, and experimentally infected mice.

Conclusions: HRM analysis using the *aap3* coding sequence as target is a relatively cheap, fast and robust strategy to detect and discriminate *Leishmania* species from all the endemic regions worldwide. The target and method proved to be useful in clinical, field and experimental samples, thus it could be used as a tool in diagnosis as well as ecological and epidemiological studies.

Keywords: PCR, HRM, Infectious diseases, *Leishmania* discrimination

Background

The leishmaniases are a group of diseases caused by *Leishmania* spp. Clinical presentations range from self-healing cutaneous lesions to potentially lethal visceral leishmaniasis [1]. It is defined by the World Health Organization (WHO) as a neglected tropical disease, meaning it is underreported, underestimated, underfunded and underprioritized by the

pharmaceutical industry and often by public health authorities alike [2]. Over 350 million people are at risk of being infected, and it is estimated that 20,000 to 40,000 die each year of the leishmaniases [3]. In some regions, it is considered as largely an anthroponotic disease, while in others it is a zoonosis. The reservoir of the parasite can be domestic, sylvatic, and in some areas, both. Over 20 different species of *Leishmania* can cause the disease in humans [1, 4]. The species that cause disease in humans are grouped into two subgenera: *Leishmania* (*Leishmania*) and *Leishmania* (*Viannia*), based on biological features of the parasites [5]. Species identification is important for clinical diagnosis,

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prognosis, treatment and follow-up management. For example, some *L. (Viannia)* species, such as *L. (V.) braziliensis*, are known to cause cutaneous lesions and may later reappear as mucocutaneous lesions, thus requiring systemic treatment, while other lesions infected by strains known to only cause cutaneous manifestations may be treated with local treatment or observation therapy [6].

The leishmaniases can be diagnosed in various ways, all with their strengths and limitations. However, there is no gold standard for diagnosing the diseases. Microscopy is useful in a high-endemic setting, but lacks sensitivity and needs a trained microscopist, not easily found in most non-endemic areas. Diagnostic tests involving serology, such as the direct agglutination test (DAT) and lateral flow immunochromatographic tests can also be useful. Especially lateral flow immunochromatographic tests can give a rapid diagnosis, are simple to use, easy to interpret and relatively cheap - all qualities which are very important in a low-resource setting in many endemic areas. However, serological tests may vary greatly in their sensitivity and specificity between endemic regions [7]. Furthermore, serology is not able to determine the species causing the disease. Diagnosis involving nucleic acid detection is valuable due the high sensitivity and specificity, and for the potential ability to quantify and identify the infecting species. There is a plethora of possible techniques and an equal amount of possible targets [8].

Real-time PCR, followed by high-resolution melting analysis (HRM), generates thermodynamic differences in the dissociation profile of amplicons resulting in specific signatures of polymorphisms due to small differences in nucleotide composition [9]. HRM is rapid, comparatively little laborious and a relatively cheap method where the post-PCR treatment is contained in the tubes with small risk for lab-contamination. HRM has been used for identification of other infectious agents [10–13]. In earlier work, we have already shown that HRM can be a valuable tool for *Leishmania* genotyping, using *hsp70* as a target [14]. AAP3 is an amino acid transporter, which mediates uptake of lysine, histidine, phenylalanine, citrulline and arginine, with the highest affinity for the last one [15–20]. AAP3 is involved in the polyamine pathway, essential for parasite replication [17, 21–23]. The coding sequence for AAP3 is conserved among *Leishmania* spp., indicating its value as the chosen target [17]. We already demonstrated *aap3* as an attractive target for detecting *Leishmania*, by a real-time PCR method, but this approach did not discriminate the species [24]. In this paper, we describe the use of the *aap3* coding sequence as target for differentiation of *Leishmania* spp., using HRM analysis. The *aap3*-HRM method showed to be a specific and sensitive tool to differentiate *Leishmania* spp., using reference strain cultures and validated using clinical samples, naturally infected sand flies and experimentally infected mice samples.

Methods

Organisms

Promastigotes of *Leishmania* spp. (see Table 1) were grown at 25 °C in M199 medium containing Earle's salts, supplemented with 10% fetal bovine serum, 40 mM HEPES (pH 7.4), 100 μM adenine, 5 mg/l hemin, 0.05 mg/ml streptomycin and 4550 U/ml penicillin. *Trypanosoma cruzi* (Y-strain), *Crithidia fasciculata* (TCC-039) and *Endotrypanum schaudinni* (MCHO/BR/80/M6159) were grown at 28 °C in liver-tryptose medium supplemented with 10% fetal bovine serum and 0.05 mg/ml streptomycin and 4550 U/ml penicillin. *Trypanosoma brucei* (Lister 427) was grown at 28 °C in SDM-79 medium (LGC Biotecnologia, Cotia, São Paulo, Brazil), supplemented with 10% fetal bovine serum, 0.05 mg/ml streptomycin and 4550 U/ml penicillin. Mammalian DNA from BALB/c mouse and Wistar rat were obtained from the DNA repository of the Laboratory of Trypanosomatidae at Physiology - IB-USP and were used as negative controls (see Additional file 1: Figure S1 and Additional file 2: Figure S2).

Naturally and experimentally infected samples

To validate the standardized protocols, samples previously identified by other diagnosis tests for the leishmaniases were used as a template in *aap3*-HRM assays [14, 26]. Human paraffin-embedded samples (from patients from Hospital das Clínicas da Universidade de São Paulo or Hospital da Irmandade da Santa Casa de Misericórdia de São Paulo), a sample from an infected cat (from Instituto de Medicina Tropical de São Paulo - USP), naturally infected sand flies (from Superintendência de Controle de Endemias de São Paulo), and samples from experimentally infected mice (from Instituto de Biociências - USP) were included.

DNA extraction

The *Leishmania* strains used as assay-standards, *T. cruzi*, *T. brucei*, *C. fasciculata*, *E. schaudinni* and the mammalian DNA was isolated using a modified salting out technique, as previously described [14]. The DNA from the additional strains used for specificity studies was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA from mouse was isolated from whole blood and DNA from rat was isolated from liver-tissue, both by using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. DNA quality and concentration were determined by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA). All DNA was stored at -20 °C until further use.

For the paraffin-embedded tissues, a first step of washing was performed with xylol heated to 95 °C to remove paraffin, followed by repetitive washes of absolute ethanol.

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Table 1 *Leishmania* reference strains and additional strains used in this study with information about international code number, clinical form and host according to the World Health Organization classification [25]

Strain	International code number	Clinical form	Isolated from	WHO reference strain
Assay standard strains				
L. (L.) donovani	MHOM/IN/80/DD8	Visceral	*Homo sapiens*	Yes
L. (L.) infantum	MCER/BR/1981/M6445	Visceral	*Cerdocyon thous*	No
L. (L.) tropica	MHOM/SU/60/OD	Cutaneous	*Homo sapiens*	No
L. (L.) major	MHOM/IL/81/Friedlin	Cutaneous	*Homo sapiens*	No
L. (L.) amazonensis	MHOM/BR/1973/M2269	Cutaneous	*Homo sapiens*	Yes
L. (L.) mexicana	MNYC/BZ/62/M379	Cutaneous	*Nyctomys sumichrasti*	Yes
L. (V.) lainsoni	MHOM/BR/81/M6426	Cutaneous	*Homo sapiens*	Yes
L. (V.) braziliensis	MHOM/BR/1975/M2903	Cutaneous	*Homo sapiens*	Yes
L. (V.) guyanensis	MHOM/BR/1975/M4147	Cutaneous	*Homo sapiens*	Yes
L. (V.) naiffi	MDAS/BR/1979/M5533	na	*Dasypus novemcinctus*	Yes
L. (V.) shawi	MCEB/BR/84/M8408	Cutaneous	*Cebus apella*	Yes
Additional strains used for specificity studies				
L. (L.) donovani	MHOM/CY/2006/CH33	Visceral	*Homo sapiens*	No
L. (L.) tropica	MHOM/SU/74/K27	Cutaneous	*Homo sapiens*	Yes
L. (L.) tropica	MHOM/MA/2000/INHW10	Cutaneous	*Homo sapiens*	No
L. (L.) major	MHOM/AF/2006/LEM5344	Cutaneous	*Homo sapiens*	No
L. (L.) major	MHOM/MA/2004/LEM4905	Cutaneous	*Homo sapiens*	No
L. (L.) major	MHOM/TN/2006/LPN296	Cutaneous	*Homo sapiens*	No
L. (L.) major	MRHO/SU/59/LV39	na	*Rhombomys opimus*	No
L. (L.) mexicana	MHOM/BZ/82/BEL21	Cutaneous	*Homo sapiens*	Yes
L. (L.) mexicana	MHOM/MX/93/CRE47	Cutaneous	*Homo sapiens*	No
L. (L.) mexicana	MHOM/MX/96/NAN01	Cutaneous	*Homo sapiens*	No
L. (V.) braziliensis	MHOM/BR/75/M2904	Cutaneous	*Homo sapiens*	Yes
L. (V.) braziliensis	MHOM/BR/87/LTB12MAR87	Mucocutaneous	*Homo sapiens*	No
L. (V.) guyanensis	MHOM/GF/94/22319	Cutaneous	*Homo sapiens*	No
L. (V.) naiffi	MHOM/GF/97/CRE88	Cutaneous	*Homo sapiens*	No

Abbreviation: na not applicable

Then DNA was purified by an organic extraction with phenol-chloroform according Uliana et al. [27]. For sand flies, cultured *Leishmania* and mice samples, DNA was purified by silica columns, using DNeasy Blood & Tissue Kit (Qiagen).

Primer design

The primers (listed in the Table 2) were designed based on the following sequences: *L.* (*L.*) *amazonensis* (HQ912026.1 and HQ912027.1), *L.* (*V.*) *braziliensis* (XM_001567050.2), *L.* (*L.*) *major* (XM_001685021.1), *L.* (*L.*) *donovani*

Table 2 Primer sequences used in this study based on the amino acid permease 3 (*aap3*) coding sequence, for amplicon 1 (Amp1), amplicon 2 (Amp2) and amplicon 3 (Amp3)

Name	Sequence (5'-3')	Length (bp)	Orientation	Amplicon size (bp)
AAP3-Amp1-F	ATCCGCTACGTCTCCGCCATCGG	23	Forward	123
AAP3-Amp1-R	CGTGGTGAAGTACTTCATGTCGC	23	Reverse	
AAP3-Amp2-F	GCCGTCGATAAACACCCGAGC	21	Forward	131
AAP3-Amp2-R	AAGCGGAAGATGATGTTGCGCCC	23	Reverse	
AAP3-Amp3-F	GGCGGTCGCCTACATCAGCG	20	Forward	140
AAP3-Amp3-R	CGGGCACCATGAACACGAGCCATA	24	Reverse	

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(AY247004.1), *L.* (*L.*) *infantum* (XM_001467313.2) from GenBank, and *L.* (*L.*) *aethiopica* (LAEL147_000015800), *L.* (*L.*) *mexicana* (L.mxM.30.0870), *L.* (*L.*) *tropica* (LTRL590_310015200) and *L.* (*V.*) *panamensis* (LPAL13_000030300) from the TriTryp Database [28]. To predict melting temperatures (Tm), Oligo Calculator version 3.27 was used [29]. The nucleotide sequences of the amplicons and primer localizations are detailed in Additional file 3. A global BLAST search was performed for the three amplicon regions using the available amplicon sequences from the strains listed above.

PCR assays

Conventional PCR with the primers (Table 2) was performed for testing amplicon specificity. PCR reactions were performed using TopTaq Master Mix (Qiagen, Hilden, Germany) in a final volume of 25 μl with 200 nM of each primer and 25 ng of genomic DNA as a template. The cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 30 s and a final extension at 72 °C for 10 min.

Real-time PCR was performed using MeltDoctor HRM Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Final reaction volume was 20 μl, including 200 nM of each primer and 25 ng of genomic DNA as a template. Real time amplification conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s and annealing/extension at 60 °C for 1 min, with the acquisition of fluorescent signals at the end of each extension step, followed by the dissociation curve for HRM analysis. All reactions were performed in a Thermocycler PikoReal96 (Thermo Fisher Scientific, Walthan, MA, USA).

High resolution melting analysis

For HRM analysis, fluorescent signals were detected at 0.2 °C intervals, with hold-time for 10 s, between 60–95 °C. Data analysis was performed using PikoReal 2.1 Software (Thermo Fisher Scientific, Walthan, MA, USA).

Sensitivity assays

For sensitivity assays we tested the performance of the three amplicons with standard strains which produced an overlapped melting profile (see Table 1 for information about the strains).

For amplicon 1, we tested *L.* (*L.*) *donovani*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis*. For amplicon 2, we tested *L.* (*L.*) *donovani*, *L.* (*L.*) *mexicana*, *L.* (*L.*) *infantum* and *L.* (*L.*) *tropica*. For amplicon 3, we tested *L.* (*L.*) *donovani*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis*. For all amplicons, a range of parasite DNA from 25 ng to 100 fg was tested, with or without

25 ng/μl of human DNA. The DNA from standard strains was purified from *in vitro* cultivated parasites, as described above. Efficiency calculations were made for each amplicon from template ranging from 25 ng to 50 pg (see Additional file 4: Figure S3).

Statistics

All samples were tested in duplicate in at least three independent experiments. One-way ANOVA was used to calculate statistical difference between the Tm's of paired species for each amplicon. The results are presented as mean differences with 95% confidence intervals, and statistical significance set to $P < 0.05$. The results were analyzed and graphs were produced using GraphPad Prism version 7 (Additional file 5).
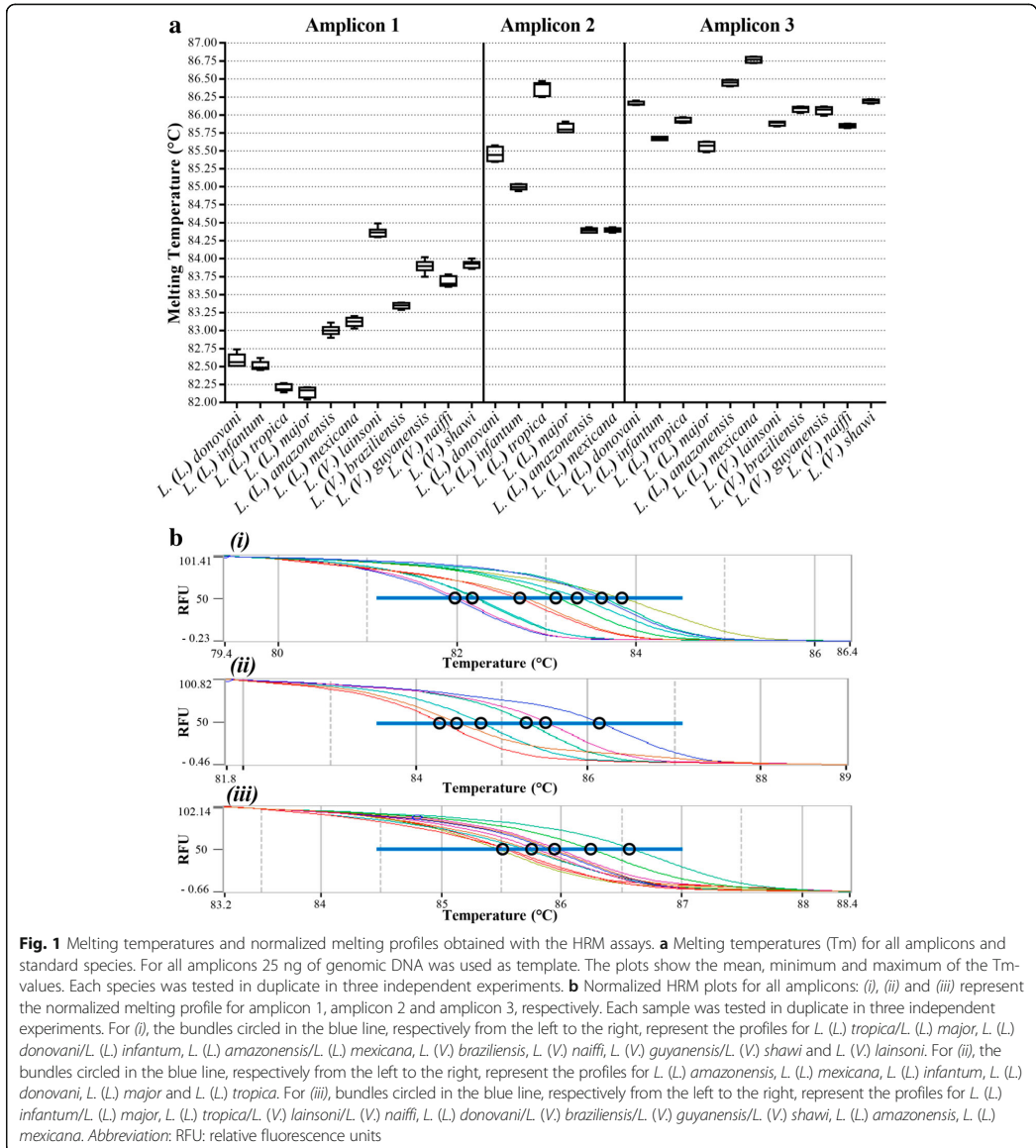
Results

Specificity

Three amplicons were designed based on available sequences from GenBank or TriTryp database [28], considering that *aap3* is present in two copies situated *in tandem* around 4 kb apart in most of *Leishmania* species [16, 47]. As expected, the BLAST search did not reveal any other hits than for *Leishmania* spp. As shown in Additional file 1: Figure S1, designed primers generated amplicons of the same size for all the species, presenting differences in nucleotide composition. To predict and to delimit potentially informative polymorphic regions, theoretical melting temperatures were calculated *in silico* using the OligoCalc tool [29] (data not shown), to guide primer design. In addition, the two copies of the gene were aligned concomitantly for the choice of primers that amplify both copies (data not shown). Although some single nucleotide polymorphism (SNP's) were detected between the two copies of the gene, it did not alter the Tm values. The alignments also allowed the selection of conserved regions for the design of oligonucleotides common to all species, which were used to generate products containing differences in nucleotide composition that were able to distinguish species or groups of species. Furthermore, we analyzed the amplification profile by conventional PCR using the same primers (Additional file 1: Figure S1). We observed specific products of the same size for all species analyzed for each set of primers: 123, 131 and 140 bp for amplicon 1, amplicon 2 and amplicon 3, respectively. No formation of dimers of primers were detected. Control samples were used and no amplification was observed for *T. brucei*, *T. cruzi*, *E. schaudinni*, rat, mouse and human DNA (Additional file 1 and Additional file 2). The analysis of amplicon 3 showed an amplification of *C. fasciculata*, a non-pathogenic and closely related organism to *Leishmania* spp., but the Cq and Tm values were able to distinguish *C. fasciculata* from all *Leishmania* spp. tested (Additional file 1: Figure S1 and

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Additional file 2: Figure S2). Individual Tm values and normalized melting profiles of 3 amplicons for reference strains are presented in Fig. 1, indicating that these differential profiles distinguished among *Leishmania* spp. To reliably distinguish different species, we only considered differences in Tm values exceeding ± 0.25 °C, an interval statistically determined as discriminatory (Additional file 5: Figure S3).

According to Tm and melting curves, amplicon 1 was able to distinguish the species into 3 clusters for subgenus *L. (Leishmania)*: the visceral *L. (L.) donovani* and *L. (L.) infantum*, cutaneous strains from Eurasia and Africa [*L. (L.) major* and *L. (L.) tropica*], and cutaneous and mucocutaneous strains from the Americas *L. (L.) amazonensis*, *L. (L.) mexicana*, and strains of subgenus *L. (Viannia)*. Furthermore, it was also able to distinguish

Fig. 1 Melting temperatures and normalized melting profiles obtained with the HRM assays. **a** Melting temperatures (Tm) for all amplicons and standard species. For all amplicons 25 ng of genomic DNA was used as template. The plots show the mean, minimum and maximum of the Tm-values. Each species was tested in duplicate in three independent experiments. **b** Normalized HRM plots for all amplicons: *(i), (ii)* and *(iii)* represent the normalized melting profile for amplicon 1, amplicon 2 and amplicon 3, respectively. Each sample was tested in duplicate in three independent experiments. For *(i)*, the bundles circled in the blue line, respectively from the left to the right, represent the profiles for *L. (L.) tropica/L. (L.) major, L. (L.) donovani/L. (L.) infantum, L. (L.) amazonensis/L. (L.) mexicana, L. (V.) braziliensis, L. (V.) naiffi, L. (V.) guyanensis/L. (V.) shawi* and *L. (V.) lainsoni*. For *(ii)*, the bundles circled in the blue line, respectively from the left to the right, represent the profiles for *L. (L.) amazonensis, L. (L.) mexicana, L. (L.) infantum, L. (L.) donovani, L. (L.) major* and *L. (L.) tropica*. For *(iii)*, bundles circled in the blue line, respectively from the left to the right, represent the profiles for *L. (L.) infantum/L. (L.) major, L. (L.) tropica/L. (V.) lainsoni, L. (V.) naiffi, L. (L.) donovani, L. (V.) braziliensis/L. (V.) guyanensis/L. (V.) shawi, L. (L.) amazonensis, L. (L.) mexicana*. Abbreviation: RFU: relative fluorescence units

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some species of the subgenus *L.* (*Viannia*) into 3 clusters: *L.* (*V.*) *lainsoni*, *L.* (*V.*) *braziliensis* from the other species in that subgenus (Fig. 1).

Amplicon 2 was especially designed for strains of the subgenus *L.* (*Leishmania*). No amplification for subgenus *L.* (*Viannia*) was observed (Additional file 1: Figure S1). It was able to further distinguish the visceral strains into two different clusters: *L.* (*L.*) *donovani* and *L.* (*L.*) *infantum*. For the cutaneous species, it was able to distinguish the Eurasian and African cutaneous strains *L.* (*L.*) *major* and *L.* (*L.*) *tropica*. However, no difference in melting temperature was detected for the American cutaneous strains *L.* (*L.*) *amazonensis* and *L.* (*L.*) *mexicana* (Fig. 1). Therefore, amplicon 3 was designed for this purpose.

Amplicon 3 was, as predicted, able to further distinguish *L.* (*L.*) *amazonensis* from *L.* (*L.*) *mexicana*. Furthermore, it was able to distinguish *L.* (*L.*) *donovani* from *L.* (*L.*) *infantum*, thereby distinguishing the two species commonly causing VL. It was also able to distinguish the more common causes of CL in Eurasia and Africa: *L.* (*L.*) *major* and *L.* (*L.*) *tropica*.

Differences in amplification efficiencies could be observed when the amplification curves from real-time PCR were analyzed. Cq (quantification cycle) data were used as a relative parameter of quantification for the 3 targets when normalized amounts of samples were compared (see Additional file 2: Figure S2). Using 25 ng as template, for all *Leishmania* species, the amplification reactions produced curves with similar Cq values for amplicon 1 and 3, indicating a good specificity for all species. For amplicon 2, no amplification of DNA from subgenus *L.* (*Viannia*) was observed. In addition, high Cq-values for *L.* (*L.*) *amazonensis*, *L.* (*L.*) *major* and *L.* (*L.*) *mexicana* indicate a lower efficiency compared with the other *L.* (*Leishmania*) species, which also could be used as a parameter of discrimination. For amplicon 1, there was an unspecific amplification of human DNA. However, both the Cq and Tm values were distinct.

For all amplicons, additional strains were tested for specificity for all species, except *L.* (*L.*) *infantum*, *L.* (*L.*) *amazonensis*, *L.* (*V.*) *lainsoni* and *L.* (*V.*) *shawi*, as we did not have additional strains for these species available. For amplicon 1, we found that most strains, except *L.* (*L.*) *major* and *L.* (*V.*) *naiffi* showed a consistent profile for their melting temperatures. For amplicon 2 a relatively large SD was found for *L.* (*L.*) *donovani* (Table 3).

Sensitivity

To evaluate the role of DNA-concentration, we tested the performance of the three amplicons in strains that presented close melting temperatures, based on the specificity assays. The limit of detection (LOD) was estimated using ten-times serial dilutions from 25 ng to 100 fg of DNA from standard strains purified from *in vitro*

Table 3 Average melting temperatures and standard deviations (SD) for standard strains and additional strains (see Table 1 for further information on all strains tested)

Species	Amplicon 1		Amplicon 2		Amplicon 3	
	Average	SD	Average	SD	Average	SD
L. (*L.*) *donovani*	82.47	0.16	85.23	0.30	86.04	0.08
L. (*L.*) *infantum*	82.51	na	85.02	na	85.67	na
L. (*L.*) *tropica*	82.22	0.02	86.47	0.08	85.91	0.07
L. (*L.*) *major*	82.29	0.24	85.90	0.11	85.57	0.04
L. (*L.*) *amazonensis*	83.00	na	84.43	na	86.45	na
L. (*L.*) *mexicana*	83.12	0.00	84.46	0.05	86.88	0.09
L. (*V.*) *lainsoni*	84.39	na	na	na	85.88	na
L. (*V.*) *braziliensis*	83.44	0.18	na	na	85.97	0.04
L. (*V.*) *guyanensis*	83.83	0.15	na	na	86.14	0.11
L. (*V.*) *naiffi*	83.89	0.26	na	na	86.00	0.21
L. (*V.*) *shawi*	83.92	na	na	na	86.20	na

Abbreviation: na not applicable, where only one strain was tested

cultured parasites. For amplicon 1 the LOD was 100 fg for all species tested except *L.* (*L.*) *mexicana*, which had a LOD of 250 fg. For amplicon 2 the LOD was 100 fg for all species tested except for *L.* (*L.*) *mexicana*, which had a LOD of 50 pg. For amplicon 3, the LOD was lower for all species, with 500 fg for *L.* (*L.*) *donovani*, *L.* (*L.*) *mexicana* and *L.* (*L.*) *guyanensis*, while for *L.* (*V.*) *braziliensis* the LOD was 50 pg. The LOD was the same for all amplicons and all species in mixtures of *Leishmania* and human DNA (data not shown). Considering that the *Leishmania* genome sizes range between 29 Mb and 33 Mb, varying from 34 to 36 chromosomes [30], the estimated single-cell DNA is approximately 75 fg. Thus, we can assume that 100 fg is equivalent to just above one parasite in most of the *Leishmania* species.

We further tested if the initial amount of DNA would cause a variation in the melting temperature by serially diluting DNA from 25 ng to 100 fg with or without 25 ng/μl of human DNA. For all three amplicons, the results were the same with or without human DNA. There was a slight variation in melting temperature for all the species, only exceeding 0.2 °C for *L.* (*V.*) *guyanensis* in amplicon 1 with human DNA (Table 4). Furthermore, for amplicon 1 the difference in melting temperature between *L.* (*L.*) *mexicana* and *L.* (*V.*) *braziliensis* could potentially cause a confusion between these two species if melting temperatures were considered for this amplicon, the same can be noticed for *L.* (*L.*) *donovani*, *L.* (*L.*) *braziliensis* and *L.* (*L.*) *guyanensis* for amplicon 3 (Fig. 2).

Efficiency

The reaction efficiency for each amplicon was performed using 25 ng, 5 ng and 50 pg of DNA from each reference *Leishmania* species. Efficiency curves, slopes and R^2

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Table 4 Effect of initial amount of DNA on melting temperature. Mean and standard deviation (SD) for serial dilutions of DNA from standard strains. DNA concentration ranged from 25 ng to 100 fg. Human DNA concentration was kept constant at 25 ng/μl

Strain	Without human DNA	With human DNA
	Mean Tm ± SD (°C)	Mean Tm ± SD (°C)
Amplicon 1		
L. (L.) donovani	82.52 ± 0.10	82.64 ± 0.18
L. (L.) mexicana	83.16 ± 0.10	83.23 ± 0.13
L. (V.) braziliensis	83.31 ± 0.09	83.43 ± 0.11
L. (V.) guyanensis	83.83 ± 0.08	83.96 ± 0.34
Amplicon 2		
L. (L.) donovani	85.44 ± 0.14	85.44 ± 0.14
L. (L.) mexicana	84.40 ± 0.08	84.56 ± 0.07
L. (L.) infantum	84.92 ± 0.11	85.17 ± 0.14
L. (L.) tropica	86.33 ± 0.13	86.73 ± 0.18
Amplicon 3		
L. (L.) donovani	86.17 ± 0.12	86.15 ± 0.12
L. (L.) mexicana	86.77 ± 0.16	86.91 ± 0.16
L. (V.) braziliensis	86.00 ± 0.14	86.17 ± 0.15
L. (V.) guyanensis	85.97 ± 0.13	86.36 ± 0.13

Fig. 2 Effect of DNA-concentration on melting temperature (Tm °C). Dispersion graph of Tm values for amplicons 1, 2 and 3 from a range of 25 ng to 100 fg of DNA template. The plot shows mean, minimum and maximum Tm-values

values were calculated (see Additional file 4: Figure S3). The template points produced Cq values between 20 and 30, where the PCR reactions produced copies of template at exponential ratios and with 95–105% of efficiency. The efficiency patterns were similar for all species testes for each amplicon, except for *L. (L.) mexicana* in amplicon 2. The lower efficiency for *L. (L.) mexicana* for amplicon 2 can be explained by the presence of mismatches comparing in primers (see Additional file 3), as can also be observed in the Cq values showed in Additional file 2: Figure S2.

Validation using naturally and experimentally infected samples

To validate the *aap3*-HRM protocol with other samples than reference strain cultures, we applied this protocol in seven biological samples from naturally infected humans, naturally infected cat, naturally infected sand flies, and experimentally infected BALB/c mice (Table 5). The results of amplicon 1 showed a correlation with those obtained with other diagnostic targets; small subunit ribosomal DNA (SSU rDNA) [26], glucose-6-phosphate dehydrogenase (*g6pd*) PCR [31] and heat-shock protein 70 (*hsp70*) HRM [14]. Although amplicons 2 and 3 were not able to amplify all samples, the positive samples correlated with results obtained with other targets.

Discussion

Diagnosing *Leishmania* infection at the species level is important, as it may guide treatment options and follow-up protocols. Accurate diagnosis is also important in an ecological and epidemiological sense. Unfortunately, in most endemic regions, the leishmaniases are underreported, and the true burden of the disease remains unknown. Techniques that target genomic or mitochondrial DNA by PCR or related techniques are today the most common for detection and identification of *Leishmania* spp. However, there is no gold standard in methods and targets [8]. Common targets such as kinetoplast DNA (kDNA) and SSU rDNA gene have been used for parasite detection. On the other hand, the *g6pd* coding region, internal transcribed spacer 1 (ITS1) rRNA, and *hsp70* coding region have been more commonly used to identify the parasite at the species level [14, 32–38]. Conventional PCR and real-time PCR often followed by sequencing is increasingly being used for detection and identification of *Leishmania* spp. [6, 39]. Both methods are relatively laborious and time-consuming and they require PCR product manipulation, increasing the risk of laboratory contamination. Furthermore, the interpretation of the results requires also considerable skill and experience.

HRM is a relatively new real-time PCR coupled technique, with the first papers appearing in 2003 [40, 41]. The technique identifies nucleotide composition polymorphisms
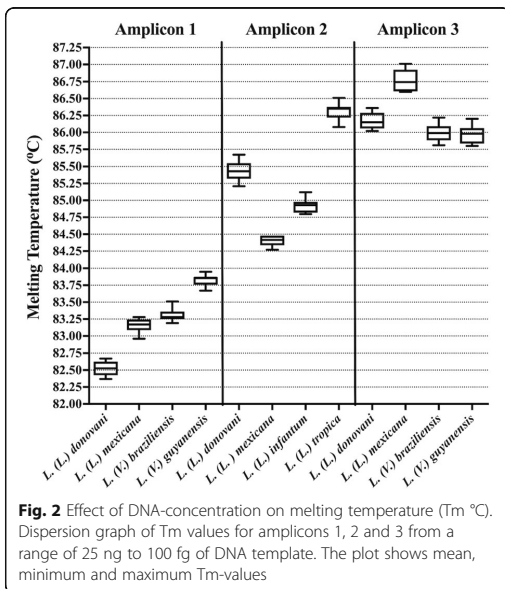
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Table 5 Identification of *Leishmania* in naturally and experimentally infected samples by HRM analysis targeting the *aap3* gene

Sample source	HRM identification			Previous diagnosis	
	Amplicon 1	Amplicon 2	Amplicon 3	Diagnostic method	Species identification
Human[a]	*L. (L.) amazonensis*	negative	negative	SSU rDNA sequencing	*L. (L.) amazonensis*
Human[b]	*L. (L.) infantum*	*L. (L.) infantum*	*L. (L.) infantum*	SSU rDNA sequencing	*L. (L.) infantum*
Cat[c]	*L. (L.) infantum*	*L. (L.) infantum*	*L. (L.) infantum*	*hsp70* HRM	*L. (L.) infantum*
Mouse[d]	*L. (L.) amazonensis*	*L. (L.) amazonensis*	*L. (L.) amazonensis*	SSU rDNA sequencing	*L. (L) amazonensis*
Mouse[d]	*L. (V.) braziliensis*	negative	*L. (V.) braziliensis*	*g6pd* PCR	*L. (V.) braziliensis*
Sand flies[e]	*L. (L.) infantum*	*L. (L.) infantum*	*L. (L.) infantum*	SSU rDNA sequencing	*L. (L.) infantum*
Sand flies[f]	Subgenus *Viannia*	negative	Subgenus *Viannia*	*g6pd* PCR	*L. (V.) braziliensis*

Note: The *aap3* amplicons 1, 2 and 3 of DNA from each sample was submitted to HRM analysis. The result was compared with previous identification performed by SSU rDNA-sequencing [26], hsp70 HRM [14] or *g6pd* PCR [31]
[a]Human paraffin-embedded tissue from Hospital das Clínicas de São Paulo
[b]Human paraffin-embedded tissue from Irmandade da Santa Casa de Misericórdia de São Paulo
[c]Isolated parasites from cat
[d]Experimentally infected BALB/c mice
[e]Naturally infected *Lutzomyia (Lutzomyia) longipalpis*
[f]Naturally infected *Lu. (Nyssomyia) whitmani*

in real-time PCR products. The HRM methodology presents several attractive features: the whole process is performed in a closed-tube system (avoiding contamination in the laboratory) and it is relatively fast and cheap. The analysis may also be automated. The melting temperature generated depends on a range of factors, where GC-content, sequence, and length of the sequence are central. The melting curves can be objectively differentiated from each other by differences in melting temperature and shape [9].

For *Leishmania*, there are few studies utilizing HRM for detection and species identification. One of the earliest reports on the usefulness of HRM in differentiation of *Leishmania* species is by Nicolas et al. [42] who utilized polymorphisms in the coding sequence for minicircle kDNA to differentiate Eurasian and African species, *L. (L.) major*, *L. (L.) donovani* and *L. (L.) tropica*, and *L. (L.) infantum*. Later, Talmi-Frank et al. [43] utilized the ITS1 rRNA region to identify, distinguish and quantify Eurasian and African species, *L. (L.) infantum/L. (L.) donovani*, *L. (L.) aethiopica*, *L. (L.) tropica* and *L. (L.) major*. Both Nicolas et al. [42] and Talmi-Frank et al. [43] only targeted Eurasian and African species, making the approaches valuable in these endemic areas, although of limited value in other endemic areas and in a non-endemic settings where species from all endemic regions could be expected. Pita-Pereira et al. [33] also utilized the minicircle kDNA to discriminate among strains from the subgenus *L. (Viannia)* and *L. (L.) infantum* and *L. (L.) amazonensis*, making it an attractive methodology in an American setting to differentiate strains commonly causing cutaneous, mucocutaneous and visceral manifestations. Ceccarelli et al. [44] also demonstrated an ability to differentiate species of the subgenus *L. (Leishmania)* from *L. (Viannia)*. HRM has also been used for detection of *Leishmania* in sand flies, where Aghaei et al. [45] used ITS1 to identify *L. (L.) tropica* in sand flies. Kuang

et al. [46] utilized the *lack* gene to differentiate five Eurasian and African species from one American species, *L. (V.) braziliensis*. Although they showed its usefulness in clinical samples, the relatively few *Leishmania* species tested made it difficult to conclude if this target could be useful in other endemic areas. With increasing travel to several endemic regions, the correct diagnosis and species identification for the leishmaniases is of paramount interest. Hernandez et al. [36] utilized the ITS1 and *hsp70* to differentiate *L. (L.) mexicana*, *L. (L.) infantum*, *L. (L.) amazonensis*, *L. (V.) panamensis*, *L. (V.) guyanensis* and *L. (V.) braziliensis*. Although they found some ambiguities in species identification between the targets, they showed that ITS1 and *hsp70* had potential as diagnostic targets utilizing HRM. Zampieri et al. [14] showed, in a recent paper, that HRM targeting several polymorphic sites on the *hsp70* coding region could successfully be used to differentiate several Eurasian, African and American species. This makes it the most attractive target reported so far, especially useful in a non-endemic setting where the patient could have travelled to several endemic areas.

In this work, we describe a method for rapid detection and discrimination of most *Leishmania* species. We used the *aap3* coding sequence as target. *Leishmania aap3* coding sequences available in the GenBank and TriTryp databases were aligned to search for relatively conserved regions but present polymorphisms that enable the identification of different species. For this, the available coding sequences of some species from the subgenus *L. (Leishmania)* and *L. (Viannia)* were analyzed *in silico*. The coding sequence for *aap3* is present in two copies and organized *in tandem* in most of the *Leishmania* spp. genomes. For *L. (L.) donovani* and *L. (L.) amazonensis* a 98% identity has been described between the copies of coding regions and a 93% identity between these two

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species [16, 47]. In addition, *aap3* appeared conserved among other *Leishmania* species [17]. Although some species present only one *aap3* gene copy, for example *L. (V.) braziliensis*, this could be due the misannotation in the genome database. Considering these observations, the polymorphisms found between the two copies of the coding region did not affect the Tm analysis.
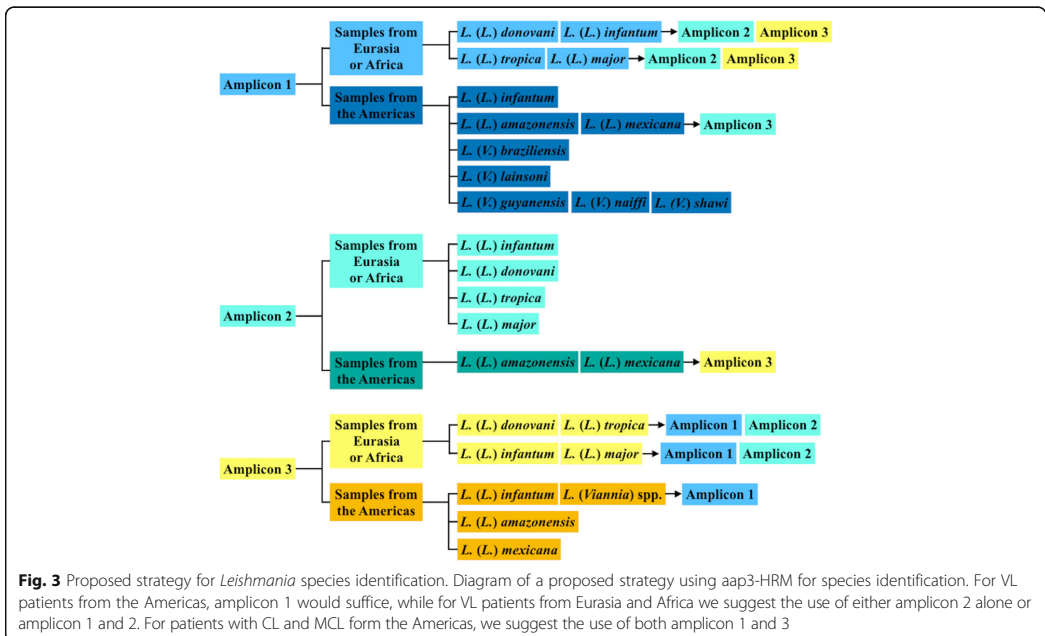
Amplicon 1 was able to discriminate the two strains causing visceral leishmaniasis from strains causing American cutaneous leishmaniasis. Furthermore, it was able to differentiate the American cutaneous strains and several of the strains of the subgenus *L. (Viannia)*. The inability to differentiate the two visceral strains *L. (L.) donovani* from *L. (L.) infantum* was compensated with these strains having distinct Tm's in amplicon 2 and amplicon 3. The same situation is true for the Eurasian cutaneous species: *L. (L.) major* and *L. (L.) tropica* were indistinguishable in amplicon 1, but had distinct Tm's in amplicons 2 and 3. *Leishmania (L.) amazonensis* and *L. (L.) mexicana* are phylogenetically closely related species [48], and the difficulty in differentiating these species for diagnostic purposes has been described elsewhere [14]. Both amplicon 1 and 2 showed similar profiles for these two species, while amplicon 3 was able to distinguish them. Amplicon 2 was specifically designed for the subgenus *L. (Leishmania)*, and there was no amplification of *L. (Viannia)* spp. High Cq values, observed for *L. (L.) amazonensis*, *L. (L.) mexicana* and *L. (L.) major* for amplicon 2, could be explained by

primer-mismatches. Some caution should be taken when analyzing the result in a diagnostic setting, especially if negative. However, taken together the results from all amplicons can be considered to strengthen the diagnosis.

Diagnosing leishmaniasis relies on patient and travel history, clinical information (symptoms and clinical findings), and results from laboratory tests. Thus, for example, the inability of amplicon 1 to reliably distinguish *L. (L.) donovani* from *L. (L.) infantum*, in a patient with suspected visceral leishmaniasis is of little importance in a clinical setting, as the patient would receive the same treatment and follow-up regime regardless. But in an epidemiological setting, it is of importance to distinguish the species from each other to generate reliable data.

In general, multi-copy genes can be expected to yield a higher sensitivity in molecular diagnostics. The assays developed by Talmi-Frank et al. [43], Hernandez et al. [36] and Zampieri et al. [14], all report a limit of detection (LOD) of less than one parasite. Despite that *aap3* only comes in two copies we report a relatively good sensitivity with a LOD of 100 fg for amplicon 1 (just above 1 parasite) to 500 fg for amplicon 3 (approximately 5 parasites). This could probably be improved with a pre-amplification step.

Some HRM assays have found little evidence that DNA concentration of the initial template influences the Tm [36, 46]. However, the initial amount of DNA for

Fig. 3 Proposed strategy for *Leishmania* species identification. Diagram of a proposed strategy using aap3-HRM for species identification. For VL patients from the Americas, amplicon 1 would suffice, while for VL patients from Eurasia and Africa we suggest the use of either amplicon 2 alone or amplicon 1 and 2. For patients with CL and MCL form the Americas, we suggest the use of both amplicon 1 and 3

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this assay influenced the Tm for some of the species. This is in concordance with the findings of Zampieri et al. [14], who also found that initial DNA applied to the assay did affect the Tm for several strains. For the amplicons investigated in our study, this could lead to a misidentification for *L. (L.) mexicana* and *L. (L.) braziliensis* for amplicon 1. However, it should be noted that we propose the use of the three amplicons in the identification to strengthen the diagnostic validity and avoid species misidentification. A strategy for *Leishmania* species identification in patients with suspected leishmaniases is proposed in Fig. 3.

The validation of the target and the technique with clinical and experimentally infected samples from human, cat, mice and sand flies indicated a good correlation with other diagnostic targets and techniques. Not all amplicons were able to yield a result for all the samples, as could be expected due to differences in specificity and sensitivity of the primers, where amplicon 2 was not produced for any species belonging to the subgenus *L. (Viannia)*. Despite the limited number of samples tested, the data indicate the potential of the *aap3*-HRM method to identify *Leishmania* species.

Conclusions

Overall, the *aap3* coding sequence can be a promising target since it is specific and conserved for *Leishmania* spp. The design of the *aap3*-HRM protocol described is a relatively rapid, simple, sensitive and specific method to identify and distinguish several *Leishmania* spp. There is no need for sequencing or gel fractionation to analyze a PCR-product, minimizing the laboratory contamination as all the reactions are performed within a closed tube. The method can be automated, dispensing a trained technician to analyze the results. It also has the potential to quantify parasites present in samples, as it is a real-time PCR technique, able to detect a small number of parasites. In conclusion, the protocol described may offer a relatively low-cost and reliable method for detection and identification of *Leishmania* in biological and clinical samples.

Additional files

Additional file 1: Figure S1. Agarose gel electrophoresis of PCR products. Reactions were performed using TopTaq Master Mix (Qiagen, Hilden, Germany) in a final volume of 25 μl with 200 nM of each primer and 25 ng of genomic DNA as a template. The PCR product was applied to a 3% agarose gel and stained with ethidium bromide. Conventional PCR products for standard strains and controls: A, amplicon 1 (expected 123 bp); B, amplicon 2 (expected 131 bp); C, amplicon 3 (expected 140 bp). (DOCX 8881 kb)

Additional file 2: Figure S2. Specificity using Cq values as parameter. Representative graph of Cq values obtained with HRM assays. The same amount of genomic DNA from all species was used as template to evaluate amplification efficiency. The samples used as negative controls are marked in red. Products generated in late Cq's (>30) were evaluated

in the PikoReal software and revealed that Tm´s and melting profiles were different than for *Leishmania*. The fluorescence generated for these samples was due to unspecific amplification or noise. (DOCX 152 kb)

Additional file 3: Alignment of nucleotide sequences of *aap3* coding regions and primer localization. The underlined sequences indicate the position of the primers used and the grey boxes represent the variable regions found among the *Leishmania* strains based on *in silico* analysis. The numbers at the top of each amplicon are based on the position of the nucleotides in relation to the whole coding sequence in *L. (L.) amazonensis*. (DOCX 21 kb)

Additional file 4: Figure S3. Efficiency curves for all amplicons. Efficiency curves, slopes and R2 were calculated from four species for each amplicon using 25 ng, 5 ng and 5 pg of DNA from each *Leishmania* species. For amplicon 1 and 3 two species of *L. (Leishmania)* and two of subgenus *L. (Viannia)* were selected. Amplicon 2 only amplified *L. (Leishmania)*, and strains from this subgenus were therefore selected. (DOCX 948 kb)

Additional file 5: Statistical analysis of melting temperatures (Tm's) for all amplicons. (XLSX 16 kb)

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