Leishmania infantum: molecular analysis for identification of potential virulence factors and genes of diagnostic use

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie
vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Igor Niederwieser
aus Fällanden (ZH)

Basel, 2004

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät der Universität Basel auf Antrag der Herren

PD Dr. Hans-Peter Beck, Prof.Dr. Niklaus Weiss und Prof.Dr. Marcel Tanner

Table of contents

List of Abbreviations

3’	3 prime end
5’	5 prime end
A	adenine
AA	amino acid
ACL	anthroponotic cutaneous leishmaniasis
AVL	anthroponotic visceral leishmaniasis
AMV	Avian Myeloblastosis Virus
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C	cytosine
CD	cluster of differentiation
cDNA	complementary (to RNA) DNA
CL	cutaneous leishmaniasis
CYT	cytochrome
DCL	diffuse cutaneous leishmaniasis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribunucleotide triphosphate mix
ds	double stranded
DTT	dithiotreitol
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EtOH	ethanol
FCS	fetal calf serum
FT	flow through
G	guanine
GST	glutathione-S transferase
GT	glucose transporter
His	histidine
HIS-	histone
HIV	human immunodeficiency virus
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HSP	heat shock protein

IFA	immunofluorescence assay
IFAT	immunofluorescence assay test
IG	immune globulin
IL	interleukin
IPTG	isopropylβ-D-1-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
L.	*Leishmania*
lacZ	gene encoding β-galactosidase
LPG	lipophosphoglycan
Lu.	*Lutzomyia*
M	marker
MAC	membrane attack complex
MAT-1	metacyclogenesis associated transcript 1
MCL	mucocutaneous leishmaniasis
MHC	major histocompatibility complex
MOPS	3-(N-Morpholino)propanesulfonic acid
miRNA	micro RNA
mRNA	messenger RNA
Mb	mega base
M-MLV	Moloney Murine Leukemia Virus
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
n.d.	not determined
NOS	nitric oxide synthase
nt	nucleotide
ORF	open reading frame
P.	*Phlebotomus*
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pfu	*Pyrococcus furiosus*
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
PKDL	post kala-azar dermal leishmaniasis
PM	peritrophic membrane
RBS	ribosome binding site
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
RP	ribosomal protein
rRNA	ribosomal RNA

RT	reverse transcriptase
S	Svedberg
Sarkosyl	N-lauroylsarcosine
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacryl amide gel electrophoresis
SHERP	small hydrophilic endoplasmic reticulum-associated protein
SL	spliced leader
SN	supernatant
SSC	saline sodium citrate buffer
ss	single stranded
SSH	suppression subtractive hybridization
SSPE	saline sodium phosphate EDTA buffer
STI	Swiss Tropical Institute
T	thymidine
T.	*Trypanosoma*
Taq	*Thermus aquaticus*
TBE	tris borate EDTA buffer
TE	tris EDTA buffer
TEM	transmission electron microscopy
Th	T helper cell
TM	transmembrane domain
tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris·Cl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
U	units
VL	visceral leishmaniasis
v/v	volume per volume
WHO	World Health Organization
w/v	weight per volume
ZCL	zoonotic cutaneous leishmaniasis
ZVL	zoonotic visceral leishmaniasis

Summary

Leishmania infantum is the causative agents of zoonotic visceral leishmaniasis, a fatal disease if left untreated. In Southern Europe, the number of HIV/*Leishmania infantum* co-infections is increasing and becomes an emerging concern for the public health. Dogs represent the main reservoir of this parasite. In order to control canine leishmaniasis, an early diagnosis system is needed because dogs are capable to transmit the parasite, before showing any symptoms of the disease. Here fore, cDNA expression libraries were screened with sera of infected dogs. We selected several antigens reacting with the sera. These were different histones, ribosomal and mitochondrial proteins. Four of them were subcloned into expression plasmids, and recombinantly expressed in *E. coli*. The purified proteins were sent to a collaborating laboratory for further examination.

Although several diagnostic polymerase chain reactions (PCRs) for *Leishmania* species causing human leishmaniasis are published, none of them is able to detect all different species and distinguish among them at the same time. Therefore, a diagnostic PCR was developed using the mini-exon gene (encoding the spliced leader) as template. A restriction fragment length polymorphism (RFLP) scheme was elaborated in order to identify different *Leishmania* species causing human leishmaniasis. The PCR and RFLP scheme is now in routine use at the STI.

In order to identify potential virulence factors, vaccine candidates and drug targets of *Leishmania infantum*, two cDNA subtractions were performed:
(1) A none-infective L. infantum strain was subtracted from a highly infective strain. Several genes which were higher or exclusively expressed in the infective strain were identified. However, association with virulence could not be shown, since the two strains differed not only in virulence but also in growth rate.
(2) Non-infective early promastigote stages were subtracted from late promastigote stages (metacyclic transmission stage) of the same strain. Several genes showing differential expression were selected including genes encoding nutrient transporters, cytochrome b5 and unknown proteins. One of them was the previously described metacyclic specific gene MAT-1. Three of the genes were recombinantly expressed in *E. coli* in order to perform immunization and preliminary localization studies.

1. Introduction

Three different projects were combined within this PhD work:

- Identification of suitable antigens for an early diagnosis system of canine leishmaniasis.
- Development of a diagnostic PCR for human leishmaniasis.
- Identification of potential virulence factors, vaccine candidates and drug targets of *Leishmania infantum*.

1.1. On the biology of *Leishmania*

1.1.1 Taxonomy

Euglenozoa

Flagellated protists

Kinetoplastidae

Kinetoplastidae contain a single mitochondrion of respectable size. Its genome consists of a huge network of maxi and mini circles (the kinetoplast), where RNA editing was first described (Benne et al., 1986). The kinetoplast is associated with the flagellar root.

Trypanosomatidae

Trypanosomatidae are diploid organism, most of them are obligate parasites. Prominent members are *Trypanosoma brucei brucei* (sleeping sickness) and *T. cruzii* (Chagas disease). *T. brucei* is the only trypanosomatid, where sexual recombination was described (reviewed by Gibson and Stevens, 1999). Therefore, clonal distribution is believed to be the rule for all other species.

Leishmania

The genus *Leishmania* contains dozens of species, all of which are obligate parasites. They are transmitted by the bite of sandflys of the genus *Phlebotomus* (Old World) and *Lutzomyia* (New World). Vector stages are flagellated (promastigotes) and live extracellularly within the insect gut, whereas host stages do not posses a flagellum (amastigotes) and multiply intracellularly within phagolysosomes of phagocytes.

1.1.2 The *Leishmania* genome

Leishmania sp. are diploid organisms, containing a genome of extraordinary plasticity: gene amplification under drug pressure yielding in extra chromosomal plasmids and mini-chromosomes was reported for *L. tropica* (Olmo et al., 1995).
The *Leishmania* genome is very GC rich (63%), relatively small (approximately 34 Mb), lacks substantial repetitive DNA, and is distributed among 36 chromosomes pairs ranging in size from 0.3 Mb to 2.5 Mb containing about 8'000 predicted genes (Myler et al., 1999).
Most of the genes are transcribed polycistronic (reviewed by Campbell et al., 2003). The mRNA capping is done by trans-splicing: a capped mini-exon, the spliced leader, is spliced within the polycistronic primary transcript yielding in capped mRNAs containing at the 5' end the 39 bases long mini-exon. The mini-exon gene itself is found in tandem repeats (head to tail) at about 200 copies per genome. This kind of trans-splicing is believed to display a primary capping mechanism (Ullu et al., 1996). Due to the polycistronic transcription, gene expression is believed to be controlled at post-transcriptional level (reviewed by Clayton, 2002).
The 28S rRNA in trypanosomatids is cleaved into two larger (α, β) and several smaller subunits. On total RNA gels therefore 3 prominent ribosomal bands are visible, instead of two as in most other organisms (figure 1).

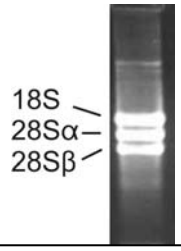


Figure 1: total *Leishmania* RNA

1.1.3. Life Cycle

These protozoan parasites are transmitted by the bite of insect vectors (*Phlebotomus* and *Lutzomyia* species) to the mammalian host, where they multiply in phagolysosomes of phagocytes as amastigotes (stages without a flagellum). In order to multiply within the host, amastigotes infect new macrophages, and horizontal distribution occurs when the insect vector ingests infected macrophages in the blood meal.
In the insect's midgut, a chitin-based peritrophic membrane (PM) is synthesized around the blood-meal. The amastigote parasites transform into procyclic promastigotes (flagellated forms), which are resistant to the digestive enzymes passing across the PM (the vector stages and vector-parasite interactions are reviewed by Sacks and Kamhawi, 2001). Rapid multiplication of the procyclic parasites occurs within the digesting blood meal. ~3 days after feeding, the procyclics transform into the highly motile and fast dividing nectanomads. Now, rupture of the

PM has begun and small amounts of blood are visible in the hindgut (figure 2). Members of the *Viannia* subgenus (containing the species *L. brasiliensis*, *L. guyanensis, L. panamensis* and *L. peruviensis*) are distinguished by the fact, that they do not enter the hindgut.

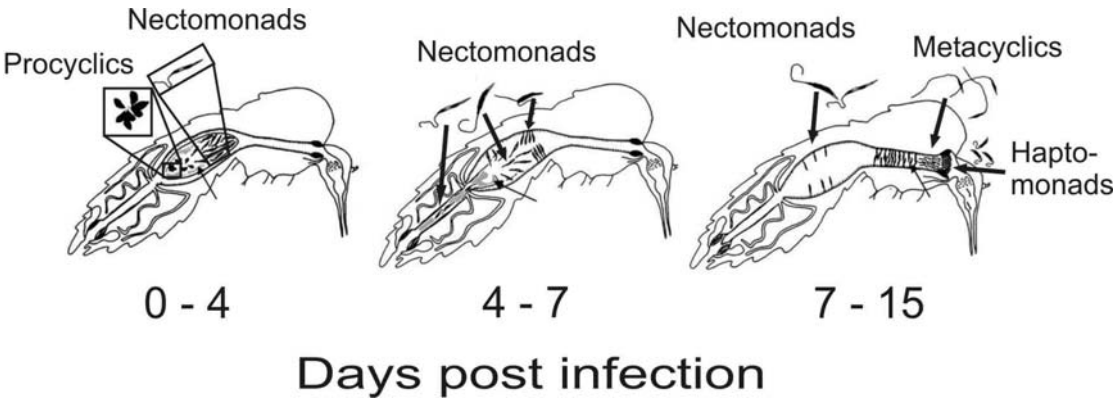


Figure 2: different parasite stages and locations within the course of a *Leishmania* infection in the sandfly vector (modified from Sacks and Kamhawi, 2001).

The parasites escape to the anterior portion of the midgut, and attach with their flagellum to microvilli of epithelial cells. The attachment prevents the parasites to be excreted together with the digested blood meal at ~day 7 after feeding. The binding is mediated by the nectanomad's lipophosphoglycans (LPG, a glycosylphosphatidylinositol-anchored polysaccharide) covering the whole surface of the parasites.

Since LPG differs not only during life cycle (McConville et al., 1992), but also among *Leishmania species*, their attachment is not successful in each vector species. This leads to the fact, that the distribution of *Leishmania species* is restricted to the geographic distribution of a susceptible vector (table 1). It has also been shown, that *Leishmania* infections develop normal in the "wrong" vector, but are excreted within the digested blood meal (Hindle, 1931). Recently, a homologue protein of galactin (a galactose-binding protein found on mammal cells) has been suggested as interaction partner of LPG (Clayton, Swiss Trypanosomatid Meeting, Leysin, 2004).

Next, nectanomads transform into haptomonads and metacyclics. By changing their LPG, they are released from the microvilli. Haptomonads cover the insect's stomodeal valve and excrete a visceous gel like matrix, which restrict their motility. They also excrete a chitinase which partially destroys the valve. The damaged valve is no longer able to close properly during blood meal which facilitates the transmission of infective metacyclic parasites to the vertebrate host (Schlein et al., 1992).

In the host's blood, they are phagocytized by 'professional' phagocytes (neutrophils, monocytes and macrophages). The transformation of metacylics to amastigotes completes the life cycle.

Table 1: The clinical manifestation, geographic distribution and vector usage of different Leishmania species (modified from Sacks and Kamhawi, 2001).

Parasite	**Clinical forms**	**Geographic distribution**	**Proven or suspected vector**
L. donovani	AVL, PKDL	China India, Nepal, Bangladesh East Afrika	*P. alexandri* *P. argentipes* *P. martini, P. orientalis*
L. infantum *L. chagasi*	ZVL, ZCL	Southern Europe Eastern Mediterranean China Central and South America	*P. ariasi, P. perniciosus* *P. perniciosus, P. langeroni* *P. chinesis P. major* *Lu. longipalis*
L. major	ZCL	Middle East South West Asia Africa	*P. papatasi* *P. papatasi* *P. papatasi, P. duboscqi*
L. tropica	ACL	Middle East South West Asia Africa	*P. sergenti* *P. sergenti* *P. sergenti, P. saevus*
L. aethiopica	CL, MCL, DCL	East Africa	*P. longipedes, P. pedifer*
L. mexicana	ZCL, DCL	Central America	*Lu. olmeca*
L. amazonensis	ZCL, DCL	Central and South America	*Lu. flaviscutellata*
L. braziliensins	CL, MCL	Central and South America	*Lu. wellcomei, Lu. carrerai*
L. guyanensis	CL	Central and South America	*Lu. umbratilis, Lu. anduzei*
L. panamensis	CL	Central and South America	*Lu. trapidoi*

AVL: anthroponotic visceral leishmaniasis
PKDL: post kala-azar dermal leishmaniasis
ZVL: zoonotic visceral leishmaniasis
ZCL: zoonotic cutaneous leishmaniasis
ACL: anthroponotic cutaneous leishmaniasis
MCL: mucocutaneous leishmaniasis
DCL: diffuse cutaneous leishmaniasis

1.1.4. Immunology

In order to survive in the vertebrate host, *Leishmania* parasites use several immune evasion strategies: the most prominent is intracellular replication, which hides the parasites by the surrounding host cell from direct contact to the immune system. Many tactics of the parasites most probably are not yet known, however a few of the known strategies are listed in table 2. While these selected strategies are by far not all, the table shows the ability of the parasite to modulate the immune response at many different levels, from complement resistance to triggering the type of immune response towards higher susceptibility of the host. The latter tactic has great impact: a common rule is, that a T_H1 type cytokine milieu yields in clearing of the parasite load, whereas a T_H2 type cytokine milieu leads to susceptibility of the host (reviewed by Alexander et al., 1999; Launois et al., 1998). This makes sense, because T_H1

cytokines are able to activate macrophages, which is the major killing mechanism of *Leishmania* parasites. T_H2 cells induce a humoral response, which has little effect for clearance of intracellular parasites. By down regulating a protective T_H1 response, the parasite therefore succeeds a dramatic intervention.
Nevertheless, antibodies against several *Leishmania* antigens are present in the blood of both, asymptomatic and symptomatic hosts.

Complement
Metacyclic parasites resist complement mediated lysis by inactivation of complement factors and shedding of the membrane attack complex (MAC, Brittingham et al., 1995; Hermoso et al., 1991; Puentes et al., 1990).

Natural killer cells (NK)
Mice deficient in NK were shown to be only modestly less capable of eliminating *L. donovani* infection than control mice; and there was no difference seen in the course of *L. major* infections (Kirkpatrick and Farrell, 1982; Kirkpatrick and Farrell, 1984). It therefore appears that NK are, if at all, only involved in the response to visceral leishmaniasis. Here, it has been shown *in vitro* that *L. donovani* parasites interact with γ/δ^+ human peripheral blood T cells and induce susceptibility to NK cell-mediated lysis (Saha et al., 1999).

Humoral response
While anti-leishmanial antibodies have been shown to lyse promastigotes *in vitro* in the presence of complement, there is little evidence for a role *in vivo*: when antibodies are mixed in large quantities with the parasites before infection, the infectivity of promastigotes was reduced; however, when injected separately, there was no effect on disease development (Anderson et al., 1983). It therefore appears that a humoral response has no central role in clearing the parasite load.

Cytotoxic T-cells (CTL, $CD8^+$ T cells)
CTL have traditionally been associated with resistance to viral infections, but they may also have an important role in immunity to intracellular microbes like *mycobacterium* (Chiplunkar et al., 1986). This may be due to direct cytotoxicity or through cytokine production and subsequent macrophage activation (some $CD8^+$ T cells produce interferon γ (INF-γ) as well tumor necrosis factor α (TNF-α), see below). However, it is unclear, whether $CD8^+$ T cells play a major role in the control of leishmaniasis. Since amastigotes multiply within phagolysosomes, presentation of processed *Leishmania* peptides is mostly restricted to MHC class II molecules. Nevertheless, some parasite proteins might be transported to the cytoplasm, from where they could be loaded to MHC class I molecules, thus allowing activation of

$CD8^+$ T cells.

$CD4^+$ T cells

There are two subsets of $CD4^+$ T cells: T_H1 cells, which provide a cytokine milieu inducing a cellular immune response, and T_H2 cells, which help to establish a humoral response. Naïve $CD4^+$ T cells differentiate into TH1 cells by activation in the presence of interleukin (IL) 12 and IFN-γ, whereas differentiation into TH2 cells occurs by activation in the presence of IL-4. The parasite's ability to repress the IL-12 gene (Carrera et al., 1996), therefore blocks the establishment of a protective T_H1 response. Most probably there are also other parasite and host factors involved in this process.

T_H1 type cytokines such as IFN-γ can activate macrophages in the presence of co-stimuli, in contrast to the inhibitory effects observed by T_H2 some cytokines.

Activated Macrophages

INF-γ (e.g. secreted by a T_H1 cell that recognizes a foreign peptide bound to a macrophage's MHC class II molecule) can activate macrophages. However, INF-γ alone is not capable of inducing leishmaniacidal activity (reviewed by Liew and O'Donnell, 1993). Auto- and parakrine acting co-stimuli (such as TNF-α) synergizing with INF-γ are needed here fore.

The antimicrobial activity is achieved by nitric oxide (NO·, produced by the inducible NO synthase iNOS) and superoxide anion ($\cdot O_2^-$) radicals. Being toxic by itself, it is unclear whether NO· is the final effector mechanism or if other molecules inflict final damage to *Leishmania* parasites. NO· can rapidly react with $\cdot O_2^-$ to form the stable peroxynirtite anion $ONOO^-$ (Beckman et al., 1990). Once protonated, as it seems likely to occur in the acidic phagolysosomes, $ONOO^-$ decays rapidly into the stable radical nitric dioxide anion ($NO_2\cdot^-$), and the highly reactive hydroxyl radical ·OH. Hydroxyl radicals react with almost all molecules found in living cells (at rate constants of between 10^9 and 10^{10} $M^{-1}s^{-1}$, Anbar and Neta, 1967). However, defense mechanisms of the parasites are able to inhibit iNOS expression (Balestieri et al., 2002), MHC-protein synthesis, peptide loading and expression of co-stimulatory molecules (De Souza Leao et al., 1995; Reiner et al., 1987) and to detoxify $\cdot O_2^-$ (superoxide dismutase).

Cytokines secreted by T_H2 cells such as IL-10 inhibit the expression of iNOS. Therefore, a T_H2 response leads to susceptibility of the host. As discussed by Rittig and Bogdan (2000), there is weak evidence that some parasites are found in the host cell's cytosol. These parasites could therefore escape macrophage activation by $CD4^+$ T cells: *Leishmania* derived peptides could no longer be presented on MHC class II, but on MHC class I molecules, which would allow killing of the host cell by $CD8^+$ T cells. However, Moore and Matlashewski (1994) showed that *Leishmania*

parasites are able to block apoptosis of the host cell. Nevertheless, only metacyclics (non-dividing parasites) have been seen within the cytosol, either by electron microscopy (harsh template preparation might result in rupture of phagolysosomes) or by video microscopy (too low resolution for visualizing membranes).

Dendritic cells (DC)

These 'professional' antigen presenting cells play a crucial role in the establishment of an immune answer, due to their ability to stimulate T lymphocytes (in addition to MHC molecules they express co-stimulatory signals to naïve T cells). Therefore, they represent key factors for the T_H1/T_H2 switch (Reid et al., 2000). Furthermore, they are capable to transport live parasites to lymph nodes (Ghosh and Bandyopadhyay, 2003). Only DCs are able to induce a vigorous T-cell immune response to *L. major in vitro* in the absence of exogenous antigen (Moll et al., 1995). Thus, their role in the immune response to *Leishmania* cannot be overestimated.

Table 2: Selected immune evasion strategies of *Leishmania* (modified from Zambrano-Villa et al., 2002)

Evasion strategy	Result	References
Inactivation of complement factors and shedding of the MAC complex	Resists complement	Brittingham et al. (1995) Hermoso et al. (1991) Puentes et al. (1990)
Inhibition of the macrophages' NOS	Inhibits the oxidative burst	Balestieri et al. (2002)
Inhibition of MHC-protein synthesis, peptide loading and expression of co-stimulatory molecules	Reduces macrophage antigen presenting ability	Reiner et al. (1987) De Souza Leao et al. (1995)
Prevention of apoptosis of infected cells	Extends the life-span of infected macrophages	Moore and Matlashewski (1994)
Repression of the IL-12 gene	Blocks the protective Th1 response	Carrera et al. (1996)

IL-12: interleukin 12
MAC: membrane attack complex
MHC: major histocompatibility complex
NOS: nitric oxide synthase

1.1.5. Leishmaniasis

The disease

Leishmaniasis (briefly reviewed by Herwaldt, 1999) is endemic in areas of the tropic, subtropic and southern Europe and from rural to periurban areas. The clinical manifestation ranges from cutanous and mucocutanous leishmaniasis (replication of the parasite within macrophages located in the dermis, and naso-oropharyngeal mucosa, respectively) to visceral leishmaniasis (replication of the parasite within the bone marrow, spleen or liver). Both, cutaneous and visceral leishmaniasis, are seen in a broad range of severity and manifestations.

Cutaneous leishmaniasis is manifested as skin lesions, whereas visceral leishmaniasis (also known as kala-azar) is associated with fever, hepato-splenomegaly, anaemia and many more life threatening symptoms. Post kala-azar dermal leishmaniasis, a syndrome that develops at variable times after resolution of kala-azar, is manifested by skin lesions and can be associated with relapse of visceral leishmaniasis. People suffering from this disease serve as reservoir hosts.

Some forms of leishmaniasis represent zoonotic disease (as *L .infantum*), whereas others represent anthroponotic disease (as *L. donovani*). Infection of different species results in different symptoms (table 1). However, in immunosuppressed patients, the disease may develop unusual manifestations (as example the patient

described by Angarano et al., 1998). In 1990, VL was the fourth most common opportunistic parasitic disease in HIV-positive individuals in Spain after pneumocystosis, toxoplasmosis, and cryptosporidiosis (Montalban et al., 1990).

Diagnosis

There are many tools for diagnosis of leishmaniasis, each differing in sensitivity, specificity and cost intensity (reviewed by Guerin et al., 2002; Singh and Sivakumar, 2003):

- Detection of parasites, either directly by microscopy (low sensitivity) or after cultivation *in vitro* and *in vivo* (time consuming).
- Serology: IFATs and ELISAs using pro- and amastigotes (better specificity) as antigens have been successfully evaluated; however antigen production is cost intensive. Several recombinant parasite proteins have been tested as antigens in ELISAs and direct agglutination tests. Recombinant K39 (a parasite homologue of kinesin) showed best results, and is commercially available for diagnosis of VL. While sensitivity and specificity appears to be lower than when using whole parasites as antigens, the cheap production costs represent a great advantage.
- PCR using ribosomal genes, repetitive DNA elements, kDNA or other repetitive genes have been successfully used with good sensitivity and specificity.

Since parasites are not equally distributed within the host's body, detection of parasites by microscopy (with or without cultivation) or by PCR is dependent on biopsies, which can be painful and dangerous for VL patients. However, in immunosuppressed individuals, parasites are found with relatively high frequency within the blood, thus facilitating the diagnosis by PCR.

Treatment

Yet obtainable drugs and drugs in stages of testing are reviewed by Berman, (2003), Croft and Coombs (2003), Rosenthal and Marty (2003). Both, VL and CL is traditionally treated with pentavalent antimonials over a time course of 20-40 days, yielding in adverse reaction of these toxic compounds, especially in the presence of HIV co-infections. Antimonial resistance has strongly arisen in some areas in India, where VL is endemic (Sundar, 2001). There are alternative drugs such as Amphotericin B (need for intravenous infusion), Pentamidine (increasing treatment failures in India) and Miltefosine (the only oral antileishmanial agent available so far). Adverse reactions caused by all these drugs, high treatment costs (lowest for Miltefosine) and increasing drug resistance demand the development of new drugs, some of which are in the pipeline. However, in respect to drug resistance, there

cannot be enough alternative drugs.

Vaccines

At present, there is no vaccine in routine use; however, several are in different stages of testing (reviewed by Ghosh and Bandyopadhyay, 2003). It is crucial for a successful vaccine, that a protective T_H1 type immune answer is induced (either by the adjuvant or by the vaccine itself).

- Live vaccines

  In the age of genetically engineering, a practice used for centuries might have to potential for a come back: 'leishmanization'. People were inoculated with live *L. major* at an aesthetically acceptable site resulting in live long protection from CL caused by *L. major* and *L. tropica* (Modabber, 1989; Nadim et al., 1983). A major draw back of leishmanization was the high risk of complication and the development of a lesion and a scar after healing at the site of inoculation. This problem might be overcome by using genetically altered parasites. Two approaches have already successfully been performed: introduction of suicidal cassettes (Titus et al., 1995) and/or markers which are responsive to external signals for their destruction (Sah et al., 2002; Yan et al., 2001). Another problem when using a live *Leishmania* vaccine, are the high production costs. Whether leishmanization works also for VL has not yet been shown. Here, development of a clinical episode would be fatal.

- Killed vaccines

  Killed promastigotes have been used for CL with different success: while there was no protection in Iran, there were promising results in Ecuador and Brazil.

- Recombinant antigens

  The first tested antigen was GP63 and showed promising results in the animal model, whereas responses of human T-cells were variable. The leishmanial elongation initiation factor (LeiF) is considered to be a promising vaccine candidate due to its ability to induce TH1 cytokines in humans, as well as the *Leishmania* homologue of the receptor for activated C kinase (LACK, in particular when IL-12 is used as adjuvant).

- Synthetic peptides

  Several peptides have been successfully used in the animal model, such as peptides derived from GP63.

- DNA vaccines

  Various naked DNA vaccines have been tested in animal models yielding in promising results, such as the glucose regulated protein, a member of the 70kDa heat-shock protein family.

However, in most experimental systems, adjuvants are essential to induce protective immunity. Unfortunately, the most effective adjuvants generally cause strong inflammation, which may be needed for adjanticity, but may exclude their use in humans due to unacceptable side effects. This problem might be overcome when using dendritic cell vaccination (Moll, 2003).

1.2. Basic molecular biological methods used during the PhD project

Most of the routine methods were performed after the standard protocols described by Sambrook and Russel (2001).

1.2.1. RNA

Isolation

RNA was either harvested according to the classical method of Chomczynski and Sacchi (1987) or using TRIZOL (Invitrogen) reagent. For long term storage, RNA was stored as a precipitate at -20°C, otherwise, RNA was dissolved in TE (10mM Tris·Cl pH 8, 0.5mM EDTA) and stored at -80°C.

Northern blots

Northern blots were performed using either formaldehyde, or preferentially glyoxal/DMSO as denaturating agents. For highly expressed genes, such as histones, even non-denaturating agarose gels worked very well (figure 3).

PEG purified (refer appendix 6.A4) dsDNA probes were ^{32}P-labeled using the High Prime Kit (Roche) and α^{32}P-dCTP and hybridized overnight at 42°C. Beside Ultrahyb (Ambion), the two different hybridization buffers described in appendix 6.A1 were successfully used.

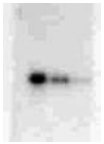


Figure 3: Northern blot of histone H2A using a non-denaturating agarose gel.

Reverse Transcription

Three different derivates from Moloney Murine Leukemia Virus (M-MLV) RT were successfully used: although unmodified M-MLV exhibits a weak RNase H activity, it showed to be the better choice for obtaining spliced leader (SL) cDNA compared to Superscript II (Invitrogen). Superscript II is M-MLV containing a deletion of the amino acids responsible for RNase H activity. Unfortunately, the deletion not only eliminates

the RNase H activity, but also lowers the reverse transcriptase activity. Best results were achieved when using an RT where the RNase H activity was destroyed by a single point mutation, as in M-MLV, RNase H^- (Finnzymes). RNase A and H digestion after reverse transcription substantially increased the yield of a subsequent PCR.
The protocol for reverse transcription and PCR amplification of SL cDNA is shown in appendix 6.A2.

1.2.2. DNA

Minipreps

Minipreps were performed according to the alkaline lysis method. If plasmids were harvested from nuclease rich *E. coli* strains (as expression strains like BL21, Stratagene), a phenol extraction was performed prior to ethanol precipitation, and otherwise this step was omitted.

Isolation of genomic DNA

Parasite DNA (from patients and in vitro cultures) was isolated using SDS mediated cell lysis, proteinase K digestion, phenol extraction and ethanol precipitation (appendix 6.A3).

PCR

PCR was performed using Taq DNA polymerase. If blunt ended PCR products were required, a proportion of 8:1 Taq:Pfu in Taq buffer was used. Since the *Leishmania* genome is very GC rich, addition of DMSO (2-12%) was often necessary. Other melting-point reducing agents like betaine or glycerol did not show beneficial effects. For amplification of difficult templates, the concentration of PCR additives like Tween20 (0.1-1%) and BSA (0.1-1g/l) had to be optimized.

Purification of PCR products

PCR products were purified using phenol extraction and ethanol precipitation. If small fragments, such as primers or restriction fragments had to be removed (as prior to ligation or sequencing), a polyethylene glycol (PEG) precipitation was performed (appendix 6.A4). Gel purification was avoided due to its low yield.

Sequencing

Sequencing was done using Perkin Elmer's DNA sequencing kit according to the manufacturer's protocol. Due to the high GC content of the intron and non-transcribed spacer sequencing of the mini-exon gene showed to be difficult. The high GC content might yield in a stable secondary structure of the intron (since its secondary structure is required for trans-splicing). Addition of DMSO could not

improve the quality of the sequences, in contrast to cloning the PCR fragments prior to sequencing, and the adaptation of the sequencing procedure described in appendix 6.A5.
Sequencing of large inserts was done as shown appendix 6.A6: the plasmids were cut with polylinker restriction enzymes, which also cut within the inserts. After religation and retransformation, the obtained minipreps contained deletions. Therefore, it was possible to sequence large insert by solely using vector primers and without purchasing an erase a base kit. Theoretically it would be possible to sequence directly after religation, but this simplification was not tested.

Ligation

Ligations were performed using either T/A cloning kit (Promega), cycle restriction ligation (appendix 6.A7) or Quick DNA ligase (New Egland Biolabs).

E. coli Transformation

Electro-poration: since input and harvest is not very profitable using large scale preparation of electro-competent cells, a fast small scale approach was used (appendix 6.A8).
Chemo-transformation: this was the method of choice, if many transformations had to be performed with the same *E. coli* strain. The Inoue method (appendix 6.A9) was used to prepare chemo-competent cells resulting in 10^8 to 10^9 colonies/µg supercoiled pUC18 DNA. The advantages compared to electroporations were: many more aliquots of competent cells after large scale production, ligation mixes could directly be transformed without the need of purification and many samples could be transformed at the same time with less handling.

1.2.3. Proteins

Expression

Most of the proteins were expressed as glutathione-S transferase (GST) fusion proteins. However, none of them was in the soluble fraction. Neither lowering the IPTG concentration for induction, nor the temperature for protein expression, nor adding ethanol for chaperon induction, nor combining all variations could change this feature.
For expression of genes selected during the cDNA subtraction, a modified pGEX (GST fusion) vector was used (appendix 6.A10).

Purification

Recombinant proteins containing a His-tag were preliminary purified as inclusion bodies (appendix 6.A11). Since renaturation did not work for any protein, the inclusion bodies were subsequently purified using Ni-NTA agarose (Qiagen) under denaturing conditions. If the protein was badly soluble in urea, 8M urea was replaced with 6M guanidine chloride in all solutions.
Recombinant MAT-1 was affinity purified under mild denaturing conditions using sarkosyl and glutathion sepharose (appendix 6.A12).

Immunofluorescence assays (IFAs)

Parasite cultures were spun, resuspended in fetal calf serum (FCS) and spread on glass slides. After drying, the slides were fixed for 10min at -20°C using acetone alone or 1:1 methanol:acetone. The slides were partitioned using a hydrophobic pen allowing the use of only 10µl of serum or secondary antibody.

2. Development of an early diagnosis test system for canine leishmaniasis caused by *L. infantum*

2.1. Background

In Southwest Europe, HIV and *L. infantum* co-infections are an emerging concern for public health (see table 3). Although cases of co-infection have so far been reported in 33 countries worldwide, most of the cases have been notified in south-western Europe (France, Italy, Portugal and Spain). The cases reported in these countries between January 1996 and June 1998 represent 49.8% of the total number of cases (1440) reported since 1990 (http://www.who.int/emc-documents/leishmaniasis/docs/wholeish200042.pdf).

Table 3: Leishmania/HIV co-infections, total number of reported cases.

Years	Spain	France	Italy	Portugal
1990-98	835	259	229	117
1996-98	412	132	85	88

The usual clinical features of VL such as fever, weight loss, and swelling of the liver, spleen and lymph nodes, are not always present or may be hidden by other associated opportunistic infections with similar symptoms. However, most of the co-infected patients who participated in a WHO study (84.9%) showed the usual clinical features (table 4, http://www.who.int/emc-documents/leishmaniasis/docs/wholeish200042.pdf).

Table 4: clinical manifestations in *Leishmania*-HIV co-infected patients participating in a WHO study (1990-98).

Clinical features	Number	%
Visceral – typical	736	84.89
Visceral – atypical	82	9.46
Cutaneous	36	4.15
Others	6	0.69
Mucocutaneous	4	0.46
Mixed	3	0.35
Total	867	

In the Mediterranean, dogs represent the main reservoir of this parasite (see references in chapter 2.3). Therefore, control of human leishmaniasis must not exclude control of canine leishmanisis: reduction of prevalence in the reservoir is

expected to lower the transmission rate to humans. However, due to the increasing number of infected humans, parasitized human hosts may also contribute substantially to the transmission. In order to control canine leishmaniasis, an early diagnosis system is needed because dogs are capable to transmit the parasite, before showing any symptoms of the disease.
For mass use (in the Mediterranean, there are many street dogs), diagnosis by PCR or microscopy is not feasible. Existing serological tests for canine leishmaniasis are not able to detect parasites within early stages of the disease. Furthermore, some reliable diagnostic methods (such as IFAT based on whole parasite antigens) cannot be used in masses, due their high production costs. Thus, a cheap serological test capable of diagnosis of early canine leishmaniasis is needed. In order to identify antigens suitable for such a diagnostic tool, we screened *L. infantum* cDNA expression libraries. These would be cheaply produced as recombinant proteins, which then could be used as antigens for Western blots, ELISAs or direct agglutination tests.

2.2. Methods

In order to identify antigens suitable for diagnostic use, several cDNA expression libraries were constructed and screened with sera of *L. infantum* infected dogs. Preliminary, Clontech's Capfinder Kit was used combined with SMART λ phages. (The capfinder method uses the feature of certain reverse transcriptases to add several G's at the 3' end of the cDNA when reverse transcribing capped mRNAs in the presence of Mn^{2+} ions. This allows adaptor ligation and subsequently PCR amplification of full length cDNA.) However, beside that the cDNA obtained after PCR looked strange (a smear on the gel, from bottom to top), no λ clone reacted with dog sera of *L. infantum* infected dogs. The simplest explanations for this finding were, that expression in *E. coli* failed due to presence of stop codons and absence of ribosome binding sites (RBS) within the 5' UTR of the cloned cDNAs: in prokaryotes, a defined sequence (the RBS) has to be located within a distinct distance from the ATG for proper translation. Since most of the *Leishmania* full length cDNA apparently lacked the RBS, the obtained cDNA library was not suitable for a serum screen.
Therefore, both cDNA amplification and vector were changed. We used the spliced leader as primer binding site instead of the CAP finder adaptor. Furthermore, the amplified cDNA was restriction digested and non-directionally ligated into λgt11 DNA containing 3 different adaptors (appendix 6.A10), resulting in 6 frame expression of all cDNA fragments as lacZ fusion proteins.

2.3. Identification of recombinant antigens from *Leishmania infantum* suitable as early diagnostic tool

Identification of recombinant antigens from *Leishmania infantum* suitable as early diagnostic tool.

Igor Niederwieser, Jutta Marfurt, Sylvia Steiger, Jose Alunda, Charles Jaffe, Hans-Peter Beck

Swiss Tropical Institute, Switzerland
University of Madrid, Spain
Hebrew University Jerusalem, Israel

Abstract

Early diagnosis of canine leishmanaiasis caused by the protozoan parasite *Leishmania infantum* remains an important requirement for the control of the disease. In order to identify antigens suitable for such an early immunological diagnosis system we generated a phage lambda cDNA expression library and this was screened using sera from naturally and artificially infected dogs. With this approach we selected 8 antigens which strongly reacted with naturally infected dog sera. Subsequent sequencing identified these as histones, ribosomal proteins, and a mitochondrial protein. Upon generation of recombinant protein Western blot analysis was performed to assess the specificity and reactivity of the identified antigens with sera of infected and healthy control dogs. Three of these antigens were further selected for the development of a dipstick test.

Introduction

Domestic dogs represent the main reservoir of *Leishmania infantum* (Old World) and *L. chagasi* (New World) and therefore play a key role in the transmission of the disease to humans. A way to control the disease would be to treat dogs found infected with *Leishmania* rapidly. However, dogs showing already symptoms might have been transmitting for an extended period [1, 2] and therefore a diagnostic assay is need that quickly can determine infectivity in subpatently infected dogs. Although usually transmitted by the bite of an infected sandfly vector, direct dog to dog transmission [3] and blood transfusion routes [4] have been reported.

Because the metacyclic stage of *Leishmania* parasites is responsible for the transmission from the insect vector to the vertebrate host and the establishment of a new infection, this stage is the first to be seen by the host's immune system. Once phagocytised by macrophages, metacyclic stages develop into amastigotes and multiply within phagolysosomes. In order to identify antigens suitable for an early diagnostic test, a cDNA expression library of *L. infantum* metacylic stages was generated and screened with a serum pool from *L. infantum* positive dogs.

Most of the mRNAs in trypanosomatids contain a conserved 5' sequence, the spliced leader [5]. And we exploited this feature to PCR amplify the cDNA obtained after reverse transcription of total RNA using spliced leader sequence specific primers. In order to construct an expression library, the PCR products were restriction digested and ligated into a λ phage which was previously modified with adaptors in three reading frames. Since the cDNA was not directionally cloned into the phage vector, all 6 frames of the PCR fragments were expressed in this λ library.

Screening the λ library we identified several fragments of histones, of ribosomal proteins, and a mitochondrial protein. In order to perform Western blot analysis using sera of artificially and naturally infected dogs, some of the identified antigens were sub-cloned in expression plasmids and expressed in *E. coli*, though we failed to express two of the gene fragments. However, Western blot results of the other clones were promising, in particular those obtained with clones representing epitops within the histones H2A and H4.

Materials and methods

Parasite culture

Promastigotes of the *L. infantum* strain LEM 768-A/ST were cultivated *in vitro* at 27°C in a 1:1 mixture of SDM-79 [6] and Schneider's medium [7] containing 15% [v/v] heat inactivated fetal calf serum. 10ml cultures were inoculated at a density of 10^6 parasites/ml and harvested 14 days later.

cDNA preparation

After washing the parasites in phosphate buffered saline (PBS), total RNA was extracted after the method of Chomczynski and Sacchi [8] using 0.5ml guanidinium thiocyanate solution per ml *in vitro* culture. Approximately 2µg RNA were reverse transcribed with 200 U Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) using the primer 5'-CGACTGTACGTGAATTCGC$(T)_{20}$ (all oligonucleotides were manufactured by Invitrogen). Prior to PCR, RNA was removed by RNase A (Boehringer) digestion and cDNA was PCR amplified using the primer above and the spliced leader primer 5'-AACTAACGCTATATAAGTATCAGTTTCTGTACTTTATTG. Taq DNA polymerase (Invitrogen) was added in presence of 10% dimethyl sulfoxide (DMSO) and the amplification was done under the following conditions: 25 cycles 30sec 94°C, 30sec 54°C, 2min 72°C. The PCR products (figure 1A) were NlaIII (all restriction enzymes obtained from New England Biolabs) digested and purified by phenol extraction, and precipitated with ethanol and polyethylene glycol (PEG)[9].

Lambda expression libraries

Three different adaptors to provide three reading frames were ligated with T4 DNA ligase (New England Biolabs) to EcoRI cut and dephosphorylated λ-gt11 DNA (Amersham). All adaptors contained phosphorylated 5' EcoRI overhangs, 3' NlaIII overhangs and 6x His tags. The adaptor in frame 1 was made with the two oligos 5'-P-AATTCCACCATCACCATCACCACCATG and 5'-GTGATGGTGATGGTGG, frame 2 was made with 5'-P-AATTCCACCATCACCATCACCATGCATG and 5'-CATGGTGATGGTGATGGTGG, and frame 3 was made with 5'-P-AATTCCACCATCACCATCACCACATG and 5'-P-TGGTGATGGTGATGGTGG. After the ligation, the remaining free adaptors were removed by PEG precipitation and the NlaIII digested cDNA was ligated

into the λ-gt11 DNA using T4 DNA ligase. The phages were packed with the Gigapack® III Gold Packaging Extract (Stratagene). The library was amplified in Y1090r- *E. coli* strain (Stratagene) after the protocol of Sambrook and Russel [10].

Screening of libraries

The libraries were screened with a serum pool of *L. infantum* positive dogs (kindly by Felix Grimm, University of Zürich) after the protocol of Sambrook et al. [11]. Briefly, the phages were plated at a density of 10^4 plaque forming units on 30ml Petri dishes and incubated for 4 hours at 42°C. The plates were overlaid with nitrocellulose sheets (Hybond N, Amersham) which were previously saturated in 10mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The plates were incubated for another 6 hours at 37°C. The sheets were removed afterwards and blocked with PBS containing 5% not fat milk powder. A serum pool from *L. infantum*-positive dogs was pre-adsorbed with crude *E. coli* extract and the antibody screen was conducted with 1:500 diluted sera. After 2 h, sheets were washed and incubated in a 1:2000 dilution of an alkaline phosphatase labeled goat anti-dog IgG antibody (Southern). Unbound antibody was washed away and recognized clones were visualized by a BCIP/NBT color reaction (Blotting substrates, BioRad). Areas of positive clones were identified on the master plates, phages were re-plated at a lower density, and screened again with the same serum pool in order to isolate single clones. Specificity of the recognition was assessed using a serum pool of uninfected control dogs (kindly by Felix Grimm, University of Zürich). Single clones which were negative with control sera but positive with sera from infected dogs were PCR amplified directly from the plagues. PCR was performed to amplify the inserts using the λgt11-primers 5'-GGTGGCGACGACTCCTGGAGCC and 5'-GACACCAGACCAACTGGTAATG, and the PCR products were directly sequenced on an ABI automated sequencer.

Subcloning in expression plasmids

The PCR products were either cloned into pQE32 containing a 6x His tag (Qiagen) or into the glutathion-S-transferase (GST) fusion vector pGEX-1 (Amersham). Prior to ligation into the pQE vector, PCR products of λ clones were NlaIII digested, their ends polished using *Pfu* DNA polymerase (Promega), and purified by phenol extraction with subsequent ethanol and PEG precipitation. Fragments were then cycle restriction ligated [12] using SmaI and T4 DNA ligase. For the ligation into the pGEX vector, PCR products

and vector were EcoRI cut and the vector was dephosphorylated using calf intestinal alkaline phosphatase (Promega). Before ligation with T4 ligase, both vector and insert were purified by phenol extraction and subsequently precipitated with ethanol and PEG. pQE clones were expressed in M15 cells (Qiagen) and pGEX clones in BL21 cells (Amersham).

Expression in E. coli

Expression clones were grown in Luria-Bertani medium (LB) at 37°C until the optical density reached 0.8 and protein expression was induced with 0.5mM IPTG for 4 hours at 37°C. All proteins were found in inclusion bodies. Because pGEX inserts still contained the λ adaptors, proteins expressed from pQE and pGEX both contained 6 x His tags and were purified with Ni-NTA Agarose (Qiagen) under denaturing conditions.

Western Blots

Recombinant proteins were run on Tricine-SDS polyacrylamide gels [13] manufactured by Bio-Rad (Ready Gel Tris-Tricine Gel, 10–20%). Heat shock protein 70 [14] (HSP-70, kindly obtained from C. Jaffe), and the identified ribosomal protein (RP) L7 and RP-L10 were on the same gel, as well as the selected proteins histone (HIS) H4, HIS-H2A and RP-L7a (figure 1B). After electrophoresis, proteins were transferred onto nitrocellulose membranes. After blocking with 5 % milk powder in TNT (10 mM Tris/HCl pH 8, 150mM NaCl, 0.05% Tween 20) over night at 4°C, blots were incubated for 3 hours at room temperature with a 1:500 dilution (TNT, 1% milk powder) of positive serum pool or a 1:200 dilution of single serum samples. An alkaline phosphatase labeled goat anti-dog IgG at 1:5000 (TNT, 0.1% milk powder) was used as a secondary antibody. Blots were revealed with BCIP/NBT blotting substrate (Bio-Rad).

Results and Discussion

The development of an early diagnostic assay for canine leishmaniasis would be a major step forward in the control of the disease which is transmitted by sand flies from infected dogs. Few assays are currently being tested either based on the detection of specific DNA or on the detection of antibodies against certain antigens elicited early during the infection. In order to identify

additional antigens useful for the development of an early antigen based assay, we created a cDNA expression library and screened it with sera from dogs naturally infected with *L. infantum*.

Spliced leader cDNA

For the synthesis of cDNA we made use of the fact that all transcribed messengers in the trypanosomidae contain a spliced leader sequence, which increased the yield of specific transcripts. The size distribution of the amplified spliced leader cDNA that we used for construction of the λ libraries is shown in figure 1A. Size distribution of eukaryotic mRNA usually ranges from 0.5 to many kb with most of the mRNA between 2 and 3kb. The spliced leader cDNA we prepared showed an acceptable distribution with a slight under-representation of mRNAs longer than 3kb, but still representing a large fraction of the metacyclic parasite's transcriptome, which was screened with dog sera.

Serum screen

With the screen using sera from *Leishmania* infected dogs we identified fragments of the following genes: histone (HIS) H2A, histone H3, and histone H4. Furthermore, we identified the ribosomal proteins (RP) L7 (2 fragments), L7a, L10, and the mitochondrial 2-oxoglutarate carrier. It seems to be surprising that no surface proteins have been identified, but only highly expressed cytoplasmatic, nuclear, or mitochondrial proteins. One might speculate that because most of the metacyclic stages are killed immediately after transmission [15] into the mammalian host, and because the amastigote stages are intracellular, the antibody response might be mostly induced by antigens descending from lysed parasites. Therefore one might indeed expect antibodies against common and immunogenic antigens regardless of their cellular localization. Nevertheless, protection against cutanous leishmaniasis in vervet monkeys using recombinant HIS-H1 [16] and immunity in mice against visceral leishmaniasis using DNA encoding for the ribosomal protein P0 as vaccines [17] has been reported. The positive effects of both immunizations most probably were derived from cell-mediated immunity.

Recombinant proteins

The selected epitopes of HIS-H2a, H4 and one of the two epitops of RP-L7 were successfully expressed as GST fusion proteins. The ribosomal proteins RP-L7a and L10 were expressed in the pQE system. However, sub cloning of HIS-H3 and one epitope of RP-L7 failed. Furthermore, no visible amount of

protein was obtained after induction of the mitochondrial 2-oxoglutarate carrier expression. All other proteins were expressed at high levels and found in inclusion bodies. These antigens were tested in 4 artificially infected dogs, from which sera were collected 60, 120, 180 and 330 days post infection (table 1). HSP-70 showed best results: all sera recognized this antigen 60 days post infection. Also the histones H2A and H4 showed good results, in contrast to the ribosomal proteins. Although only one strain of *L. infantum* was used to infect the dogs, the results seemed to be transferable to naturally infected dogs: here, HSP-70 and the two histones were recognized very well, in contrast to the ribosomal proteins. 5 of the western blots are shown in figure 1B. However, the starting point of the infection is unknown in naturally infected dogs.

Conclusions

In *Leishmania* diagnosis, a battery of tools exists [18] including PCR, ELISA, IFAT, dipstick or agglutination tests, but there are no gold standards. Therefore, the development of a diagnostic tool of high sensitivity, specificity and of low costs remains of high priority.

Different methods for detection of *L.infantum* have been compared [19]. PCR, ELISA and a dipstick (Leishmania RAPITEST), both based on recombinant K39 protein [20] were tested. The serological tests were more sensitive than PCR, suggesting that serology should preferently be used as method for diagnosis, also because of the lower costs and easier implementation.

IFAT using amastigotes as antigen showed great sensitivity and specificity [21]. However, high production costs contradict its application in the field.

Therefore, a diagnostic test based on dipstick, agglutination or ELISA technology using 2 or more different recombinantly expressed epitopes of *L. infantum* proteins might be the best solution. *Leishmania* possesses high variability in HIS-H2A [22] and H4 genes [23]. A diagnostic test using solely histones as antigens therefore might fail to detect all *L. infantum* strains.

Nevertheless, the two selected histones and HSP-70 showed promising results in all of our experiments, in contrast to the ribosomal proteins L7, L7a and L10. However, one epitope of RP L7 was not expressed recombinantely during this work as well as HIS-H3, which could show similar results as HIS-H2A and H4. Further, the mitochondrial oxoglutarat carrier should not be forgotten only because protein production failed in this attempt.

References

1. Molina R, Amela C, Nieto J, et al. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Trans R Soc Trop Med Hyg **1994**;88:491-3
2. Guarga JL, Lucientes J, Peribanez MA, Molina R, Gracia MJ, Castillo JA. Experimental infection of *Phlebotomus perniciosus* and determination of the natural infection rates of *Leishmania infantum* in dogs. Acta Trop **2000**;77:203-7
3. Gaskin AA, Schantz P, Jackson J, et al. Visceral leishmaniasis in a New York foxhound kennel. J Vet Intern Med **2002**;16:34-44
4. Owens SD, Oakley DA, Marryott K, et al. Transmission of visceral leishmaniasis through blood transfusions from infected English foxhounds to anemic dogs. J Am Vet Med Assoc **2001**;219:1076-83
5. Boothroyd JC, Cross GA. Transcripts coding for variant surface glycoproteins of *Trypanosoma brucei* have a short, identical exon at their 5' end. Gene **1982**;20:281-9
6. Brun R, Schonenberger. Cultivation and *in vitro* cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop **1979**;36:289-92
7. Cunningham I. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J Protozool **1977**;24:325-9
8. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **1987**;162:156-9
9. Hartley J, Bowen H. PEG precipitation for selective removal of small DNA fragments. Focus **1996**;18:27
10. Sambrook J, Russel DW. Method 2: Amplification of Libraries Constructed in λgt11, λZAP, λZipLox, and Their Derivatives. In: Irwin N, ed. Molecular Cloning: a Laboratory Manual. 3rd ed. Vol. 2. New York: Cold Spring Harbor Laboratory Press, **2001**:11.65
11. Sambrook J, Fritsch EF, Maniatis T. Immunological Screening of Expression Libraries. In: Ford N, Nolan C and Ferguson M, eds. Molecular Cloning: a Laboratory Manual. 2nd ed. Vol. 2. New York: Cold Spring Harbor Laboratory Press, **1989**:12.16-20
12. Pusch C, Schmitt H, Blin N. Increased cloning efficiency by cycle restriction-ligation (CRL). Technical Tips Online;1:T40071
13. Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem **1987**;166:368-79
14. Rico AI, Girones N, Fresno M, Alonso C, Requena JM. The heat shock proteins, Hsp70 and Hsp83, of *Leishmania infantum* are mitogens for mouse B cells. Cell Stress Chaperones **2002**;7:339-46
15. Dominguez M, Moreno I, Lopez-Trascasa M, Torano A. Complement interaction with trypanosomatid promastigotes in normal human serum. J Exp Med **2002**;195:451-9
16. Masina S, M MG, Demotz SO, Fasel NJ. Protection against cutaneous leishmaniasis in outbred vervet monkeys using a recombinant histone H1 antigen. J Infect Dis **2003**;188:1250-7
17. Iborra S, Soto M, Carrion J, et al. The Leishmania infantum acidic ribosomal protein P0 administered as a DNA vaccine confers protective immunity to Leishmania major infection in BALB/c mice. Infect Immun **2003**;71:6562-72
18. Reithinger R, Davies CR. Canine leishmaniasis: novel strategies for control. Trends Parasitol **2002**;18:289-90
19. Reithinger R, Quinnell RJ, Alexander B, Davies CR. Rapid detection of Leishmania infantum infection in dogs: comparative study using an immunochromatographic dipstick test, enzyme-linked immunosorbent assay, and PCR. J Clin Microbiol **2002**;40:2352-6
20. Burns JM, Jr., Shreffler WG, Benson DR, Ghalib HW, Badaro R, Reed SG. Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. Proc Natl Acad Sci U S A

1993;90:775-9

21. Fernandez-Perez FJ, Mendez S, de la Fuente C, Gomez-Munoz MT, Cuquerella M, Alunda JM. Short report: improved diagnosis and follow-up of canine leishmaniasis using amastigote-based indirect immunofluorescence. Am J Trop Med Hyg **1999**;61:652-3
22. Soto M, Quijada L, Larreta R, Iborra S, Alonso C, Requena JM. *Leishmania infantum* possesses a complex family of histone H2A genes: structural characterization and analysis of expression. Parasitology **2003**;127:95-105
23. Lukes J, Maslov DA. Unexpectedly high variability of the histone H4 gene in *Leishmania*. Parasitol Res **2000**;86:259-61

Table1: recognition of recombinant antigens by sera of artificially infected dogs (A-D, F) and control dog (E) respectively.

Gene	HIS-H2A	HIS-H4	RP L7	RP L7a	RP L10	HSP-70
Expression vector	pGEX	pGEX	pGEX	pQE	pQE	
A	120[1)]	120	-[2)]	-	-	60
B	120	120	-	-	-	60
C	120	120	-	-	120	60
D	60	120	-	-	60	60
E (control)	-	-	-	-	-	-
F	120	120	-	-	-	60

[1)] days post infection
[2)] no recognition at all

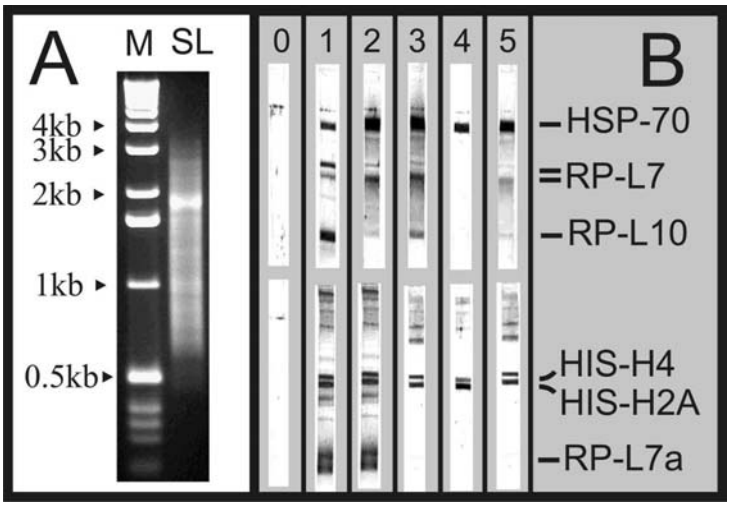


Figure 1: A: Size distribution of the spliced leader cDNA used for constructing the expression library (M: size marker, SL: spliced leader cDNA). B: Western blots using sera of naturally infected dogs (1-5) and uninfected control dog (0) respectively.

2.4. Discussion

We identified several potential antigens suitable for a diagnostic test. However, much more data are needed concerning their value to recognize early infections of different *L. infantum* strains responsible for HIV-*Leishmania* co-infections. Ongoing studies currently address these issues.

Several core histones were selected in the serum screen. Here the questions arise: why are the histones from the genus *Leishmania* highly diverse and immunogenic? What are the advantages for the parasites? So far, they have not been satisfactory answered. Nevertheless, the unusual properties of *Leishmania* histones might represent a key factor in leishmaniasis.

It remains questionable whether a diagnostic test, even when sensitive and specific, would improve the control of leishmaniasis. It would need a huge effort (personnel and cost intensive) to test and treat the majority of the canine population in Southern Europe. From this point of view, a cheap vaccine would be favorable: a vaccine might be allocated as lures (like in Switzerland for control of rabies), and there would be no need for diagnosing and treating infected animals, which most probably would also lead to treatment failures and selecting drug resistance in parasite strains. However, since no such vaccine is available yet against any parasitic disease, any other possible step has to be kept in mind.

In Brazil, there has been a study on the impact of using insecticide impregnated dog collars, which yielded satisfactory effects (Reithinger and Davies, 2002). However, this handling prevents only dogs with owners from infective sandfly bites. For street dogs, a similar approach would be to use masses of insecticides which surely has a great impact on environment and most probably also on public health.

Mass diagnosis and treatment of street dogs still appears to be a possible approach at present. However, it does not seem to be an elegant way to control leishmaniasis, especially in regard to drug resistance. Furthermore, chemotherapy of canine leishmaniasis is tagged by high relapse rates (Baneth and Shaw, 2002). Nevertheless, an early diagnostic test surely would be a useful tool for veterinarian purpose.

3. Development of a PCR assay for diagnosis of human leishmaniasis and differentiation of *Leishmania* species

3.1. Background and method

There are many different approaches for diagnosing human leishmaniasis (reviewed by Guerin et al., 2002; Singh and Sivakumar, 2003): direct detection of the parasite by microscopy (low sensitivity), or cultivation (time consuming and not all strains are equally grown *in vitro* and *in vivo*), PCR and serological methods. In Europe, most of the leishmaniasis cases represent HIV co-infections. A recent study compared several different tools for diagnosis of leishmaniasis in HIV patients (Deniau et al., 2003). In respect to sensitivity and specifity, the results suggest the use of a combination of PCR and cultivation.

Although several diagnostic PCRs for *Leishmania* species are published (reviewed by Wilson, 1995), none of them is able to detect all different species and distinguish among them at the same time. Either these PCRs amplify DNA of all species but cannot distinguish between them (as PCRs amplifying ribosomal genes), or they are only capable to amplify DNA deriving from a limited number of species (as PCRs amplifying kinetoplast DNA).

We have chosen the mini-exon gene as PCR template, because this gene is abundant in about 200 copies per genome of each *Leishmania species* (sensitivity) and shows enough polymorphism for distinguishing the different species. It consists of the highly conserved mini-exon, a semi conserved intron and a highly variable non transcribed spacer, resulting in length and sequence polymorphism of the PCR product, which allows differentiation of *Leishmania* species using restriction length polymorphism.

3.2. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP

ELSEVIER

Diagnostic Microbiology and Infectious Disease
46 (2003) 115–124
Parasitology

DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE

www.elsevier.com/locate/diagmicrobio

Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP

Jutta Marfurt, Igor Niederwieser, Ntoh Divine Makia, Hans-Peter Beck, Ingrid Felger*

Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Basel, Switzerland

Abstract

We have designed a new genotyping scheme for molecular diagnosis of the different *Leishmania* species pathogenic to humans. This scheme is based on PCR amplified sequences from the gene for the spliced leader RNA (mini-exon). This target was selected because it is present as tandem repeats (100 to 200 copies) in the genus *Leishmania* and other kinetoplastida, but is absent from the mammalian hosts and the sandfly vectors. The exon is highly conserved, whereas the intron and non-transcribed spacer region vary in size and sequence among different species. Thus, it was possible to amplify DNA from both Old and New World pathogenic *Leishmania* complexes using a single pair of primers deriving from the conserved region of the mini-exon tandem repeat. Species identification was performed by digesting mini-exon PCR products with one or two different restriction enzymes. Restriction fragment length polymorphism (RFLP) generated species-specific patterns of bands visualized in agarose gels, which allowed to differentiate each species unequivocally.

1. Introduction

Leishmaniasis is a parasitic disease which is associated with a wide spectrum of clinical manifestations depending on the species of the parasite, the host's immune response and the saliva of the sandfly vector (Grimaldi and Tesh, 1993; Gradoni and Gramiccia, 1994). The variety of symptoms range from self-curing ulcerative or diffuse, non-ulcerative cutaneous lesions, to persistent and often disfiguring mucocutaneous lesions or the potentially fatal visceral form of the disease. The assignment of the parasite species based alone on geographic location or the site of infection is not satisfactory. Accordingly, correct diagnosis and classification of a pathogenic *Leishmania* isolate is essential to determine the clinical prognosis and a species-specific therapeutic approach (Navin *et al.*, 1992; Berman, 1997; Romero *et al.*, 2001). In addition, epidemiologic studies as well as the usage of reference strains in laboratory experiments greatly depend on correct species-identification.

The genus *Leishmania* is divided into the two subgenera *Viannia* and *Leishmania* according to their development in the sandfly vector. Specification within the subgenera depends on several factors such as the geographical distribution of an isolate, the clinical presentation of the disease, and the epidemiology of the vector and the animal reservoir (Lainson and Shaw, 1987; Pearson *et al.*, 2001). Since morphologic differentiation of *Leishmania* species is not possible, a variety of biochemical, immunologic, or molecular criteria were introduced for classification of pathogenic species, such as characterization by isoenzyme electrophoresis (zymodeme analysis) (Kreutzer and Christensen, 1980), by monoclonal antibodies (serodeme) (Grimaldi *et al.*, 1987) or by hybridization with species-specific probes such as minicircle DNA probes (Wirth *et al.*, 1989).

With the advent of the PCR technology, several PCR-based assays for species differentiation were developed. As targets for amplification served either nuclear DNA, such as the SSU rRNA gene (van Eys *et al.*, 1992), repetitive sequences (Piarroux *et al.*, 1995), internal transcribed spacer (ITS) regions (Cupolillo *et al.*, 1995, Eisenberger and Jaffe, 1999), the tubulin gene (Luis *et al.*, 1998), the gp63 gene locus (Victoir *et al.*, 1998), microsatellite DNA (Russell *et al.*, 1999), or extrachromosomal DNA, such as the repetitive kinetoplast DNA (kDNA) minicircles (de Bruijn and Barker, 1992; Belli *et al.*, 1998).

While all these different approaches offer a multitude of valid taxonomic characters for species identification, a simple assay (one single PCR) which is also comprehensive (all *Leishmania* species) as well as reliable and applicable in the routine diagnostic laboratory, has been missing to date.

* Corresponding author. Tel.: +41-61-284-81-17; fax: +41-61-271-86-54.

E-mail address: ingrid.felger@unibas.ch (I. Felger).

0732-8893/03/$ – see front matter
doi:10.1016/S0732-8893(03)00040-3

Most molecular diagnostic methods developed for genotyping *Leishmania* species were based on the polymorphic kDNA minicircle, which is considered a prime candidate for a sensitive assay because of the presence of 10000 to 20000 minicircles per cell. In a first attempt to device a genotyping assay comprising all *Leishmania* species pathogenic to humans, we performed a sequence analysis of kDNA which revealed too great an amount of sequence variation between kDNAs of different New and Old World species, thus impeding the design of universal primers. An alternative kDNA approach based on multiplex PCR has been developed (Belli *et al.,* 1998). However, the yield of amplification products is generally compromised in multiplex PCR by primer dimer formation resulting in a loss of sensitivity of the assay.

Therefore, we chose the mini-exon (or spliced leader (SL)) gene found in all kinetoplastida as target sequence for a genotyping assay. The mini-exon gene, which is involved in the trans-splicing process in kintoplastid protozoa, is present 100 to 200 times per nuclear genome as tandemly repeated copies, and it is absent from the vertebrate host or invertebrate vector. A detailed study on sequence variation in the mini-exon gene repeat of human pathogenic *Leishmania* species had previously shown that the diversity detected in the non-transcribed spacers represents an informative phylogenetic marker (Fernandes *et al.,* 1994).

These features, together with a species-specific diversity, which conforms roughly with the species boundaries within the genus, designate the mini-exon for being exploited as a genotyping marker. We have used a PCR approach similar to that of Fernandes et al. (1994), but combined it with restriction digests of the PCR product, a method which is routinely used for other genotyping tasks (Singh, 1997). The resulting patterns of restriction fragments were characteristic for each species. Thus, we achieve a high resolution and high discrimination power, while the assay is still based on a single PCR reaction with universal primers.

2. Materials and methods

2.1. Parasite strains

The strains representative for the different *Leishmania* species, which were used in this study are listed in Table 1. In addition, 35 isolates from patients attending the polyclinic of the Swiss Tropical Institute (STI) were genotyped.

2.2. In vitro *cultivation of* Leishmania

The promastigote forms were cultivated at 27°C without gas phase in a 1:1 mixture of Schneider's modified *Drosophila* medium (SDM-79) (Brun and Schönenberger, 1979) and Schneider's medium (SM) (Cunningham, 1977) complemented with 15% heat-inactivated fetal bovine serum. *Leishmania* from a 5-day-old culture were harvested and counted with the Cell Counter and Analyser System CASY® 1 (Schärfe System GmbH, Reutlingen, FRG). The cells were washed twice with 0.15 M phosphate-buffered saline (PBS) pH 7.4, pelleted by centrifugation, resuspended in the appropriate buffer depending on further experiments, and processed immediately.

Table 1
Parasite strains

Species	International code	Source
L. aethiopica	MHOM/ET/72/L100	2
L. amazonensis	MPRO/BR/72/M1845	1
L. braziliensis	MHOM/PE/85/LEM772	2
L. chagasi	MHOM/BR/74/PP75	1
L. colombiensis	IGOM/PA/85/E582.34	1
L. donovani	MHOM/IN/80/DD8	2
L. donovani	MHOM/CN/0000/WangJie1	1
L. donovani	MHOM/KE/1967/MRC(L)3	1
L. donovani	IMAR/KE/1962/LRC-L57	1
L. donovani	MHOM/SD/1982/Gilani	1
L. donovani	MHOM/ET/0000/Hussen	1
L. guyanensis	MHOM/GF/99/LEM3713	3
L. guyanensis	MHOM/GF/2000/LEM3952	3
L. infantum	MHOM/FR/78/LEM75	2
L. killicki	MHOM/TN/80/LEM163	1
L. major	MHOM/IL/85/LEM769	2
L. mexicana	MNYC/BZ/62/M379	1
L. naiffi	MDAS/BR/79/M5533	1
L. panamensis	MHOM/PA/71/LS94	1
L. peruviana	MHOM/PE/84/LC39	1
L. tropica	MHOM/SU/74/K27	2
L. venezuelensis	MHOM/VE/74/PM-H3	1

The parasites were provided by:
[1] The WHO Leishmania Reference Strain Collection at the London School of Hygiene and Tropical Medicine (LSHTM), London, UK.
[2] Prof. Dr. Reto Brun from the Laboratory of Protozoology at the Swiss Tropical Institute in Basel, CH.
[3] Dr. Renaud Piarroux, Laboratoire de Parasitologie-Mycologie, Hopital Jean Minjoz, Université de France-Comté, Besancon, F.

2.3. DNA preparation

Cultured cells, corresponding to about 2×10^8 cells, were transferred into 500 μl digestion buffer (10 mM Tris/HCl pH 8; 5 mM EDTA; 0.5% SDS). Proteinase K (Sigma-Aldrich, Inc., CH) was added to a final concentration of 0.5 mg/ml and the sample was incubated over night at 56°C. DNA was extracted successively with one volume of phenol pH 8.0, one volume of phenol/chloroform, and one volume of chloroform according to standard procedures (Sambrook and Russell, 2001). DNA was precipitated from the aqueous phase with ethanol, the pellet washed with 75% ethanol, air dried and finally resuspended in 100 μl $H_2O_{dest.}$

2.4. PCR

The location of the forward primer Fme (5′-TAT TGG TAT GCG AAA CTT CCG-3′) and reverse primer Rme (5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′) within

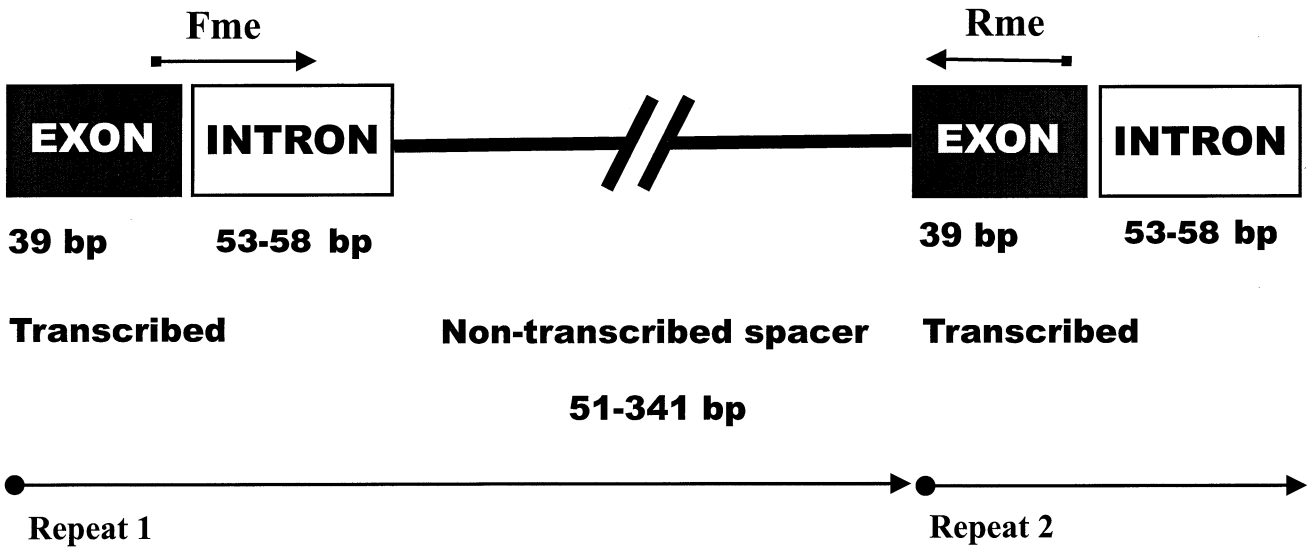


Fig. 1. The mini-exon repeat of *Leishmania*. Each repeat contains a highly conserved exon (39 bp), a moderately variable transcribed intron region (55 to 101 bp), and a highly variable non-transcribed spacer sequence (51 to 341 bp). Location of primer sites (Fme and Rme) are indicated with arrows.

the mini-exon gene repeat is shown in Fig. 1. Two μl of DNA solution were amplified in a 100 μl reaction containing 50 mM KCl, 20 mM Tris-HCl pH 8. 4 (GIBCO™, Invitrogen™ Life Technologies, CH), 0.2 mM dNTPs (Amersham Biosciences Europe, CH), 12% DMSO (Fluka Chemika, CH), 40 mM tetramethylammonium chloride (Carl Roth GmbH, FRG), 1. 5 mM $MgCl_2$ (GIBCO™), 0.5 μM of each primer (MWG Biotech AG, CH), and 1 U *Taq* polymerase (GIBCO™). The PCR conditions were 5 min at 94°C followed by 25 to 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 45 sec at 72°C. The number of cycles depended on the origin and concentration of the template. PCR products were separated on a 1.5% agarose gel.

2.5. Sensitivity titration assays

A) Crude *Leishmania* extracts: To test whether samples can be directly used in the PCR assay, cultured cells were resuspended in PBS pH 7. 4 and tenfold serial dilutions of 5×10^6 to 2.5×10^2 cells per millilitre (ml) were prepared, yielding a concentration range from 10000 to 0.5 parasites per 2 μl. The dilutions were subjected to three freeze-thaw cycles, incubated at 95°C for 10 min and 2 μl of the solutions were directly used in the PCR reaction.

B) Purified *Leishmania* DNA: DNA concentrations were determined by measuring the optical density (OD) at 260 nm with a GeneQuant® RNA/DNA Calculator (Amersham Biosciences Europe GmbH, CH). Tenfold serial dilutions of 0.15 mg to 1. 5 pg per ml were prepared, yielding a concentration range from 300 ng to 3 fg per 2 μl which were used in the PCR assay.

2.6. Specificity assays

The specificity of the mini-exon PCR assay was determined by testing different concentrations of purified DNA from the human host and other pathogens in the assay. Human DNA was extracted from peripheral blood mononuclear cells (PBMCs) from a volunteer as described in section 3. Genomic DNA from *Mycobacterium spp.* (i.e., *M. tuberculosis* and *M. ulcerans*) and *Plasmodium falciparum* was kindly provided by the STI laboratories of Molecular Immunology and Molecular Parasitology, respectively. Cultured organisms from other kinetoplastida (i.e., *Crithidia spp.* and *Trypanosoma spp.*) were kindly provided by the laboratory of Protozoology at STI. DNA was extracted and purified as described in section 3.

2.7. Restriction digests

Ten μl of the PCR products were digested with 1U *Eae* I (New England Biolabs®, Inc., USA) and at least one additional enzyme according to the genotyping scheme (Fig. 2). The restriction enzymes and the corresponding reaction buffers and conditions are summarized in Table 2. Resulting restriction fragments were separated on a 2. 5% agarose or 10% polyacrylamide gel. Fragment sizes were estimated by comparison with bands of a DNA length standard (1 kb ladder DNA size marker, GIBCO™). In cases of uncertainty, restriction fragments of a given strain were run in parallel with digested PCR products of known culture strains.

2.8. Sequencing of the mini-exon gene

PCR products were purified by size-selective polyethyleneglycol (PEG) precipitation (Lis, 1980). Briefly: the PCR reaction was mixed with 1. 5 volumes of a 20% PEG solution in 1 M NaCl in a microtube and incubated for 10 min at room temperature. The tube was centrifuged for 20 min at 14000× g in a microfuge. The precipitated PCR

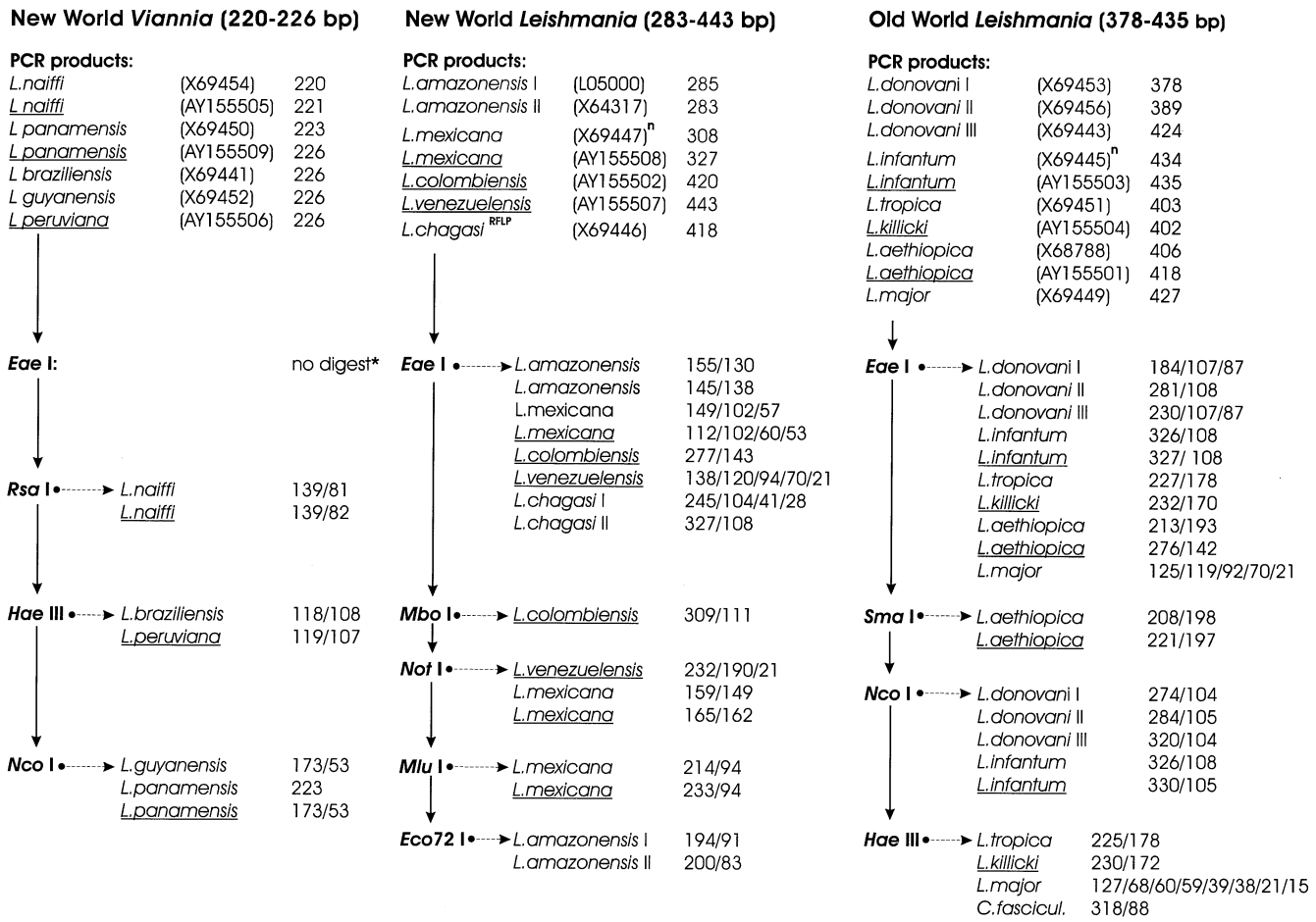


Fig. 2. The mini-exon PCR-RFLP genotyping scheme. GenBank accession numbers are given in brackets. Vertical arrows denote the consecutive processing of the samples; horizontal, dashed arrows denote the expected fragment sizes (bp) of the given restriction enzyme digest. n, undetermined nucleotides in the sequence; RFLP, confirmed by RFLP analysis; *, mini-exon sequences from the subgenus *Viannia* lack an *Eae* I restriction site.

products were washed with 1 volume of 75% ethanol (centrifugation for 5 min at 14000× g), air dried and resuspended in 15 μl $H_2O_{dest.}$ The PCR products were cloned by TA cloning (Zhou *et al.*, 1995) using the pGEM®-T Vector System (Promega, Madison, USA) according to the manufacturer's instructions. Bacterial colonies harboring plasmids containing the mini-exon genes were identified by PCR screening with vector primers SP6 (5′-ATT TAG GTG ACA CTA TAG-3′) and T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′) using the same PCR conditions as described

Table 2
Restriction enzymes, buffers and reaction conditions used for RFLP analysis

Enzyme*	Source	Recognition site	Reaction buffer*	Reaction conditions
Eae I[a]	*Enterobacter aerogenes*	5′-Py▼GGCCPu-3′	NEB® 1	1 h at 37°C
*Eco*72 I[b]	*Escherichia coli*	5′-CAC▼GTG-3′	Y^+/Tango™	1 h at 37°C
Hae III[a]	*Haemophilus aegypticus*	5′-GG▼CC-3′	NEB® 2	1 h at 37°C
Mbo I[a]	*Moraxella bovis*	5′-▼GATC-3′	NEB® 3	1 h at 37°C
Mlu I[a]	*Micrococcus luteus*	5′-A▼CGCGT-3′	NEB® 3	1 h at 37°C
Nco I[a]	*Nocardia corallina*	5′-C▼CATGG-3′	NEB® 4	1 h at 37°C
Not I[a]	*Nocardia otitidis-cavarium*	5′-GC▼GGCCGC-3′	NEB® 3 + BSA	1 h at 37°C
Rsa I[a]	*Rhodopseudomonas sphaeroides*	5′-GT▼AC-3′	NEB® 1	1 h at 37°C
Sma I[a]	*Stenotrophomonas maltophilia*	5′-CCC▼GGG-3′	NEB® 4	1 h at 25°C

* Enzymes and ready-to-use buffers were purchased from:
[a] New England Biolabs (NEB), BioConcept Laboratories, CH,
[b] MBI Fermentas GmbH, Labforce AG, CH.

above. The CONCERT™ Rapid Plasmid Miniprep System (GIBCO™, Invitrogen™ Life Technologies, CH) was used to purify the plasmid DNA. 100-200 ng of the purified plasmid DNA was used for the sequencing reaction which was performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosciences, CH) based on the dideoxy termination method (Sanger *et al.*, 1977). Mini-exons were sequenced twice in both directions using vector primers SP6 and T7 and PCR primers Fme and Rme. Cycle sequencing (25 cycles of 96°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min) was performed in a GeneAmp PCR System 2400 (Perkin Elmer) and sequences were analyzed using the ABI PRISM™ 310 Genetic Analyzer (Perkin Elmer) and the ABI PRISM™ software.

3. Results

3.1. The mini-exon PCR assay

To achieve highest PCR sensitivity and efficiency (high yield of specific amplicons, no primer artifacts), conditions were optimized by adding DMSO and tetramethylammonium chloride to the reaction mix and determining the optimal annealing temperature of 54°C in a Mastercycler® gradient 5331 (Eppendorf, Hamburg, FRG). Amplification products varied in size which was indicative of the different *Leishmania* complexes. Fig. 3 shows a selection of mini-exon PCR products of varying fragment sizes from different *Leishmania* species. The smallest fragments of 223 to 226 bp were obtained by the New World species belonging to the subgenus*Viannia* (i.e., the complexes *L. braziliensis* (Fig. 3, lane 7) and *L. guyanensis* (lane 8)), whereas those belonging to the subgenus *Leishmania* produced longer products, ranging from 283 to 327 bp (i.e., *L. mexicana* complex (lanes 5 and 6)) or from 420 to 443 bp (New World *Leishmania* species unassigned on complex level (not shown in Fig. 3)). Old World species (i.e., the complexes *L. donovani* (lane 2), *L. major* (lane 3), *L. tropica* (lane 4) and *L. aethiopica* (not shown in Fig. 3)) gave rise to amplification products ranging from 378 to 435 bp. In one of our *L. donovani* isolates, amplification of genomic DNA produced three PCR fragments of slightly different sizes. This finding was confirmed by a GenBank (EMBL) search which also revealed three different mini-exon sequences (Accession numbers: X69453, X69456, and X69443, respectively). This suggested the presence of three mini-exon repeats in *L. donovani*, detectable by PCR at least in one of the *L. donovani* strains (MHOM/IN/80/DD8).

Sensitivity titration experiments were performed with selected strains representing the size range of the expected mini-exon PCR products. Whereas the assay detected 50 cells of the species *L. infantum*, *L. major* (Fig. 4), *L. mexicana* and *L. tropica*, amplification products of *L. brasiliensis* and *L. guyanensis* were already detected with 10 parasites in the PCR reaction. Assuming that the DNA content of *Leishmania* is approximately 100 fg per genome (Myler and Stuart, 2000), the results obtained with crude extracts corresponded well with those observed with purified DNA.

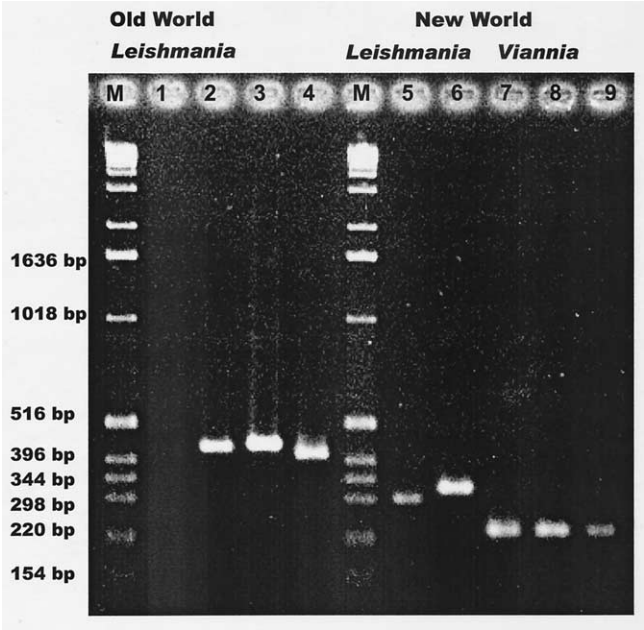


Fig. 3. PCR amplification products of different *Leishmania* species. Lanes: M, 1 kb ladder DNA size marker; 1, Negative control (H_2O_{dest}); 2. *L. infantum* (MHOM/FR/, 78/LEM75); 3, *L. major* (MHOM/IL/85/LEM769); 4, *L. tropica* (MHOM/SU/74/K27); 5, *L. amazonensis* (MPRO/BR/72/M1845); 6, *L. mexicana* (MNYC/BZ/62/M379); 7, *L. braziliensis* (MHOM/PE/85/LEM772); 8, *L. guyanensis* (MHOM/GF/99/LEM3713); 9, *L. guyanensis* (MHOM/GF/2000/LEM3952).

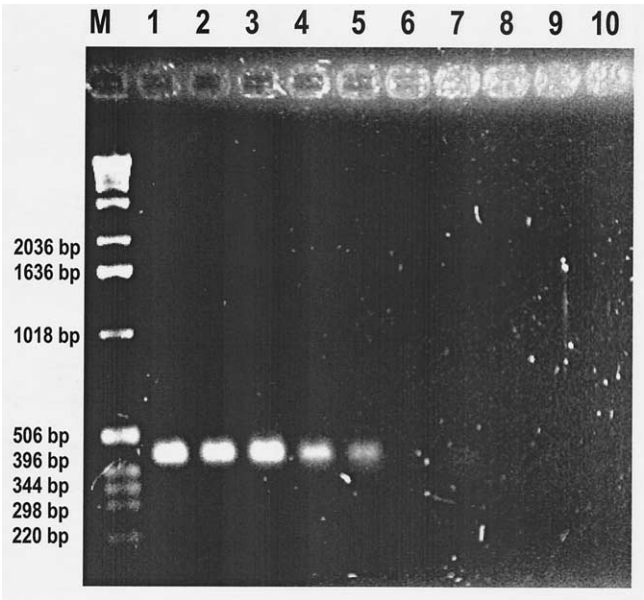


Fig. 4. Sensitivity of the mini-exon PCR assay. Crude extracts from cultured *L. major* organisms were tested in the PCR assay. Lanes: M, 1 kb ladder DNA size marker; 1, 10,000 parasites (p); 2, 1000 p; 3, 500 p; 4, 100 p; 5, 50 p; 6, 10 p; 7, 5 p; 8, 1 p; 9, 0.5 p; 10, $H_2O_{dest.}$

The detection limit was 3 pg DNA with the species yielding amplification products larger than 250 bp, but reached 300 fg with those producing the smallest PCR fragments (223 to 226 bp).

To assess the specificity of our PCR assay, we tested DNA from the human host as well as from other pathogens and related kinetoplastida in the PCR reaction. Whereas the mini-exon sequences of *Trypanosoma brucei rhodesiense, T. b. gambiense* and *T. cruzi* were not amplified with these primers, the other kinetoplastida (i.e., *Crithidia bombi, C. lucilia* and *C. fasciculata*) revealed PCR products between 400 and 430 bp in size. The PCR yield was lower with *Crithidia* than with *Leishmania* species, indicating mismatches between *Crithidia* mini-exon and our primer sequences. However, *Crithidia* species were discernible by the absence of an *Eae* I restriction site and the production of a unique pattern of fragments (318 and 88 bp) with the *Hae* III digest which was not found with any *Leishmania* species (data not shown).

3.2. PCR-RFLP genotyping scheme

The observed size difference of PCR products identified the different *Leishmania* complexes, but did not discriminate between individual species. Therefore, we chose to subsequently digest the PCR products with restriction enzymes in order to produce a species-specific RFLP pattern in an agarose gel. The resulting pattern of bands depends on both, mutations in the recognition sequence of the chosen restriction enzyme, and thus on the number of the fragments generated, and on size variation of the internal non-transcribed spacer fragment. In order to establish such a RFLP genotyping scheme, we initially performed a GenBank (EMBL) search. The diversity of the mini-exon genes within the *Leishmania* and *Viannia* subgenus was determined by sequence alignments (ClustalW, BCM Search Launcher, http://searchlauncher.bcm.tmc.edu/), and restriction enzymes differentiating between the individual species were identified using the Webcutter version 2.0 program (http://www.firstmarket.com/firstmarket/cutter/cut2.html). Using this information, restriction maps of the mini-exon repeat of different *Leishmania* species were produced. As a result, we defined different digests which allowed species discrimination: *Eae* I proved to be the most informative restriction enzyme, because it digested all mini-exon PCR products of the *Leishmania* subgenus in a species-specific manner, but it did not cut the mini-exon of the species of the subgenus *Viannia.* The latter PCR products were digested with *Hae* III, *Nco* I, *or Rsa* I which identified each species unequivocally. Whereas the species belonging to all other complexes were differentiated by a second round of restriction digests as shown in the flow diagram in Fig. 2. This diagram depicts the mini-exon RFLP genotyping scheme and indicates the fragment sizes expected from the nucleotide sequences submitted to GenBank. This virtual sequence analysis suggested that all *Leishmania* species can be distinguished by mini-exon PCR-RFLP.

3.3. Validation of the genotyping scheme with cultured Leishmania *reference strains*

The genotyping scheme deduced from sequence information from GenBank was validated with culture strains representative for different species. Fig. 5 shows the RFLP pattern of different species on an agarose gel after diagnostic restriction digests with *Eae* I and *Hae* III. Two different *L. donovani* strains (lane 1 and 2 of Fig. 5) produced different RFLP patterns. This is in agreement with the fact that 3 different mini-exon sequences of *L. donovani* have been found in the GenBank search. The analyzed isolates correspond to the restriction map of mini-exon sequence accession numbers X69443 and X69453, respectively.

L. panamensis in lane 11 remains uncut by *Hae* III and therefore reflects the PCR product. The 468 bp band reflects occasional amplification of two consecutive mini-exon repeat units. This PCR artifact is due to the mini-exon's arrangement as tandem repeats and is more likely to occur in small *Viannia*-type repeat units. The 403 bp band in lane 4 represents the PCR band of *L. tropica* which occurred due to partial digest of the amplified DNA. The unexpected 215 bp fragment of *L. mexicana* in lane 8 might either be an artifact due to partial digest (*Eae* I site at position 112), or suggests the co-existence of two different mini-exon repeat units within this species.

Next we examined whether this new genotyping scheme based on GenBank sequences can be used to characterize field isolates or applied in molecular epidemiologic studies. 23 isolates of *L. infantum* and 7 isolates of *L. major* (formerly typed by isoenzyme analysis) deriving from bone marrow or skin biopsies of patients attending the polyclinic of the STI were analyzed. All showed the expected restriction pattern according to the genotyping scheme (results not shown). In the course of genotyping *Leishmania* laboratory strains representative for given species from different origins (see Table 1), we encountered several inconsistencies between the observed RFLP pattern and the genotyping scheme. These might well reflect existing problems in *Leishmania* taxonomy. The *L. chagasi* strain (MHOM/BR/74/PP75) used in our study could not be differentiated from *L. infantum* (MHOM/FR/78/LEM75) since both strains produced identical RFLP patterns.

In some cases, the expected RFLP pattern based on GenBank sequences could not be confirmed (i.e., for *L. aethiopica, L. infantum, L. mexicana, L. naiffi,* and *L. panamensis*). We therefore performed sequence analysis of the mini-exon gene amplified from reference strains of these species (Accession numbers: AY155501, AY155503, AY155508, AY155505, and AY155509, respectively). Fig. 2 represents the final genotyping scheme, which is confirmed by RFLP and sequence data from laboratory strains available to us (Mini-exon genes sequenced in our labora-

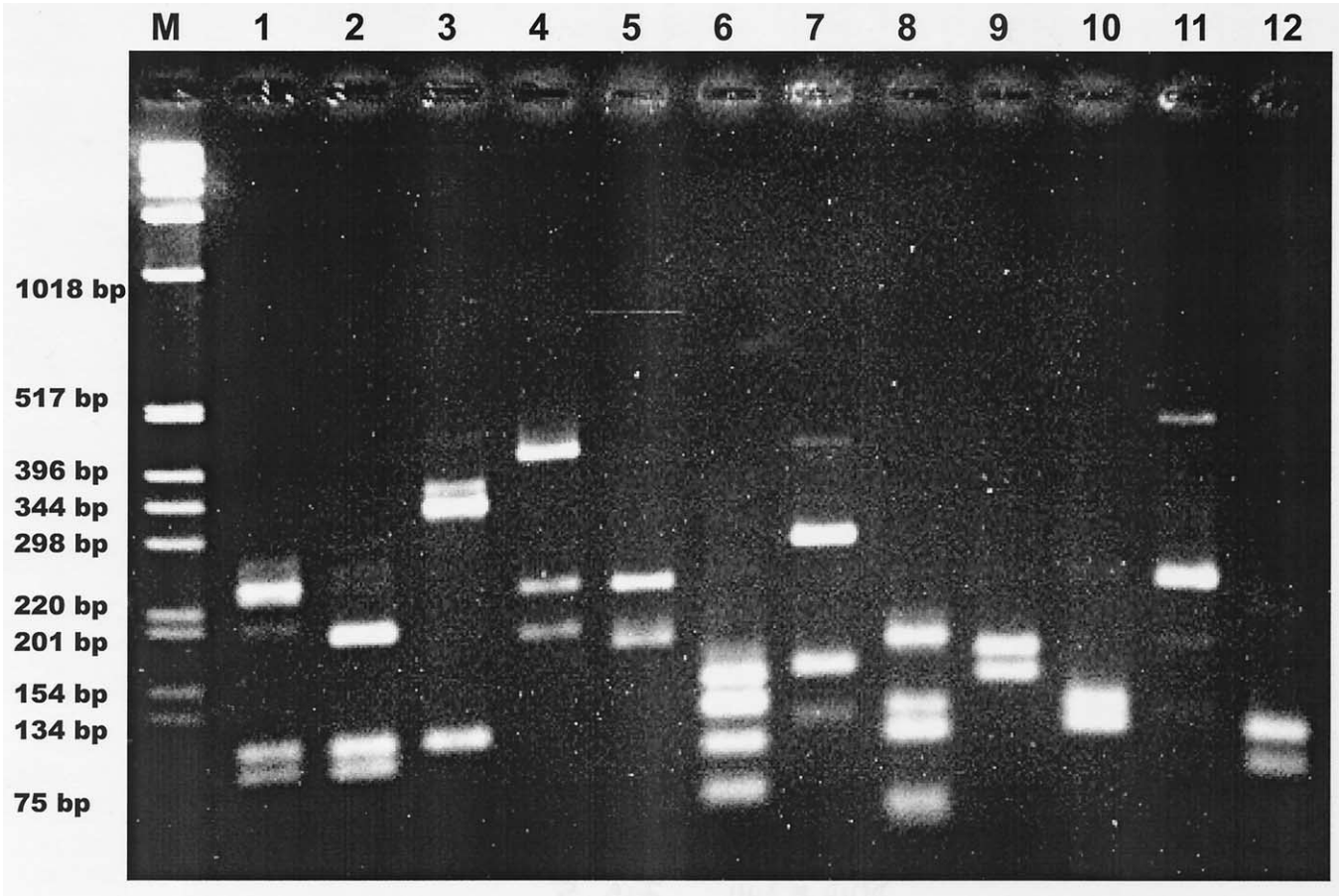


Fig. 5. RFLP patterns of different *Leishmania* species after restriction enzyme digest with *Eae* I and *Hae* III**.** *Eae* I digest: lanes 1 to 9; *Hae* III digest: lanes 10 to 12. Lanes: M, 1 kb ladder DNA size marker; 1, *L. donovani* (MHOM/CN/0000/Wang Jie1); 2, *L. donovani* (MHOM/IN/80/DD8); 3, *L. infantum* (MHOM/FR/78/LEM75); 4, *L. tropica* (MHOM/SU/74/K27); 5, *L. killicki* (MHOM/TN/80/LEM163); 6, *L. major* (MHOM/IL/85/LEM769); 7, *L. aethiopica* (MHOM/ET/72/L100); 8, *L. mexicana* (MNYC/BZ/62/M379); 9, *L. amazonensis* (MPRO/BR/72/M1845); 10, *L. braziliensis* (MHOM/PE/85/LEM772); 11, *L. panamensis* (MHOM/PA/71/LS94); 12, *L. naiffi* (MDAS/BR/79/M5533).

tory appear underlined in Fig. 2). Additionally, mini-exon genes from *Leishmania* strains whose sequences were not available in GenBank (i.e., *L. colombiensis, L. killicki, L. peruviana,* and *L. venezuelensis*), were sequenced (Accession numbers: AY155502, AY155504, AY155506, and AY155507, respectively) in order to complete the initial genotyping scheme.

4. Discussion

The present genotyping assay is based on amplification of the mini-exon gene and was shown to be sensitive due to the repetitive character of this template. An added technical and cost-reducing advantage is the use of a single instead of a nested PCR for the amplification of all *Leishmania* species. Therefore, there is no need for multiplex PCR with species-specific sets of primers which is likely to result in a loss of sensitivity.

The detection limit was 50 parasites for *Leishmania* species producing amplification products ranging from 300 to 450 bp, whereas the sensitivity for the species belonging to the *Viannia* subgenus was 10 organisms. The enhanced sensitivity in the latter subgenus can be attributed to the smaller size (220 to 227 bp) and the higher copy number of these mini-exon sequences. However, our assay does not reach the sensitivity reported for the amplification of SSU rRNA or kDNA (Meredith et al., 1993; Rodgers et al., 1990), where detection limits from 1 to 10 parasites are probably due to a hundredfold higher copy number of these molecules per haploide *Leishmania* genome.

We have shown that the assay is specific to the *Leishmania* genus and correctly identifies species representing a broad taxonomical and geographical diversity. There were no products amplified with DNA from other microorganisms which might show clinical manifestations similar to those of leishmaniasis (e.g., *Plasmodium spp.* and *Mycobacterium spp.*). The test was also negative for the related kinetoplastida *Trypanosoma brucei gambiense, T. b. rhodesiense* and *T. cruzi*. These are important features because mixed infections of *Leishmania* and *T. cruzi* are known to occur (Chiaramonte *et al.*, 1996) and there are geographical

regions where leishmaniasis and malaria or mycobacterial infections co-exist. However, the assay generated PCR products with DNA from the kinetoplastida *Crithidia spp.* Though a contamination of the laboratory or a leishmanial lesion is theoretically possible, the risk is low and *Crithidia spp.* can be differentiated from *Leishmania spp.* by the production of a characteristic RFLP pattern (Fig. 2). We also demonstrated that no amplicons were produced with human DNA (data not shown) and the sensitivity was not compromised when PCR reactions were conducted with crude *Leishmania* extracts. This is promising since the assay could be directly applied to patient specimens. Thus, in vitro cultivation of the parasite, which is limited in sensitivity because of special growth requirements of certain species and the risk of contamination (Berman, 1997), could be avoided.

Two criteria are essential for a species-specific and sensitive diagnostic test: 1) a repetitive target sequence for PCR, since sensitivity increases with the copy number of the amplified sequence, and 2) an adequate degree of polymorphism showing variation between species but not between different isolates belonging to one species. With respect to species differentiation, the mini-exon repeat proved to be an ideal target for RFLP genotyping, because it shows a fair but finite amount of sequence variation between individual species. While the genotyping scheme, which was initially based on sequences derived from the GenBank, could be validated with most of the laboratory strains, we also encountered several inconsistencies, in particular in the discrimination of closely related species. The WHO isolate of *L. chagasi* showed the same RFLP genotype as *L. infantum,* despite the fact that a distinct restriction pattern was expected according to the GenBank entry. This allows several interpretations: 1) the reference culture and the isolate used for sequencing were not the same species, 2) there was an initial mix up of *L. infantum* and *L. chagasi* samples, 3) *L. chagasi* is portrayed by two distinct genotypes, one of which corresponds to that of *L. infantum,* 4) the mini-exon genotypes do not segregate according to species boundaries, and 5) both species are not only closely related but still can recombine. This problem can only be resolved by analyzing a number of different isolates of both species. However, since there has already been some doubt about the distinctness of these two species, the analysis with several molecular methods suggest that *L. chagasi* might simply be *L. infantum* imported into the New World (Mauricio *et al.*, 1999). In cases where many isolates of the same species were available to us, such as 23 *L. infantum* strains from patients, the predicted RFLP genotype was confirmed in 22 cases. In one case, a misclassification was found. The isolate, earlier characterized as *L. donovani,* was found to be *L. infantum.* This was a plausible result, because the sample has originally been isolated from *Phlebotomus ariasi,* a vector typical for *L. infantum.*

The restriction maps derived from GenBank sequences of *L. aethiopica* and *L. mexicana* could not be confirmed with our reference strains. Therefore, the mini-exon genes were sequenced in our laboratory and confirmed by PCR-RFLP analysis. These differences in the sequences and thus in the RFLP patterns may be due to: 1) intra-specific variability within the mini-exon gene, 2) the existence of two different repeat units within a species such as in *L. donovani,* or 3) sequencing errors in the GenBank sequence. (The *L. mexicana* sequence (Accession number: X69447) contained several undetermined nucleotides). Good quality nucleotide sequences could not be obtained easily. Cloning the PCR products instead of direct sequencing improved results. The sequencing problem might be due to secondary structures and the high GC content of this fragment. This is also reflected by the fact that PCR amplification was optimal at DMSO concentrations as high as 12%.

The mini-exons of the Old World species *L. killicki* and the New World species *L. colombiensis, L. peruviana* and *L. venezuelensis* were sequenced for the first time. Since the RFLP pattern could be confirmed for all four species, they are included in the genotyping scheme. It is worth mentioning, that the mini-exon sequences of *L. venezuelensis* and *L. major* are very similar and are hardly distinguishable by RFLP analysis. Recently, the close relationship of these two species has also been revealed by molecular analysis of the ITS region and the SSU rRNA gene (Berzunza-Cruz *et al.,* 2002). Alternatively, the *L. venezuelensis* reference strain might simply represent a member of *L. major*-like strains known to occur in several regions in the New World. Such strains were characterized by isoenzyme and schizodeme analysis in earlier studies and are believed to correspond to *L. major* populations imported from the Old World into the Americas (Momen *et al.*, 1993; Yamasaki *et al.*, 1994).

Though GenBank entries seemed to enable the discrimination between the individual species within the *L. braziliensis* complex (i.e., *L. b. braziliensis* and *L. b. peruviana*) and the *L. guyanensis* complex (i.e., *L. g. guyanensis* and *L. g. panamensis*), our sequence analysis revealed that the homology of the mini-exon genes within each of the two complexes was very high and we failed to identify a differentiating restriction site. Thus, sequencing was required to discriminate between the single species within the *L. braziliensis* and the *L. guyanensis* complex. However, these results were not surprising since the status of the individual species within these two *Leishmania* complexes still remains controversial (Banuls et al., 2002).

The few problems we encountered during this project might well reflect common difficulties in *Leishmania* taxonomy. In part, they occurred because of ambiguities in the starting material. Tracing the origin of samples and confirming the identity, as well a misclassification or even mislabelling of culture lines complicated the molecular determination. This is also reflected in the mini-exon sequences submitted to GenBank of which many revealed inadequately defined sources and were incomplete. A clearly identified set of reference DNAs or culture lines of all clinically relevant *Leishmania* species would greatly

assist in the establishment of a reliable and robust diagnostic tool. These observations call for an inter-laboratory collaboration aiming at the establishment of a set of reference samples for the unequivocal determination of each species by all available techniques.

In conclusion, our PCR-RFLP based genotyping assay is cheap, reliable with high sensitivity and specificity, easy to perform, and enables differentiation of all the clinically relevant *Leishmania* species within a reasonable timeframe.

Acknowledgments

We wish to thank Debbie Nolder at LSHTM for the provision of strains from the WHO *Leishmania* Reference Strain Collection, as well as Renaud Piarroux and Reto Brun who kindly provided *Leishmania* culture lines from their collections. The project was supported by the European Community grant No. FAIR-CT 98-4104, and the Swiss insurance company 'National', Basel, Switzerland.

References

Banuls, A. L., Hide, M., & Tibayrenc, M. (2002). Evolutionary genetics and molecular diagnosis of *Leishmania* species. *Trans R Soc Trop Med Hyg 96 (Suppl 1)*, S9–13.

Belli, A., Rodriguez, B., Aviles, H., & Harris, E. (1998). Simplified polymerase chain reaction detection of New World *Leishmania* in clinical specimens of cutaneous leishmaniasis. *Am J Trop Med Hyg 58*, 102–109.

Berman, J. D. (1997). Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. *Clin Infect Dis 24*, 684–703.

Berzunza-Cruz, M., Cabrera, N., Crippa-Rossi, M., Sosa, C. T., Perez-Montfort, R., & Becker, I. (2002). Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*. *Parasitol Res 88*, 918–925.

Brun, R., Schönenberger M. (1979). Cultivation and *in vitro* cloning of procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. *Acta Tropica 6*, 289-292.

Chiaramonte, M. G., Zwirner, N. W., Caropresi, S. L., Taranto, N. J., & Malchiodi, E. L. (1996). *Trypanosoma cruzi* and *Leishmania spp.* human mixed infection. *Am J Trop Med Hyg 54*, 271–273.

Cunningham, I. (1977). New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. *J Protozool 24*, 325–329.

Cupolillo, E., Grimaldi, J. G., Momen, H., & Beverley, S. M. (1995). Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of *Leishmania*. *Mol Biochem Parasitol 73*, 145–155.

De Bruijn, M. H., & Barker, D. C. (1992). Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. *Acta Tropica 52*, 45–58.

Eisenberger, C. L., & Jaffe, C. L. (1999). *Leishmania*: identification of Old World species using a permissively primed intergenic polymorphic-polymerase chain reaction. *Exp Parasitol 91*, 70–77.

Fernandes, O., Murthy, V. K., Kurath, U., Degrave, W. M., & Campbell, D. A. (1994). Mini-exon gene variation in human pathogenic *Leishmania* species. *Mol Biochem Parasitol 66*, 261–271.

Gradoni, L., & Gramiccia, M. (1994). *Leishmania infantum* tropism: Strain genotype or host immune status. *Parasitology Today 10*, 264–267.

Grimaldi, G. Jr, David, J. R., & McMahon-Pratt, D. (1987). Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. *Am J Trop Med Hyg 36*, 270–287.

Grimaldi, G. Jr, & Tesh, R. B. (1993). Leishmaniases of the New World: current concepts and implications for future research. *Clin Microbiol Rev 6*, 230–250.

Kreutzer, R. D., & Christensen, H. A. (1980). Characterization of *Leishmania spp.* by isozyme electrophoresis. *Am J Trop Med Hyg 29*, 199–208.

Lainson, R., & Shaw, J. J. (1987). Evolution, classification and geographical distribution. In: W. Peters, & R. Killick-Kendrick (Eds), *The Leishmaniases in Biology and Medicine*. *Orlando: Academic Press*, pp 1–120.

Lis, J. T. (1980). Fractionation of DNA fragments by polyethylene glycol induced precipitation. *Methods Enzymol 65*, 347–353.

Luis, L., Ramirez, A., Aguilar, C. M., Eresh, S., Barker, D. C., & Mendoza-Leon, A. (1998). The genomic fingerprinting of the coding region of the β-tubulin gene in *Leishmania* identification. *Acta Tropica 69*, 193–204.

Mauricio, I. L., Howard, M. K., Stothard, J. R., & Miles, M. A. (1999). Genomic diversity in the *Leishmania donovani* complex. *Parasitology 119 (Pt 3)*, 237–246.

Meredith, S. E., Zijlstra, E. E., Schoone, G. J., Kroon, C. C., Van Eys, G. J., Schaeffer, K. U., el Hassan, A. M., & Lawyer, P. G. (1993). Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. *Arch de l'Institut Pasteur de Tunis 70*, 419–431.

Momen, H., Pacheco, R. S., Cupolillo, E., & Grimaldi, J. G. (1993). Molecular evidence for the importation of Old World *Leishmania* into the Americas. *Biol Res 26*, 249–255.

Myler, P. J., & Stuart, K. D. (2000). Recent developments from the *Leishmania* genome project. *Curr Opin Microbiol 3(4)*, 412–416.

Navin, T. R., Arana, B. A., Arana, F. E., Berman, J. D., & Chajon, J. F. (1992). Placebo-controlled clinical trial of sodium stibogluconate (Pentostam) versus ketoconazole for treating cutaneous leishmaniasis in Guatemala. *J Infect Dis 165*, 528–534.

Pearson, R. D., De Queiroz Sousa, A., & Jeronimo, S. M. B. (2001). *Leishmania* Species: Visceral (Kala-Azar), Cutaneous and Mucosal Leishmaniasis. In: G. L. Mandell, J. E. Bennett, & R. Dolin (Eds), *Principles and practice of infectious diseases*. New York: Churchill Livingstone, pp 2831–2845.

Piarroux, R., Fontes, M., Perasso, R., Gambarelli, F., Joblet, C., Dumon, H., & Quilici, M. (1995). Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. *Mol Biochem Parasitol 73*, 249–252.

Rodgers, M. R., Popper, S. J., & Wirth, D. F. (1990). Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Exp Parasitol 71*, 267–275.

Romero, G. A., Guerra, M. V., Paes, M. G., & Macedo, V. O. (2001). Comparison of cutaneous leishmaniasis due to *Leishmania (Viannia) braziliensis* and *L. (V.) guyanensis* in Brazil: therapeutic response to meglumine antimoniate. *Am J Trop Med Hyg 65*, 456–465.

Russell, R., Iribar, M. P., Lambson, B., Brewster, S., Blackwell, J. M., Dye, C., & Ajioka, J. W. (1999). Intra and inter-specific microsatellite variation in the *Leishmania* subgenus. *Viannia Mol Biochem Parasitol 103*, 71–77.

Sambrook, J., & Russell, D. W (2001). Molecular Cloning, A Laboratory Manual. Cold Spring Harbor, New York: CSHL Press.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences 74*, 5463–5467.

Singh, B. (1997). Molecular methods for diagnosis and epidemiological studies of parasitic infections. *Int J Parasitol 27*, 1135–1145.

van Eys, G. J., Schoone, G. J., Kroon, N. C., & Ebeling, S. B. (1992). Sequence analysis of small subunit ribosomal RNA genes and its use

for detection and identification of *Leishmania* parasites. *Mol Biochem Parasitol 51*, 133–142.

Victoir, K., Banuls, A. L., Arevalo, J., Llanos-Cuentas, A., Hamers, R., Noel, S., De Doncker, S., Le Ray, D., Tibayrenc, M., & Dujardin, J. C. (1998). The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus. *Viannia Parasitology 117*, 1–13 (Pt 1).

Wirth, D. F., Rogers, W. O., Barker, R., Dourado, H., Suesbang, L., & Albuquerque, B. (1989). Leishmaniasis and malaria: DNA probes for diagnosis and epidemiologic analysis. *Ann NY Acad Sci 569*, 183–192.

Yamasaki, H., Agatsuma, T., Pavon, B., Moran, M., Furuya, M., & Aoki, T. (1994). *Leishmania major*-like parasite, a pathogenic agent of cutaneous leishmaniasis in Paraguay. *Am J Trop Med Hyg 51*, 749–757.

Zhou, M. Y., Clark, S. E., & Gomez-Sanchez, C. E. (1995). Universal cloning method by TA strategy. *Biotechniques 19*, 34–35.

3.3 Identification and Differentiation of *Leishmania* Species in Clinical Samples by PCR Amplification of the Miniexon Sequence and Subsequent Restriction Fragment Length Polymorphism Analysis

JOURNAL OF CLINICAL MICROBIOLOGY, July 2003, p. 3147–3153 Vol. 41, No. 7
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.7.3147–3153.2003

Identification and Differentiation of *Leishmania* Species in Clinical Samples by PCR Amplification of the Miniexon Sequence and Subsequent Restriction Fragment Length Polymorphism Analysis

Jutta Marfurt,[1] Abed Nasereddin,[2] Igor Niederwieser,[1] Charles L. Jaffe,[2] Hans-Peter Beck,[1] and Ingrid Felger[1]*

Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Basel, Switzerland,[1] and Department of Parasitology, Hadassah Medical School, Hebrew University, Jerusalem, Israel[2]

Received 19 November 2002/Returned for modification 12 January 2003/Accepted 28 April 2003

We recently developed a new PCR-restriction fragment length polymorphism (RFLP)-based assay using the miniexon sequence from the genus *Leishmania*. Here we report the application of this new genotyping method to naturally infected clinical samples for the differentiation of New and Old World *Leishmania* species. Of the newly developed assay and four currently applied diagnostic tests (i.e., in vitro cultivation, serology, and two other molecular assays using either the small subunit-internal transcribed spacer sequence or a repetitive genomic sequence), the miniexon assay showed the highest sensitivity, 89.7%, compared to 70.6, 57.1, 51.7, and 79.3%, respectively. Species differentiation was robust and reliable compared with that by two other *Leishmania* genotyping techniques. The assay provides a valuable tool for the identification of *Leishmania* directly from clinical samples and enables determination of the infecting species by a facile technique with high discrimination power. Since *Leishmania* causes a broad spectrum of diseases distinguished by different parasite and host factors, detection and characterization of the infecting species is crucial for the confirmation of a diagnosis as well as the establishment of the clinical prognosis and the initiation of an adequate therapeutic approach. The miniexon PCR-RFLP assay will facilitate such determination and might improve diagnosis and treatment of leishmaniasis.

Diagnosis of leishmaniasis can be made on the basis of clinical and epidemiological data but has to be confirmed by the demonstration of the parasite to avoid potential misdiagnosis (29). Because of differences among the *Leishmania* species in levels of virulence and in responses to the various chemotherapeutic regimens, correct identification is essential in order to determine the clinical prognosis and prescribe an appropriate species-specific therapeutic regimen (3).

At present, the classical methods used for the direct detection of the parasite include the visualization of amastigotes by microscopic examination of Giemsa-stained smears and in vitro culture of the parasite. Though microscopic examination is rapid, cheap, and easy to perform, it lacks sensitivity due to the generally low number of parasites in tissue samples (42). Culture techniques are more sensitive but require a sophisticated laboratory setup, are time-consuming, and harbor the risk of contamination (3). Indirect methods based on the analysis of specific immune responses cannot distinguish between past and current infections, and depending on the antigens used in serological assays, diagnosis can be complicated by cross-reaction of antibodies with other pathogens. Another drawback is the limited sensitivity because antibody titers may vary with the infecting species, tissue tropism, and the immunocompetence of the host (1).

Species discrimination is important not only for epidemiological reasons but also for clinical reasons. *Leishmania* species were originally classified by their geographical distributions and the clinical presentations of the diseases they cause, as well as by epidemiology, sand fly vectors, or animal reservoirs (20). Since morphological discrimination of *Leishmania* species is not possible, a variety of biochemical, immunological, and molecular tools have been developed for the differentiation of the pathogenic species, such as isoenzyme, serodeme, and schizodeme analysis and hybridization techniques with species-specific DNA probes (16, 43).

With the advances in molecular techniques, a number of molecular markers and PCR protocols for the detection or identification of *Leishmania* on different taxonomical levels (genus, complex, and species) have been reported (11, 43). Target sequences for characterization include either nuclear DNA, such as the small subunit rRNA (SSU rRNA) gene (40), a repetitive genomic sequence (30), the miniexon (spliced leader) gene repeat (18), the beta-tubulin gene region (22), the gp63 gene locus (41), and internal transcribed spacer (ITS) regions (9); microsatellite DNA (36, 37); or kinetoplast DNA, such as minicircle sequences (34).

While all the above-mentioned approaches provide a multitude of valid taxonomic characters for differentiation, they are accompanied by a number of factors which limit their use in a routine diagnostic laboratory. Some of the assays are restricted to the identification of *Leishmania* at the genus level (5, 25) and do not allow species differentiation, whereas others are adapted for individual complex characterization (10, 13), which limits their applicability to restricted geographical areas. Other

* Corresponding author. Mailing address: Swiss Tropical Institute, Socinstrasse 57, Postfach, CH-4002 Basel, Switzerland. Phone: 41 61 284 81 17. Fax: 41 61 271 86 54. E-mail: ingrid.felger@unibas.ch.

molecular approaches involve the combination of PCR amplification with DNA probe hybridization to enhance the sensitivity of the assay (33, 35), an elaborate and time-consuming procedure. The use of random amplified polymorphic DNA analysis of genomic DNA (12, 38) is limited since it requires purified parasite DNA and highly standardized PCR conditions to guarantee specificity. In addition, the results may be difficult to interpret. Assays based on the multiplex PCR technique (17) represent yet another approach but harbor the risk of reduced sensitivity caused by primer dimer formation.

The miniexon gene of kinetoplastid protozoa, which is involved in the transsplicing process of nuclear mRNA, is present as 100 to 200 tandemly repeated copies per nuclear genome. Each repeat consists of three major parts: a transcribed region comprising a highly conserved 39-nucleotide exon; a moderately conserved intron, approximately 55 to 101 bp; and a nontranscribed, intergenic spacer of variable length (51 to 1,350 bp) depending on the genus and species (39). These features allow the miniexon to be utilized as a genotyping marker. Based on these characteristics, we recently established a PCR assay similar to that described by Fernandes et al. (14). This PCR assay amplifies all the miniexon sequences in a single reaction using universal primers, allowing preliminary discrimination between the major complexes (i.e., Old World *Leishmania*, New World *Leishmania*, and New World *Viannia* complexes) as a result of the variability in sizes of the amplification products. After restriction enzyme digestion of the PCR product, a characteristic restriction fragment length polymorphism (RFLP) pattern is produced that depends on size variations in the polymorphic spacer regions as well as mutations in the recognition sites of a chosen restriction enzyme. The miniexon PCR-RFLP genotyping scheme was validated with cultured World Health Organization reference strains of *Leishmania* and cultured isolates from patients attending the polyclinic of the Swiss Tropical Institute (STI). It has proven to be a valuable tool showing high resolution, sensitivity, and an ability to discriminate between all clinically relevant *Leishmania* species (24).

The aim of this study was to optimize the miniexon PCR assay for routine diagnosis of infections in naturally infected clinical samples without the need for cultivation and to compare our results with those obtained by using current methods for diagnosis of leishmaniasis. Moreover, genotyping results of the miniexon RFLP system were validated by parallel analysis of the samples with two other PCR-RFLP typing schemes which are based on an uncharacterized repetitive genomic sequence of Old World *Leishmania* (30) or the ITS region of the rRNA gene (37a).

MATERIALS AND METHODS

Clinical samples. A total of 50 samples from patients presumed to have leishmaniasis were used in this study. The samples were either sent to our diagnostic unit or obtained from patients returning from areas where the disease is endemic who attended the STI polyclinic with either cutaneous signs or a tentative diagnosis of visceral leishmaniasis. Three samples were from patients with a concurrent HIV infection. The samples consisted of 25 skin biopsy specimens, 16 bone marrow biopsy specimens, 2 lesion aspirates, 4 blood samples (anticoagulated with EDTA), 2 duodenal biopsy specimens, and 1 gastric biopsy specimen. Four of the 25 skin biopsy specimens arrived as paraffin-embedded tissue samples. Samples were split on arrival; one part was used for in vitro cultivation, except in the case of paraffin-embedded tissue samples, and the other part was processed for PCR analysis. Corresponding serum samples were available from 16 patients, and they were tested for *Leishmania*-specific antibodies by an indirect fluorescent-antibody test (IFAT). Of these, four samples were derived from patients who had traveled to the Americas, six samples were derived from patients for whom there was no information on travel history, and 19 were derived from patients with a history of travel in the Old World. In this study, a sample was considered positive when the results of at least one of the assays performed were positive. Assays were repeated independently to exclude contamination.

Specimen processing and DNA preparation. Skin, duodenal, and gastric biopsy specimens arrived in 0.9% NaCl or another physiological transport medium and were first cut into smaller pieces with a sterile scalpel in a petri dish. They were then transferred with the medium into a 2-ml tube, pelleted by centrifugation, and finally resuspended in 500 μl of lysis buffer (10 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% sodium dodecyl sulfate).

Lesion aspirates were centrifuged at 8,000 × *g* for 10 min and resuspended in 500 μl of lysis buffer.

Anticoagulated bone marrow samples were centrifuged for 15 min at 800 × *g*. The buffy coat was transferred to a new tube, washed with phosphate-buffered saline (PBS), pH 7.4, and resuspended in 500 μl of lysis buffer. Coagulated clots of bone marrow samples were treated like skin biopsy samples as described above.

EDTA-anticoagulated blood was mixed with an equal volume of PBS, pH 7.4, layered over Ficoll-Paque PLUS (density, 1.077 g/liter; Amersham Biosciences Europe GmbH, Dübendorf, Switzerland), and spun for 30 min at 400 × *g* The interphase containing peripheral blood mononuclear cells was carefully transferred to a fresh tube, washed twice with PBS, pH 7.4, and resuspended in 500 μl of lysis buffer.

Small sections (not more than 25 mg) of paraffin-embedded tissue samples were scratched with a sterile scalpel into a 1.5-ml tube. Paraffin was removed by being washed once with 1.2 ml of xylene and two times with 1.2 ml of ethanol. Residual ethanol was evaporated by incubating the tube for 15 min in a heating block at 37°C. The pellet was resuspended in 180 μl of buffer ATL (QIAamp DNA Mini Kit; Qiagen AG, Basel, Switzerland), and DNA was extracted and purified by using the QIAamp DNA Mini Kit according to the QIAamp DNA Mini Tissue protocol.

Generation of the positive control template. A 498-bp fragment of the cysteine protease *cpb2* gene from *Leishmania mexicana* (accession no. AJ319727; nucleotides 1302 to 1800), designated as the positive internal control in the miniexon PCR assay (6) was amplified by PCR by using primers cpb2fw (5′-TGA TCA CCA CGA AGG AGT GC-3′) and cpb2rev (5′-ACG CGA GGG CAC GCG AGG-3′). Miniexon primer sites were introduced by subsequent nested PCR with the composite primers cpb2Mifw (5′-TAT TGG TAT GCG AAA CTT CCG TGA TCA CCA CGA AGG AGT GC-3′) and cpbMirev (5′-ACA GAA ACT GAT ACT TAT ATA GCG ACG CGA GGG CAC GCG AGG-3′). PCR mixtures (50 μl) and cycling conditions for all the reactions were the same as those for the miniexon PCR described below. PCR products were purified by size-selective polyethylene glycol precipitation and cloned by using the pGEM-T vector system (Promega, Madison, Wis.) according to the manufacturer's instructions. Plasmid DNA containing the positive control template was purified by using the CONCERT Rapid Plasmid Miniprep system (Invitrogen Life Technologies, Basel, Switzerland) according to the manufacturer's manual. Purified plasmid DNA was stored in Tris-EDTA, pH 8.0, containing 0.05% W-1 (Invitrogen Life Technologies).

PCR assays. For repetitive sequence PCR (*Leishmania* genotyping [LEG] PCR) (28), primers T2 (5′-CGG CTT CGC ACC ATG CGG TG-3′) and B4 (5′-ACA TCC CTG CCC ACA TAC GC-3′) were used to amplify a repetitive DNA sequence from Old World *Leishmania*. Two microliters of DNA solution was amplified in a 100-μl reaction mixture containing 50 mM PCR buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 μM (each) primers, and 0.5 U of *Taq* DNA polymerase (Invitrogen Life Technologies). PCR amplification was carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Boston, Mass.). After an initial denaturation step (5 min at 96°C), 40 cycles of 96°C for 30 s, 56°C for 45 s (annealing), and 72°C for 60 s (extension) were performed. For miniexon PCR, primers Fme (5′-TAT TGG TAT GCG AAA CTT CCG-3′) and Rme (5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′) were used to amplify the nontranscribed spacer region of the miniexon sequence of *Leishmania* (24). Two microliters of DNA solution was amplified in a 50-μl reaction mixture containing 50 mM PCR buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 40 mM tetramethylammonium chloride, 12% dimethyl sulfoxide, 0.5 μM (each) primers, and 0.5 U of *Taq* DNA polymerase. The positive control template was added to a final concentration of 250 fg (corresponding to approximately 70,000 plasmid copies) per reaction mix. The PCR amplification was

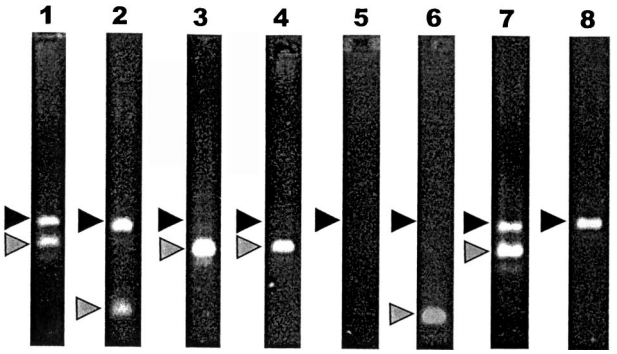


FIG. 1. Coamplification of *Leishmania* DNA from clinical samples and a positive control template. Black triangles denote the 540-bp band of the positive control template, and grey triangles correspond to the *Leishmania*-specific PCR signal. Lanes 1 and 2, positive samples. The PCR products of the target DNA and the positive control template are visible on the agarose gel. Lanes 3 and 4, positive samples. Target DNA outcompeted the positive control template. Lane 5, PCR inhibition is indicated because the positive control template is not visible. Lane 6, previously inhibited sample after DNA dilution. Lane 7, previously inhibited sample after subsequent purification. Lane 8, negative sample with only control template visible.

carried out in a Mastercycler gradient 5331 (Eppendorf, Hamburg, Federal Republic of Germany). After an initial denaturation (5 min at 94°C), 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 45 s were performed.

Genotyping. Typing of the *Leishmania* species was achieved by RFLP analysis of the amplified miniexon gene. Ten microliters of the miniexon PCR products was digested with the restriction enzymes according to the genotyping scheme described elsewhere (24).

In order to confirm our genotyping results, DNA aliquots from all the samples found to be positive by PCR were analyzed by two other PCR-RFLP genotyping schemes based on the sequence differences in the genomic repeat of Old World *Leishmania* (30) and the ITS region of the SSU rRNA gene (37a).

Cultivation of parasites. The culture medium, a 1:1 mixture of SDM-79 (7) and SM (8) complemented with 15% heat-inactivated fetal bovine serum, was supplemented with an anticontamination cocktail consisting of penicillin G (60 μg/ml), kanamycin (100 μg/ml), chloramphenicol (10 μg/ml), and flucytosine (50 μg/ml). The medium was directly inoculated with the patient specimens and incubated at 27°C without gas phase in T-25 tissue culture flasks (Falcon, BD Biosciences, Allschwil, Switzerland). Cultures were checked for the presence of promastigote forms of *Leishmania* by examination with an inverted microscope. Microscopic examination was done every 2 days for up to 15 days.

Serology. Serum samples were tested for anti-*Leishmania* antigen antibodies by using an IFAT. Twelve-well slides were coated with imprint preparations of an *L. donovani*-infected hamster spleen and stored at −80°C. Slides were defrosted, fixed with acetone for 10 min at room temperature, and dried under ventilation. The wells on the slides were covered with 25 μl of serial twofold dilutions (1:40 to 1:1,280 in PBS, pH 7.2) of the sera and incubated in a humid chamber for 30 min at 37°C. After three washes with PBS, pH 7.2, 25 μl of fluorescein isothiocyanate-labeled goat anti-human immunoglobulin G (Biomérieux, Marcy l'Etoile, France) diluted 1:80 in Evansblue (0.1 mg/ml of PBS, pH 7.2) was applied to each well. After incubation in a humid chamber for 30 min at 37°C and three washes with PBS, pH 7.2, the slides were dried under ventilation, mounted with 50% (vol/vol) glycerol-PBS, and covered with a coverslip. Staining of the samples was assessed by examination with a fluorescence microscope and compared with that of positive and negative controls. Samples showing fluorescence with serum dilutions higher than 1:80 were regarded as positive.

RESULTS

Application of the miniexon PCR assay to clinical samples. In order to identify inhibitory effects in the PCR due to impurities in the sample and to assess tube-to-tube variations in PCR efficiencies, the test was set up with an internal positive control. The sensitivity of the assay was not compromised by the coamplification of the positive control template (data not shown), and a concentration of 250 fg of plasmid DNA (corresponding to about 70,000 plasmid copies) per reaction mix produced a clear band in the PCR assay. From a total number of 50 clinical samples, DNA was purified and tested with the miniexon PCR assay. DNA from each sample was coamplified along with the positive control template. Of the 26 DNA samples which gave a positive result, the majority outcompeted the positive control template (Fig. 1, lanes 3 and 4), whereas in the remaining samples the signals of both the *Leishmania* and the positive control were visible on the agarose gel (Fig. 1, lanes 1 and 2). The other 24 samples were negative in the assay (Fig. 1, lane 8). PCR inhibition was observed in 6 of the 50 samples (Fig. 1, lane 5). Inhibition was resolved in two samples after a 10-fold dilution, after which one sample became positive (Fig. 1, lane 6) and one was determined to be negative. The remaining four samples had to be purified again by using the QIAamp DNA Mini Kit, after which two became positive (Fig. 1, lane 7) and two were determined to be negative.

Comparison of the diagnostic assays. The miniexon PCR assay was compared to the methods currently applied at the STI for the diagnosis of leishmaniasis (i.e., in vitro cultivation and serology) and a PCR assay which is based on the amplification of a repetitive genomic sequence of Old World *Leishmania* (LEG PCR assay) (28). All tissue samples, which were obtained from patients with a tentative diagnosis of leishmaniasis on clinical grounds, were tested with the LEG and the miniexon PCR assays, and 38 of them were inoculated into culture. Corresponding serum samples, available from 16 patients, were analyzed for anti-*Leishmania* antigen antibodies by IFAT. Results are summarized in Table 1. For a limited comparison, only positive samples were tested by the PCR assay using the ITS region of the SSU rRNA gene (37a).

No single test could serve as a "gold standard" against which the different assays could be evaluated. Therefore, assessment of test performance (Table 2) was based on the assumptions that (i) a patient was positive for leishmaniasis when the infection was detected by at least one of the diagnostic assays and (ii) a patient was negative for leishmaniasis when results of all four assays (including serology) were negative. A *Leishmania* infection was diagnosed in 29 patients. Two infections were detected by all four methods, 12 infections were detected by

TABLE 1. Diagnostic results for clinical samples

Specimen type	Total no. of positive samples[a]	No. of samples positive by:				
		Culture	LEG PCR	Miniexon PCR	Serology[b]	SSU-ITS PCR
Skin biopsy	12	6	8	12	4	6
Bone marrow	7	2	7	4	3	3
Lesion aspirate	1	0	1	1	0	1
Blood[c]	2	2	2	2	0	1
Duodenal biopsy[d]	2	1	2	2	0	1
Gastric biopsy[d]	1	1	1	1	0	1
Paraffin-embedded skin biopsy	4	0	2	4	1	1
Total	29	12	23	26	8	15

[a] Leishmaniasis confirmed by at least one of the diagnostic tests.
[b] For patients from whom a corresponding serum sample was available.
[c] Supplemented with EDTA.
[d] From patients positive for HIV.

TABLE 2. Comparison of the diagnostic assays

Assay (total)[a]	No. of positive[b] samples/no. of tested samples							Sensitivity (%)
	Skin biopsy specimens	Bone marrow samples	Blood samples	Duodenal biopsy specimens[c]	Gastric biopsy specimens	Lesion aspirates	Total	
In vitro cultivation (38)	6/9	3/5	1/1	1/1	1/1	NA	12/17	70.59
Serology (16)	5/9	3/4	0/1	NA	NA	NA	8/14	57.14
LEG PCR (50)	10/16[d]	7/7	2/2	2/2	1/1	1/1	23/29	79.31 (100)[d]
SSU-ITS PCR (29)[e]	7/16	3/7	1/2	2/2	1/1	1/1	15/29	51.71
Miniexon PCR (50)	16/16	4/7	2/2	2/2	1/1	1/1	26/29	89.66

[a] Total number of samples subjected to each assay.
[b] Leishmaniasis confirmed by at least one of the diagnostic tests.
[c] NA, not applicable.
[d] All negative samples were subsequently shown to be infected with New World species; therefore, sensitivity for Old World species was adjusted to 100%.
[e] Only samples that were previously identified as positive by other assays were tested.

three methods, and 10 infections were detected by two methods. In five patients, the presence of a *Leishmania* infection was confirmed by one method only (four cases were confirmed by the miniexon PCR assay, and one was confirmed by the LEG PCR assay).

In vitro cultivation was performed for a total of 38 samples. No cultures were set up from the four paraffin-embedded specimens, and results for eight specimens are missing because either they were lost due to contamination or there was no request for this test. Twelve of 17 *Leishmania*-positive samples were detected by cultivation, of which one infection was confirmed by one other test, 13 were confirmed by two other tests, and three were confirmed by three other tests, resulting in a sensitivity of 70.6%. The remaining 21 samples with a negative culture result were also negative by both PCR assays, giving a specificity of 100%.

Sixteen serum samples were available to test by IFAT for anti-*Leishmania* antigen antibodies. This test identified eight positive samples from 14 patients whose infections were confirmed by other assays (two were confirmed by three other tests, three were confirmed by two other tests, and three were confirmed by one other test) but failed to diagnose the infection in six samples determined to be positive by other assays, giving a sensitivity of 57.1%. Two serum samples available from patients without an infection, as confirmed by all other tests, were also negative (specificity, 100%).

The LEG PCR detected *Leishmania* in 23 of the 29 positive samples, giving a sensitivity of 79.1%, but failed to diagnose the infection in six specimens that were confirmed to be positive by other tests. Parasites in all six samples for which no PCR product was obtained were subsequently identified by miniexon RFLP analysis as *Leishmania* species from the New World, resulting in a sensitivity of 100% for Old World species only. Specificity of the LEG PCR assay was 100% with all samples which were also negative by all other tests, but one sample was positive only with this test and was not confirmed to be positive by any of the other tests.

The PCR-RFLP genotyping assay based on the ITS region of the SSU rRNA gene detected a *Leishmania* infection in 15 of the 29 samples and gave a sensitivity of 51.7%. Because we tested only samples previously identified as positive, we were not able to determine the specificity of the assay.

The miniexon PCR detected *Leishmania* in 26 of the 29 positive samples and gave a sensitivity of 89.7% but failed to diagnose the infection in three specimens, of which two infections were confirmed by the LEG PCR assay and positive serology and one was confirmed by the LEG PCR assay only. It is worth mentioning that these three DNA samples were all derived from bone marrow specimens and were freeze-thawed several times. All the 21 *Leishmania*-negative patients were negative by the miniexon PCR assay (specificity, 100%), but it should be noted that four of the 29 positive results were not confirmed by any other test.

Genotyping of *Leishmania* spp. by RFLP analysis. All samples positive by the miniexon PCR assay were subjected to RFLP analysis of the amplification product (24). No unusual or ambiguous RFLP patterns occurred, and *Leishmania* from all positive samples could be assigned to a given species according to the typing scheme. The species in 15 of the 26 positive samples was identified as *L. infantum*, that in four samples was identified as *L. major*, those in one sample each were identified as *L. tropica* and *L. mexicana*, that in three samples was identified as *L. braziliensis*, and that in two samples was identified as *L. guyanensis*.

Species in the 23 samples positive by the LEG PCR assay were typed by RFLP analysis as belonging to the Old World species *L. infantum* ($n = 14$), *L. donovani* ($n = 4$), *L. major* ($n = 4$), and *L. tropica* ($n = 1$).

DNA aliquots of the samples from the 29 *Leishmania*-positive patients were also analyzed by the PCR-RFLP genotyping assay, which is based on sequence variations in the ITS region of the SSU rRNA gene (37a). With this assay, species from positive samples were typed at the complex level. They were assigned to the *L. donovani* ($n = 7$), *L. major* ($n = 6$), *L. tropica* ($n = 1$), and *L. braziliensis* ($n = 1$) complexes.

A summary of the genotyping results is given in Table 3. There was a good correlation between the results obtained with the LEG and miniexon genotyping schemes. However, the LEG PCR system cannot detect any of the New World species, and a species from four samples typed as *L. donovani* with the LEG PCR system was typed as *L. infantum* with the miniexon system. There was also a good correlation between the results of the miniexon and ITS genotyping schemes, except that a species from two samples typed as *L. infantum* by the miniexon system was assigned to the *L. major* complex by the ITS system. These two samples were two of the four samples with a species typed as *L. donovani* by the LEG PCR system.

TABLE 3. Results from three PCR-RFLP genotyping schemes for *Leishmania* spp.

No. of patients	Species or complex identified by[a]:		
	LEG-RFLP typing	Miniexon RFLP typing	ITS-RFLP typing
1	ND	*L. braziliensis*	*L. braziliensis* complex
2	ND	*L. braziliensis*	ND
2	ND	*L. guyanensis*	ND
1	ND	*L. mexicana*	ND
1	*L. tropica*	*L. tropica*	*L. tropica* complex
4	*L. major*	*L. major*	*L. major* complex
7	*L. infantum*	*L. infantum*	*L. donovani* complex
4	*L. infantum*	*L. infantum*	ND
3	*L. infantum*	ND	ND
2[b]	*L. donovani*	*L. infantum*	ND
2[b]	*L. donovani*	*L. infantum*	*L. major* complex

[a] ND, not determined (PCR test failed to detect the infection).
[b] Discordant results.

DISCUSSION

Detection of *Leishmania* parasites in a clinical sample is necessary to confirm a suspected case of leishmaniasis. Most commonly used methods for the direct detection of the parasite (e.g., microscopic examination of Giemsa-stained smears and in vitro cultivation) lack sensitivity because of the paucity of *Leishmania* parasites in some specimens (42) or are hampered by the problem of contamination (3). Indirect methods (e.g., detection of anti-*Leishmania* antigen antibodies by serological methods) are also limited in sensitivity and are not able to discriminate between past and current infections. The advent of the PCR technology has opened new avenues in the diagnosis of leishmaniasis, and several approaches have been developed during the last two decades (11, 43). Most of these assays are based on highly repetitive genomic gene loci or extrachromosomal kinetoplast DNA sequences, but their use is confined to the detection of the parasite at genus (5, 25) or complex (10, 13) level, and therefore, their application is limited to restricted geographical areas and diagnostic settings.

Our assay was developed for use in a diagnostic setting where patients with a history of global travel are seen. It was the aim of this study to optimize and evaluate a recently developed PCR-RFLP-based genotyping assay for *Leishmania* species. The assay targets the miniexon gene of the parasite and has proven to be simple and cheap; i.e., the detection of all *Leishmania* spp. is accomplished by one PCR with all-encompassing primers. It has a sufficient sensitivity, high specificity, and the ability to differentiate between all clinically relevant *Leishmania* species by RFLP analysis of the PCR product (24).

The inclusion of a positive control template makes it possible to detect inhibitory effects in the PCR mix and also controls for tube-to-tube variation both within and between different PCR experiments. Several methods are available for the design and construction of an internal positive control, some of which were successfully used in PCR assays for *Leishmania*. These include the coamplification of the human β-globin gene in a multiplex PCR approach (27), the use of a competitive PCR assay with heterologous plasmid sequences (25), and the use of synthetic probes in the reaction (4). We used a competitive PCR assay targeting a 498-bp fragment of the *cpb2* gene of *L. mexicana*. This fragment is slightly larger than our target sequence, has a G+C content similar to that of the miniexon sequence, and is free of any restriction sites required for subsequent RFLP analysis of the miniexon sequence. In all assays, this positive control template yielded a clearly distinguishable PCR product, and coamplification did not affect assay sensitivity or compromise amplification of the miniexon sequence and never interfered with subsequent RFLP analysis (data not shown).

Fifty patient samples were available for the validation of the assay, and samples were subjected in parallel to a second PCR assay (LEG PCR) (28). When possible, samples were cultured, and serum samples were tested for *Leishmania*-specific antibodies. As expected, PCR assays were by far more sensitive than the currently applied diagnostic methods. Successful culture is dependent on the presence of viable organisms, and growth requirements are known to differ between species (3). IFAT failed to detect six infections (those of four patients with cutaneous manifestations and those of two patients suspected to have visceral leishmaniasis) which were all confirmed by at least two other tests. This failure might reflect the generally lower sensitivity of serological assays for detection of cutaneous forms of the disease (21) or might be due to a lack of cross-reactivity with the antigens on which the assay is based (26). Furthermore, humoral responses in patients with a concurrent HIV infection or other causes of immunosuppression may be moderate or even absent (1). Indeed, all three samples obtained from HIV-positive patients were negative by the serological assay.

The LEG PCR assay was tailored for the characterization of Old World *Leishmania* and consequently failed to detect six infections by parasites subsequently typed as New World species by the miniexon assay. On the other hand, the miniexon assay failed to detect three infections by parasites typed as Old World species by the LEG assay, which might be due to lower sensitivity of the miniexon PCR. However, storage of DNA from these three patients might also have compromised the results of the miniexon PCR.

Proper sampling is crucial for the establishment of a reliable diagnostic assay for leishmaniasis, and basic questions, such as what the optimal site of sample collection is (32) and whether blood samples are sufficient for a reliable diagnosis, remain open. Hematogenous dissemination has been described for *L. donovani*, *L. infantum*, *L. tropica*, and *L. braziliensis* (J. Kubar, J. F. Quaranta, P. Marty, A. Lelievre, Y. Le Fichoux, and J. P. Aufeuvre, Letter, Nat. Med. **3:**368, 1997), and PCR-based assays on blood samples have proven useful for the diagnosis and monitoring of visceral leishmaniasis in adults and children in the Mediterranean region, as well as in HIV-coinfected patients (15, 19). The four blood samples in our study were processed only after the request for an additional tissue sample, and their analysis confirmed the results obtained with the other analyzed specimens.

The miniexon PCR assay was more sensitive than the conventional diagnostic methods and capable of detecting infection in a wide range of clinical samples, including paraffin-embedded tissue samples. This is of advantage since tissue biopsy specimens are often fixed and embedded in paraffin for histological examinations. Application of the PCR assay to paraffin-embedded specimens prevents repeated invasive sam-

pling if a differential diagnosis of leishmaniasis is required and might be useful for the investigation of archived samples. It is noteworthy that other PCR-based assays have reported a higher sensitivity. Yet, these assays detect the presence of *Leishmania* parasites rather than individual *Leishmania* species. Although we found the miniexon assay to show a high sensitivity, other assays might be preferable for samples in which very low densities might be expected.

Prior to the development of biochemical or molecular tools for the characterization of *Leishmania*, diagnosis of leishmaniasis relied on clinical and epidemiological criteria. Over the past few years, the reports of atypical clinical manifestations have accumulated, challenging this traditional classification. This includes reports of visceral leishmaniasis caused by the dermotropic species *L. tropica* and *L. mexicana* (23) and the occurrence of cutaneous manifestations caused by *L. chagasi* and *L. infantum*, which usually cause visceral leishmaniasis (31; J. A. Rioux and G. Lanotte, Letter, Trans. R. Soc. Trop. Med. Hyg. **84:**898, 1990).

Therefore, correct classification of infecting *Leishmania* species can provide important complementary information for the clinician that may result in modified clinical prognosis, as well as initiation of species-specific therapeutic approaches (3). Furthermore, identification of the infecting species is important in monitoring therapy. In areas where different species are sympatrically transmitted, discrimination between relapse of a latent infection and reinfection by another species is important in cases of reemerging leishmaniasis.

In order to appraise the validity of the miniexon genotyping scheme, we typed the samples with two other PCR-RFLP-based methods, the LEG and the ITS system. Although no unusual patterns were encountered with any of the genotyping schemes and the correlation between the results of all the systems was satisfying (Table 3), some discrepancies were observed. Because the biological species concept is difficult to apply to *Leishmania* and there is as yet no agreement on the definition of the species boundaries within the genus (2), none of the genotyping systems could actually serve as a gold standard for the evaluation of the others. We observed four discrepancies, two in which data obtained with the miniexon PCR suggested the presence of *L. infantum* but LEG PCR identified *L. donovani*. Although *Leishmania* has been thought to be clonal (2), hybrid forms within the *L. donovani* complex might occur. Using independent single marker genes cannot exclude this possibility. Discrepant identification of *L. infantum* and *L. major* in two cases cannot be explained but might reflect a laboratory artifact. Previously, the miniexon genotyping system has been tested with both World Health Organization reference strains and culture isolates from the STI, all independently characterized by isoenzyme analysis, and the results were in good yet not perfect concordance with the species definitions (24).

In conclusion, the miniexon PCR-RFLP-based genotyping assay has proven to be a reliable test for the detection of *Leishmania* in a wide range of clinical samples obtained from a travel clinic. The standardization of the test as a competitive assay including the coamplification of an internal positive control template has turned out to be a valuable tool for the assessment of PCR performance. We have demonstrated that the assay is quick and easy to perform and shows in this study a higher sensitivity than conventional diagnostic methods. Moreover, it enables the characterization of the parasite at species level by a simple method within a reasonable time frame and provides a useful tool for the characterization of clinically relevant *Leishmania* species, which can help the clinician choose an adequate therapeutic approach.

ACKNOWLEDGMENTS

The project was supported by the European Community grant no. FAIR-CT 98-4104. This work also obtained support from the Schweizerische Nationalversicherung.

We thank all the members of the Diagnostic Centre at the Swiss Tropical Institute for their collaboration and support.

REFERENCES

1. **Alvar, J., C. Canavate, B. Gutierrez-Solar, M. Jimenez, F. Laguna, R. Lopez-Velez, R. Molina, and J. Moreno.** 1997. *Leishmania* and human immunodeficiency virus coinfection: the first 10 years. Clin. Microbiol. Rev. **10:**298–319.
2. **Banuls, A. L., M. Hide, and M. Tibayrenc.** 1999. Molecular epidemiology and evolutionary genetics of *Leishmania* parasites. Int. J. Parasitol. **29:**1137–1147.
3. **Berman, J. D.** 1997. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin. Infect. Dis. **24:**684–703.
4. **Bezold, G., M. Lange, K. Gethoffer, H. Pillekamp, H. Reindl, C. Richter, G. Schonian, L. Weber, and R. U. Peter.** 2001. Competitive polymerase chain reaction used to diagnose cutaneous leishmaniasis in German soldiers infected during military exercises in French Guiana. Eur. J. Clin. Microbiol. Infect. Dis. **20:**421–424.
5. **Bhattacharyya, R., K. Das, S. Sen, S. Roy, and H. K. Majumder.** 1996. Development of a genus specific primer set for detection of *Leishmania* parasites by polymerase chain reaction. FEMS Microbiol. Lett. **135:**195–200.
6. **Brooks, D. R., H. Denise, G. D. Westrop, G. H. Coombs, and J. C. Mottram.** 2001. The stage-regulated expression of *Leishmania mexicana* CPB cysteine proteases is mediated by an intercistronic sequence element. J. Biol. Chem. **276:**47061–47069.
7. **Brun, R., and M. Schonenberger.** 1979. Cultivation and *in vitro* cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop. **36:**289–292.
8. **Cunningham, I.** 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. **24:**325–329.
9. **Davila, A. M., and H. Momen.** 2000. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within *Leishmania*. Ann. Trop. Med. Parasitol. **94:**651–654.
10. **De Bruijn, M. H., and D. C. Barker.** 1992. Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Trop. **52:**45–58.
11. **Degrave, W., O. Fernandes, D. Campbell, M. Bozza, and U. Lopes.** 1994. Use of molecular probes and PCR for detection and typing of *Leishmania*—a mini-review. Mem. Inst. Oswaldo Cruz **89:**463–469.
12. **Eisenberger, C. L., and C. L. Jaffe.** 1999. *Leishmania*: identification of Old World species using a permissively primed intergenic polymorphic-polymerase chain reaction. Exp. Parasitol. **91:**70–77.
13. **Eresh, S., S. M. McCallum, and D. C. Barker.** 1994. Identification and diagnosis of *Leishmania mexicana* complex isolates by polymerase chain reaction. Parasitology **109**(Pt. 4)**:**423–433.
14. **Fernandes, O., V. K. Murthy, U. Kurath, W. M. Degrave, and D. A. Campbell.** 1994. Mini-exon gene variation in human pathogenic *Leishmania* species. Mol. Biochem. Parasitol. **66:**261–271.
15. **Fisa, R., C. Riera, E. Ribera, M. Gallego, and M. Portus.** 2002. A nested polymerase chain reaction for diagnosis and follow-up of human visceral leishmaniasis patients using blood samples. Trans. R. Soc. Trop. Med. Hyg. **96**(Suppl. 1)**:**S191–S194.
16. **Grimaldi, G., Jr., J. R. David, and D. McMahon-Pratt.** 1987. Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. Am. J. Trop. Med. Hyg. **36:**270–287.
17. **Harris, E., G. Kropp, A. Belli, B. Rodriguez, and N. Agabian.** 1998. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. J. Clin. Microbiol. **36:**1989–1995.
18. **Hassan, M. Q., A. Ghosh, S. S. Ghosh, M. Gupta, D. Basu, K. K. Mallik, and S. Adhya.** 1993. Enzymatic amplification of mini-exon-derived RNA gene spacers of *Leishmania donovani*: primers and probes for DNA diagnosis. Parasitology **107**(Pt. 5)**:**509–517.
19. **Lachaud, L., J. Dereure, E. Chabbert, J. Reynes, J. M. Mauboussin, E. Oziol, J. P. Dedet, and P. Bastien.** 2000. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. J. Clin. Microbiol. **38:**236–240.

20. **Lainson, R., and J. J. Shaw.** 1987. Evolution, classification and geographical distribution, p. 1–120. *In* W. Peters and R. Killick-Kendrick (ed.), The leishmaniases in biology and medicine. Academic Press, Orlando, Fla.
21. **Liew, F. Y., and C. A. O'Donnell.** 1993. Immunology of leishmaniasis. Adv. Parasitol. **32:**161–181.
22. **Luis, L., A. Ramirez, C. M. Aguilar, S. Eresh, D. C. Barker, and A. Mendoza-Leon.** 1998. The genomic fingerprinting of the coding region of the beta-tubulin gene in *Leishmania* identification. Acta Trop. **69:**193–204.
23. **Magill, A. J., M. Grogl, R. A. Gasser, Jr., W. Sun, and C. N. Oster.** 1993. Visceral infection caused by *Leishmania tropica* in veterans of Operation Desert Storm. N. Engl. J. Med. **328:**1383–1387.
24. **Marfurt, J., I. Niederwieser, D. Makia, H. P. Beck, and I. Felger.** 2003. Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn. Microbiol. Infect. Dis. **46:**115–124.
25. **Mathis, A., and P. Deplazes.** 1995. PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. J. Clin. Microbiol. **33:**1145–1149.
26. **Medrano, F. J., C. Canavate, M. Leal, C. Rey, E. Lissen, and J. Alvar.** 1998. The role of serology in the diagnosis and prognosis of visceral leishmaniasis in patients coinfected with human immunodeficiency virus type-1. Am. J. Trop. Med. Hyg. **59:**155–162.
27. **Meredith, S. E., E. E. Zijlstra, G. J. Schoone, C. C. Kroon, G. J. Van Eys, K. U. Schaeffer, A. M. el Hassan, and P. G. Lawyer.** 1993. Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. Arch. Inst. Pasteur Tunis **70:**419–431.
28. **Minodier, P., R. Piarroux, F. Gambarelli, C. Joblet, and H. Dumon.** 1997. Rapid identification of causative species in patients with Old World leishmaniasis. J. Clin. Microbiol. **35:**2551–2555.
29. **Pearson, R. D., A. De Queiroz Sousa, and S. M. B. Jeronimo.** 2001. Leishmania species: visceral (kala-azar), cutaneous and mucosal leishmaniasis, p. 2831–2845. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York, N.Y.
30. **Piarroux, R., M. Fontes, R. Perasso, F. Gambarelli, C. Joblet, H. Dumon, and M. Quilici.** 1995. Phylogenetic relationships between Old World *Leishmania* strains revealed by analysis of a repetitive DNA sequence. Mol. Biochem. Parasitol. **73:**249–252.
31. **Ponce, C., E. Ponce, A. Morrison, A. Cruz, R. Kreutzer, D. McMahon-Pratt, and F. Neva.** 1991. *Leishmania donovani chagasi*: new clinical variant of cutaneous leishmaniasis in Honduras. Lancet **337:**67–70.
32. **Ramirez, J. R., S. Agudelo, C. Muskus, J. F. Alzate, C. Berberich, D. Barker, and I. D. Velez.** 2000. Diagnosis of cutaneous leishmaniasis in Colombia: the sampling site within lesions influences the sensitivity of parasitologic diagnosis. J. Clin. Microbiol. **38:**3768–3773.
33. **Ramos, A., D. A. Maslov, O. Fernandes, D. A. Campbell, and L. Simpson.** 1996. Detection and identification of human pathogenic *Leishmania* and *Trypanosoma* species by hybridization of PCR-amplified mini-exon repeats. Exp. Parasitol. **82:**242–250.
34. **Rodgers, M. R., S. J. Popper, and D. F. Wirth.** 1990. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. Exp. Parasitol. **71:**267–275.
35. **Rodriguez, N., B. Guzman, A. Rodas, H. Takiff, B. R. Bloom, and J. Convit.** 1994. Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridization. J. Clin. Microbiol. **32:**2246–2252.
36. **Rossi, V., P. Wincker, C. Ravel, C. Blaineau, M. Pages, and P. Bastien.** 1994. Structural organisation of microsatellite families in the *Leishmania* genome and polymorphisms at two (CA)n loci. Mol. Biochem. Parasitol. **65:**271–282.
37. **Russell, R., M. P. Iribar, B. Lambson, S. Brewster, J. M. Blackwell, C. Dye, and J. W. Ajioka.** 1999. Intra and inter-specific microsatellite variation in the *Leishmania* subgenus *Viannia*. Mol. Biochem. Parasitol. **103:**71–77.

37a. **Schonian, G., A. Nasereddin, N. Dinse, C. Schweynoch, D. F. H. Schallig, W. Presber, and C. L. Jaffe.** A PCR method for the diagnosis and characterization of *Leishmania* in local and imported clinical samples. Diagn. Microbiol. Infect. Dis., in press.

38. **Schonian, G., C. Schweynoch, K. Zlateva, L. Oskam, N. Kroon, Y. Graser, and W. Presber.** 1996. Identification and determination of the relationships of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. Mol. Biochem. Parasitol. **77:**19–29.
39. **Ullu, E., C. Tschudi, and A. Günzl.** 1996. Trans-splicing in trypanosomatid protozoa, p. 115–133. *In* D. F. Smith and M. Parsons (ed.), Molecular biology of parasitic protozoa. IRL Press at Oxford University Press, Oxford, England.
40. **Van Eys, G. J., G. J. Schoone, N. C. Kroon, and S. B. Ebeling.** 1992. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. Mol. Biochem. Parasitol. **51:**133–142.
41. **Victoir, K., A. L. Banuls, J. Arevalo, A. Llanos-Cuentas, R. Hamers, S. Noel, S. De Doncker, D. Le Ray, M. Tibayrenc, and J. C. Dujardin.** 1998. The gp63 gene locus, a target for genetic characterization of *Leishmania* belonging to subgenus *Viannia*. Parasitology **117**(Pt. 1)**:**1–13.
42. **Weigle, K. A., M. de Davalos, P. Heredia, R. Molineros, N. G. Saravia, and A. D'Alessandro.** 1987. Diagnosis of cutaneous and mucocutaneous leishmaniasis in Colombia: a comparison of seven methods. Am. J. Trop. Med. Hyg. **36:**489–496.
43. **Wilson, S. M.** 1995. DNA-based methods in the detection of *Leishmania* parasites: field applications and practicalities. Ann. Trop. Med. Parasitol. **89**(Suppl. 1)**:**95–100.

3.4. Discussion

Sexual reproduction has not yet been shown in *Leishmania*, so the term ‘species’ might not be correctly used for all known strains. As example, *L. infantum* and *L. chagasi*, as well as *L. major* and *L. venezuelensis* are believed to be genetically identical, only the geographic distribution differs. Therefore, *L. chagasi* and *L. venezuelensis* most probably derived from dogs infected by *L. infantum* and *L. major* respectively, which had been imported during settlement of the Americas by Europeans. Sequencing of the mini-exon genes supports this hypothesis. Furthermore, the mini-exon genes of *L. colombiensis* and *L. naiffi* showed high homology.

Although the mini-exon gene is present in kinetoplatids, nematodes and even chordates, the specifity of the PCR for *Leishmania* is given, since DNA of *Trypanosoma* and nematodes lacks proper binding sites of our primers. However, PCR using DNA of *Chritidia* (parasitic kinetoplastids, living in insects) as template yielded an amplification of the mini-exon gene but the resulting restriction patterns of the PCR products were completely different from *Leishmania* patterns.

The mini-exon PCR proved to be a great tool for confirming an suspected *Leishmania* infection and distinguishing the infecting species at the same time. However, it is important to note that when diagnosing *Viannia species* a single point mutation within the restriction site can change the interpretation of the results. As *L. brasiliensis* and *L. guyanensis* differ in the required treatment, but their mini-exon genes differ solely by a few point mutations, another genetic marker should be used for these species.

However, the mini-exon PCR was not designed as solely tool used for diagnosis, but as an additional method to confirm interpreting of the symptoms and classical diagnostic tools, such as cultivation of the parasites.

4. Identification of genes encoding potential virulence factors, drug targets and vaccine candidates of *L. infantum*.

4.1. Background

For establishment of a successful infection in the vertebrate host, *Leishmania* parasites are involved in the following events:

1. Evasion of the humoral lytic factors.
2. Attachment to macrophages following endocytosis of the parasites.
3. Survival within the harsh conditions of phagolysosomes.
4. Differentiation from pro- to amastigotes.
5. Replication of the amastigotes.
6. Infection of 'empty' macrophages by amastigotes.

These events yield in escape from the host's innate immune system by intracellular parasitism. Furthermore, the parasite enhances its pathogenity by triggering the immune answer towards a non-protective T_H2 type answer, resulting in establishment of a long lasting infection. All parasite factors involved in these processes can be defined as virulence factors. However, in a broader range, virulence can not solely be enhanced by factors involved in host-parasite interactions but also by unspecific processes as high replication rate. The whole complexity of this area is shown in figure 4.

While some *Leishmania* virulence factors are known - as GP63 (inhibition of complement mediated lysis, reviewed by Yao et al. (2003) or LPG (survival within phagolysosomes, Spath et al., 2003) -, many others may have not yet been discovered.

In order to identify potential virulence factors, a cDNA subtraction was performed using metacyclic (transmission) stage cDNA from a strain of low infectivity and a strain of high infectivity:

highly infective strain
- low infective strain
potential virulence factors

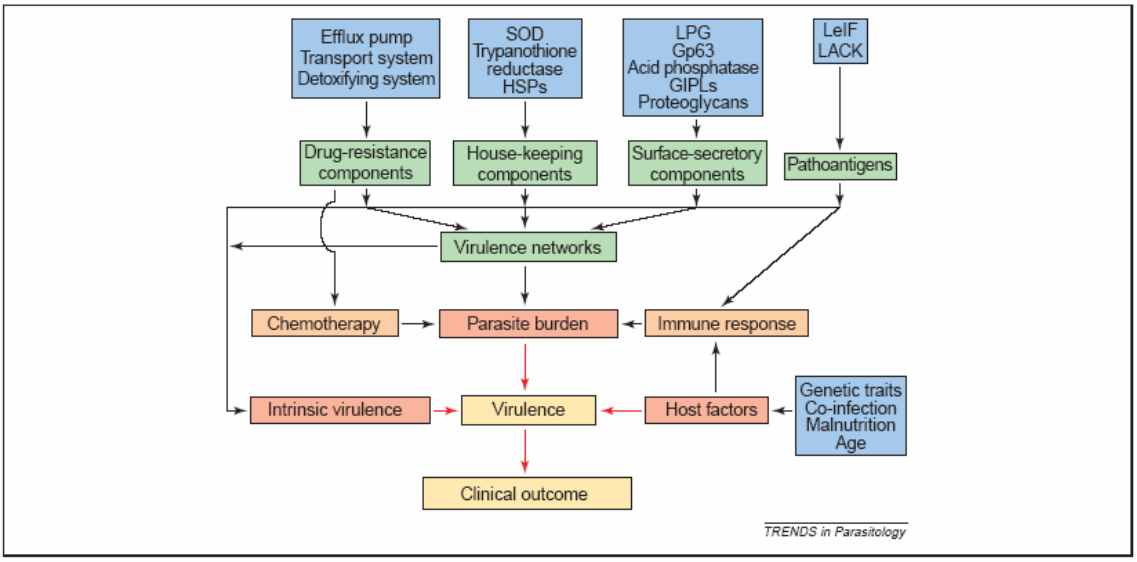


Figure 4 (from (Rivas et al., 2004). A global view of virulence. Clinical outcome is a direct consequence of virulence, when considered in a broad sense. There are three main virulence factors (indicated in red): intrinsic virulence; parasite burden; and host factors. Intrinsic virulence is the addition of single actuations of the different groups of virulence factors, together with intertwined actions among them, grouped under the term virulence networks. Some of the representative examples of the corresponding groups are indicated in blue. At the same time, the host plays a major role in clearing parasite burden either through the immune response or chemotherapy (indicated in orange).This figure is partially based on the concepts developed by Chang et al. (2003). In the case of natural infections, chemotherapy will be deleted and, from an epidemiological background, other factors, such as virulence inside the vector or the vector–mammal relationship, must be incorporated. Abbreviations: GIPL, glycoinositolphospholipid; Gp63, glycoprotein 63 KDa (leishmaniolysin); HSP, heat-shock protein; LACK, Leishmania homologue of receptors for activated C-kinase; LeiF *Leishmania* elongation initiation factor; LPG, lipophosphoglycan; RACKs, receptors for activated C kinase; SOD,.superoxide dismutase.

However, the two strains differed not solely in infectivity, but also in growth rate (which of course has a great impact on virulence). Nevertheless, higher growth rate is achieved by up regulation of many genes, for which association with virulence can hardly be determined. Although late stationary phase cultures (consisting mostly of non-dividing metacyclics) were used for the subtraction, many of the genes we selected were indeed up regulated in the highly infective strain, but could not be put into relation with virulence - as translation elongation factors and histone H1.

Therefore, the experiment was redesigned. cDNA of non-infective vector stages was subtracted from the infective vector stage of the same strain:

infective stage

\- non-infective stages

potential virulence factors

Since the latest vector stage, the metacyclic stage is the only vector stage capable of infecting macrophages, early vector stages were subtracted from late stages. For successful transmission, metacyclis escape from the complement, and have to be phagocytized by macrophages, where they must survive within phagolysosomes and differentiate into amastigotes. Furthermore, one could speculate that metacyclic

parasites are also involved in triggering the host's immune answer towards a non-protective direction, since metacyclics are the first parasites seen by the immune system. Consequently, many virulence factors can be assumed to be expressed within this parasite stage.
For the same reason, transmission blocking vaccine candidates might be identified, if putative membrane proteins expressed at metacyclic's surface would be discovered (anti-bodies against a flagellum specific epitope were already shown to inhibit adherence of promastigotes to phagocytes, Singla and Vinayak, 1994).
Furthermore, identification of proteins of metacyclic parasites which show no homologies to mammal proteins might be regarded as potential drug targets for both, prophylaxis and treatment (for the latter purpose only when these proteins are also expressed in amastigotes).

4.2. Methods

We used suppression subtractive hybridization (SSH, Diatchenko et al., 1996) as described below. Since most of the mRNAs in *Leishmania* contain a 5' conserved sequence, two simplifications of the SSH method were tested.

SSH
Using this method (figures 5 and 6A), ds cDNA was the starting material for both, tester and driver (the driver is subtracted from the tester). The ds cDNA was cut with a suitable restriction enzyme leading to fragment sizes of 100 to 500 bases. In contrast to the driver, half of the tester was ligated to adaptor1, the remaining tester to adaptor2. In two hybridization steps, pre-subtracted tester1 and 2 were finally combined. This led to formation of a hybrid of tester 1 and 2. Prior to PCR, the ends were filled in. For selection of the tester hybrid, a suppression PCR was performed using adaptor primers. Since the 5' ends of both adaptors consisted of a palindromic sequence, DNA strands containing the same adaptors at both ends could form a loop and thus were not efficiently amplified (suppression PCR). In contrast, the tester hybrid containing 2 different adaptors at both ends was amplified exponentially and could be subsequently ligated into a cloning vector.

Modification1
In this method (figures 5 and 6B), tester and driver were obtained by generating cDNA using the spliced leader sequence. Tester 1 was amplified using a short spliced leader primer and RT-primer1. For Tester 2, a long spliced leader primer and RT-primer2 was used, and amplification of the driver was done using the short

spliced leader primer and RT-primer1. The hybridization scheme was the same as for SSH above. The hybrid of tester 1 and 2 could be selected by PCR (using the very 5'end of the mini-exon as forward and RT-primer1 as reverse primer).

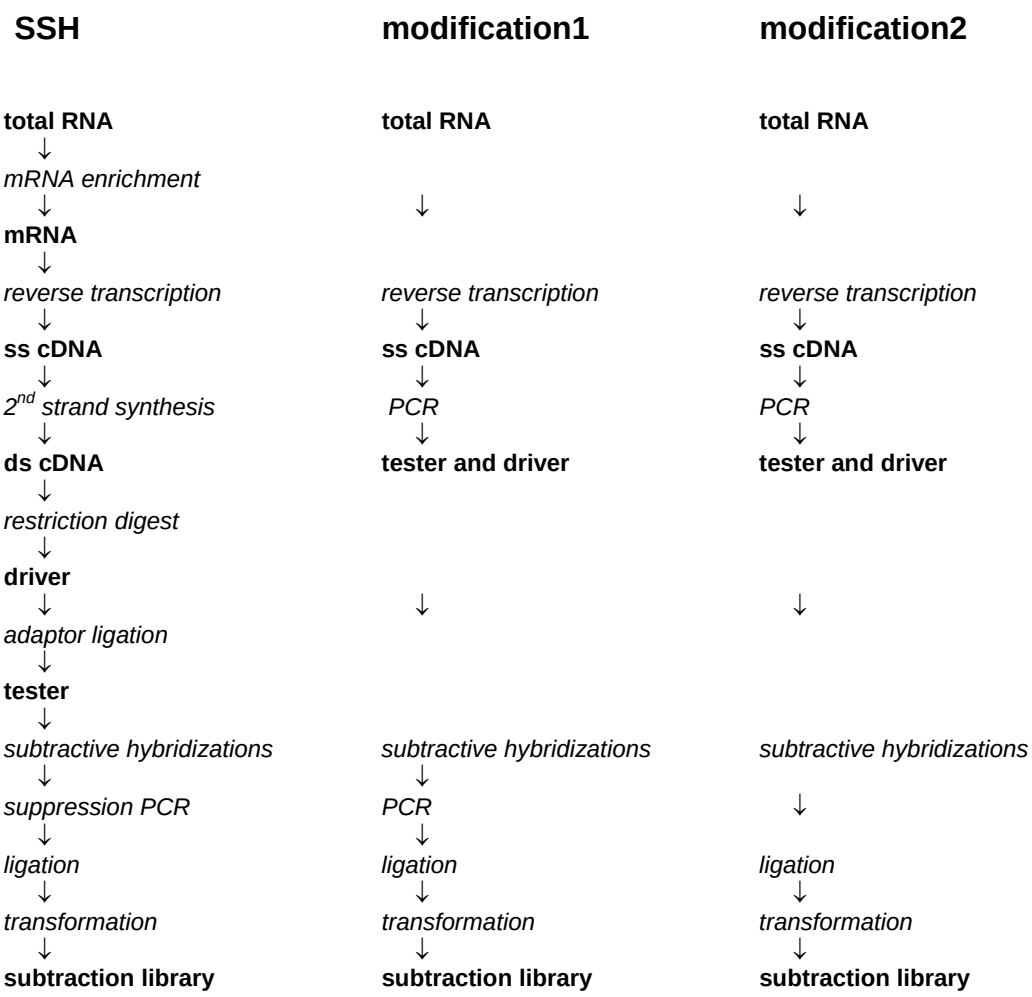


Figure 5: production steps required for SSH and its modifications.

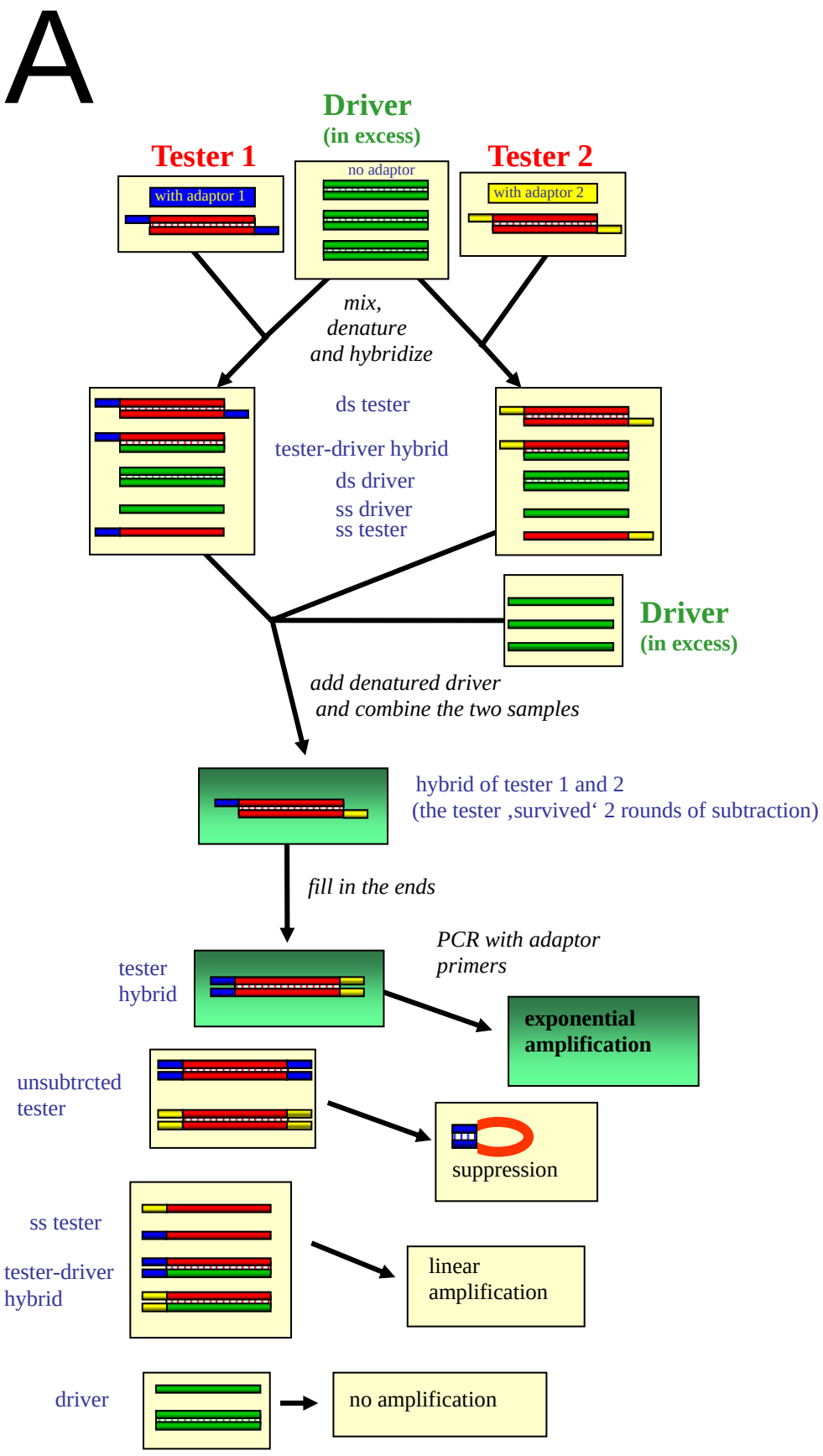
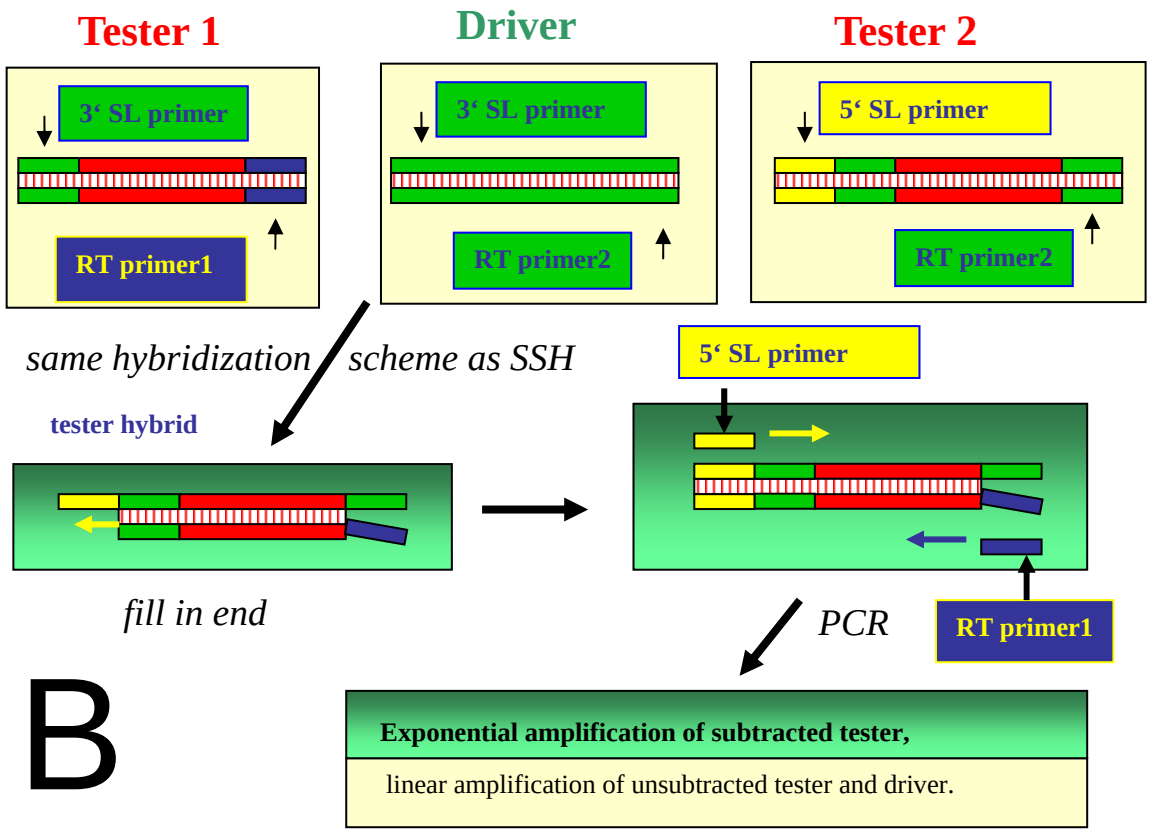


Figure 6: details of SSH (A) and modifications (B and C, next page)



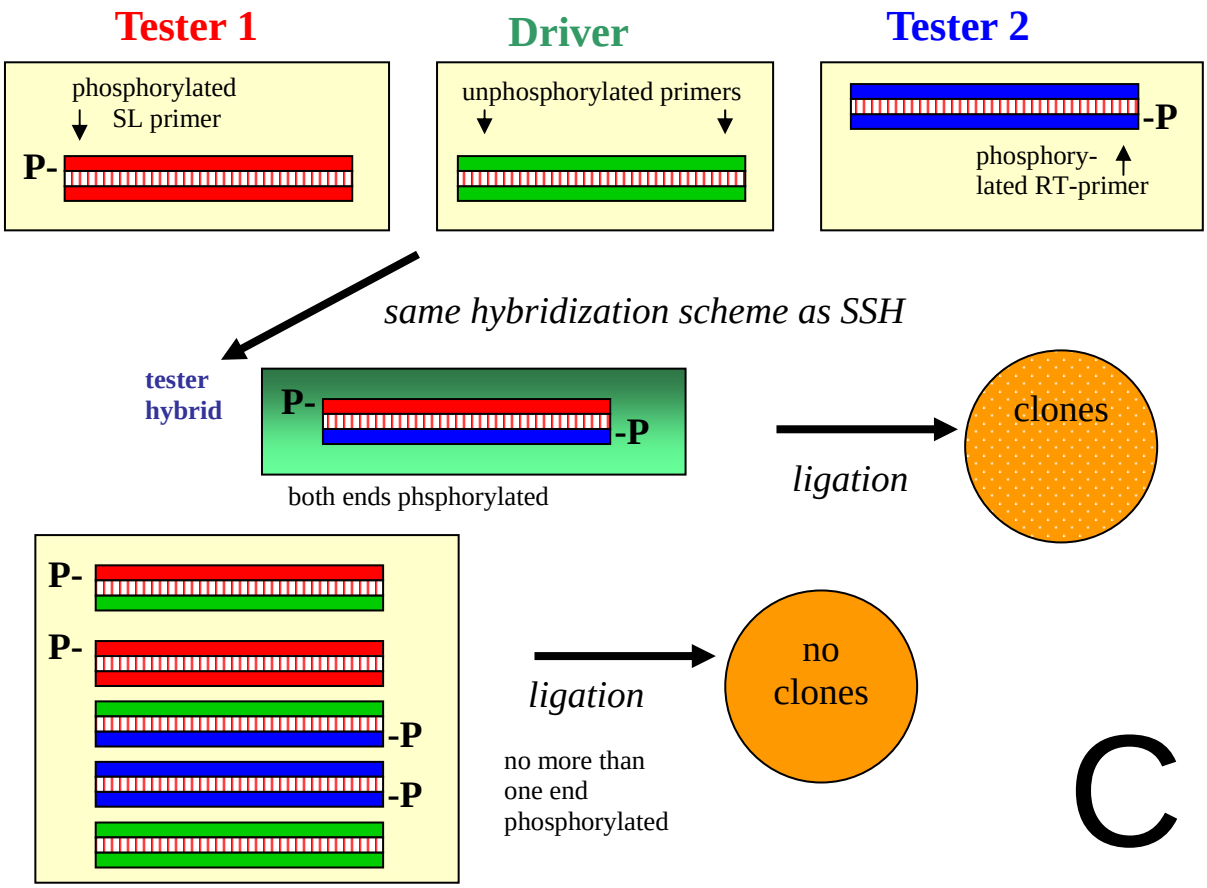


Figure 6: details of SSH (A, previous page), modification1 (B) and modification2 (C)

Again, tester and driver were obtained using spliced leader RT-PCR (figures 5 and 6C). For tester1, the spliced leader primer but not the RT-primer was phophorylated, in contrast to tester2, where the RT-primer but not the spliced leader primer was phosphorylated. The driver was amplified using non-phosphorylated primers. Because the hybrid of tester1 and 2 was the only ds DNA in the hybridizytion mix which was phosphorylated at both ends, it could be selected by ligation. It could either be directly ligated into a blunt end cut and dephosphorylated vector, or ligated to non-phosphorylated adaptors (using subsequently PCR with adaptor primers as method for selection of the subtracted tester).

The advantages of both modifications would be:

- total RNA (not only mRNA) could be used as starting material
- PCR instead of second strand synthesis
  - subtraction using limited amounts of RNA possible
- no restriction digest of tester and driver
  - subtraction library would contain full length cDNA resulting in simplification of gene identification.
    Furthermore, selecting of different fragments belonging to the same gene would be avoided.
- no adaptor ligation prior to subtraction.
  - Blunt end ligation, as used in SSH, usually lacks high efficiency.

However, changes in hybridization conditions (DNA concentration, buffer, temperature) yielded in completely different band patterns after the final PCR using the modified subtraction methods. Since too many optimization steps would have been necessary to get to work both modifications in a reliable manner, both methods were not developed beyond the state of testing. Nevertheless, the potential of the second modification was promising, due to its simplicity.

SSH was used for both strain and stage subtractions. For the strain subtraction, amplified spliced leader cDNA was the starting material, whereas the protocol of Clontech's PCR select subtraction kit was followed for the stage subtraction.
The differentially expression patterns of the clones were performed by Northern blot analysis and their sequence was analyzed using motif scans and basic local alignment search tools (BLAST). In order to perform localization studies, three clones of the stage subtraction library were recombinantly expressed in *E. coli*, and the purified proteins were used to immunize mice.

4.3. Differentially expressed genes in *Leishmania infantum* promastigotes

Submitted to Experimental Parasitology

Differentially expressed genes in *Leishmania infantum* promastigotes

Igor Niederwieser, Jutta Marfurt, Hans-Peter Beck*

Swiss Tropical Institute
Socinstrasse 57
CH 4002 Basel
Switzerland

* Corresponding author:
Tel: +41-61-284 8116
Fax: +41-61-271 8654
e-mail: hans-peter.beck@unibas.ch

Keywords: suppression subtractive hybridization, metacyclic stages, stage specific regulation, MAT-1, transporter genes, CytochromeB5-like gene

Note: Sequence data reported in this paper are available at GenBank™ under the accession numbers: ……..
SSH obtained short sequence fragments were submitted separately from sequences obtained by primer walking.

Abstract

Suppression subtractive hybridization technology was used to select *Leishmania infantum* promastigote genes that show differential expression in early logarithmic phase and stationary phase parasites. Several genes showing differential expression were identified and eight were selected for further analysis. Increased expression in stationary phase parasites was observed for 7 genes, while one selected gene was strongly down regulated in this phase. The selected genes encoded putatively for a glucose transporter, an amino acid transporter, a short chain dehydrogenase, a cytochrome b5 like protein, a protein probably involved in protein-protein interactions, proteins with unknown functions and the previously identified metacylogenesis associated transcript 1 (MAT-1). Larger sequences of the selected genes were generated using primer walking, and the cellular localization of the glucose transporter was determined by immunofluorescence. In this report we show that suppression subtractive hybridization can be used to identify growth stage specific genes of *Leishmania*, and that this approach can potentially identify drug or vaccine targets.

Introduction

Visceral leishmaniasis (VL) caused by the protozoan parasite *Leishmania infantum* is usually fatal if not rapidly diagnosed and treated. Existing first line drugs for the treatment of VL, such as Pentostam and Glucamtin, are highly toxic and may cause severe side effects. Over the last ten years, there has been a sharp increase in the number of recorded cases and distribution of this disease (WHO, http://www.who.int/emc/diseases/leish/leishmaniasis.pdf), highlighting the need for better intervention strategies. *Leishmania* are transmitted as promastigotes by sandflies (*Phlebotomus sp*. and *Lutzomyia sp*.) to vertebrate hosts where they are endocytosed by polymorphonuclear phagocytes and dendritic cells. Following phagocytosis they differentiate inside phagolysosomes into amastigotes where they multiply and are responsible for disease. Several types of promastigote forms have been described in sandflies and culture, including dividing parasites (procyclic and nectomonad forms) and non-dividing parasites (metacyclic forms). Metacyclic parasites are of special interest since they are the infectious promastigote form transmitted by insect vectors to the mammalian host (reviewed by Killick-Kendrick, 1990).

Because metacyclic parasites are responsible for the establishment of a new infection, it is likely that genes responsible for virulence may be up regulated during this phase of promastigote growth. Three prominent parasite virulence factors, lipophosphoglycan (LPG), the major surface protease (MSP or gp63) and the amastigotes specific gene family A2, have been identified (Descoteaux and Turco, 2002). LPG covers most of the metacylic form's surface and has been shown to be structural different from LPG expressed on the parasite during other stages of growth (McConville et al., 1992). *lpg1*$^{-}$ mutants are highly susceptible to human complement, have lost the ability to inhibit phagolysosomal fusion transiently, and are oxidant sensitive (Spath et al., 2003). MSP is also considered a virulence factor (reviewed by Yao et al., 2003). This protein is encoded for by a gene family of which several show stage specific expression. MSP plays an important role in resistance to complement mediated lysis by converting the complement factor C3b to the inactive form C3bi (Brittingham et al., 1995) and may show additional functions as discussed by Yao et al. (2003). Finally Zhang and Matlashewski (1997) showed that products of the amastigote specific gene family A2 are required

for virulence of *L. donovani* in mice.

Since metacyclic promastigotes are exposed to environments seen by both stages of the parasite such as the sandfly vector, the vertebrate host's blood, and macrophage phagolysosome, one might expect that they share potential drug targets with intracellular amastigotes. Therefore, identification metacyclic specific genes could reveal proteins that are essential for the infection of and survival in the macrophages. These proteins might represent interesting new targets for the development of new drugs or vaccines. As yet, no vaccine against any *Leishmania* species is in routine use, though several experimental vaccines are currently being tested (Ghosh and Bandyopadhyay, 2003). Because metacyclic parasites are exposed to the complement system and important in inducing initial B- and T-cell responses, surface molecules expressed at this stage might be exploited as potential vaccine candidates. Futhermore, resistance appears to be increasing against most drugs used to treat VL today and new drugs are urgently needed.

Several techniques have been used successfully to identify metacyclic specific genes including differential screening of poly(A)+ RNA (Coulson and Smith, 1990), vectorette PCR amplified 3' untranslated sequences (Coulson et al., 1997) and microarray analysis (Saxena et al., 2003). Within *in vitro* cultures, metacyclic forms are primarily found in stationary phase cultures. Since metacyclics appear to represent the most virulent promastigotes, we enriched for genes differentially expressed in this stage by suppression subtraction cDNA hybridization (SSH) technique (Diatchenko et al., 1996) of cDNA synthesized in late logarithmic and stationary phase cultures. Using SSH we were able to identify several differentially expressed genes not yet described. These included two genes encoding transporters, a protein containing leucine rich repeats and an open reading frame (ORF) lacking homology to any known or hypothetical protein. Furthermore, the previously described MAT-1 (Brodin et al., 1992) encoding the small hydrophilic endoplasmic reticulum associated protein (SHERP, Knuepfer et al., 2001) was also selected by the SSH technique. Several of these genes were further analyzed, and two were expressed for immunization and localization studies. In addition, differentially expressed genes were compared by SSH using weakly infective and highly infective *L. infantum* strains.

.

Materials and Methods

Parasites

Two strains of *L. infantum* strains were used. Promastigotes of the strain LEM 287-D/ST (low infectivity) and LEM 768-A/ST (high infectivity, Grimm et al., 1991) were cultured at 27°C in a 1:1 mixture of SDM-79 (Brun and Schonenberger, 1979) and Schneider's medium (Cunningham, 1977) containing 10% [v/v] heat inactivated fetal calf serum (FCS). Parasite densities and parasite volumes were quantified with a CASY®1 Cell Counter + Analyser Model TT (Schaerfe System GmbH). Cultures were inoculated at a density of $5x10^5$ parasites/ml. Stationary phase parasites obtained 15 days (LEM 287) and 10 days (LEM 768) after inoculation were used for cDNA SSH. For SSH experiments using logarithmic and stationary phase parasites, cultures of the LEM 768 were harvested at different time points (table 1).

RNA preparation

Promastigotes (6ml of an *in vitro* culture) were centrifuged, the pellet washed in phosphate buffered saline (PBS) and total RNA isolated using 10ml TRIZOL (Invitrogen). After extraction and isopropanol precipitation, the pellet was suspended in 1ml TE (10mM TrisCl pH 8, 0.5mM EDTA). Another 10ml TRIZOL was added to the resuspended RNA, RNA extracted, and precipitated again. The final RNA pellet was washed with 75% ethanol, suspended in 0.25ml TE. RNA and either stored in TE at -80°C or as a precipitate at -20°C. Total RNA (1/100 of the preparation) was examined by non-denaturing agarose gel (reference table 1) and mRNA was isolated using oligo(dT) cellulose (Micro-Fast Track kit, Invitrogen) according to the supplier's instructions from half the remaining total RNA.

cDNA Subtraction

Differential cDNA subtraction was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech), based on SSH technique (Diatchenko et al., 1996). For the strain subtraction, we took advantage of the fact that most trypanosomatids mRNAs contain a conserved 5' region, the spliced leader, first described by Boothroyd and Cross (1982). Therefore, we used PCR amplified spliced leader cDNA of strain LEM 768 (infective) as tester and cDNA of LEM 287 (low infective)

as driver. Total RNA (~2µg) of both strains was reverse transcribed using 200u M-MLV reverse transcriptase (Invitrogen) and the RT-primer (table 2). Subsequently, RNA was removed by RNase A (Boehringer) digestion. The cDNA was diluted 1:10 and PCR amplified using the RT-primer and the SL-primer (table 2) according to the following program: 25 cycles 30sec 94°C, 30sec 54°C, 2min 72°C using Taq DNA polymerase (Qiagen) in presence of 10% [v/v] dimethyl sulfoxide (DMSO). These PCR products replaced the double stranded cDNA used in the PCR-Select™ cDNA Subtraction Kit, whose instructions were followed subsequently.
For the stage subtraction, ~0.5µg mRNA from late logarithmic phase promastigotes (day 2) was used as starting material for the driver and mid-stationary phase promastigotes (day 5) as starting material for the tester. The subtraction was performed according to the manufacturer's protocol with a minor modification. After the first 2 rounds of subtractive hybridization, the hybridization mix was diluted 1:200. And instead of using only one dilution as suggested in the kit's manual, the mix was diluted further (undiluted, 1:10, 1:100) and was amplified in a primary PCR (5min 75°C and 27 cycles 30sec 94°C, 30sec 66°C, 1min 30sec 72°C) using Advantage® cDNA Polymerase Mix (Clontech). Primary products were diluted 1:10 and amplified in a nested PCR (5min 94°C and 10 to 28 cycles 30sec 94°C, 30sec 68°C, 1min 30sec 72°C) using Taq DNA polymerase (Qiagen). For the construction of 3 libraries we chose the following PCR products: undiluted hybridization mix with 16 nested cycles, 1:10 dilution with 19 cycles, 1:100 dilution with 25 cycles.
The PCR products obtained by both subtraction experiments were cloned into pUC18 (Amersham). Several clones were grown for mini prep plasmid preparations which were subsequently sequenced.

Northern blot

RNA was denatured in glyoxal, run on agarose gels after the method of Burnett (1997), and transferred to Hybond XL membranes (Amersham) using a vacuum blotter (Appligene). For labeling, mini preps were diluted 1:100 and the inserts were PCR amplified with SP6 and T7 primers (all primers obtained from Qiagen). The products were purified by polyethylene glycol (PEG) precipitation (Hartley and Bowen, 1996) and labeled with α-^{32}P dCTP (Amersham) using the High Prime kit (Roche Biochemicals). The hybridization was carried out overnight in a hybridization oven at 42°C in 1M NaCl, 50mM 4-morpholinepropanesulfonic acid

(MOPS) pH 7, 50% [w/v] formamide, 0.5% [w/v] sodium dodecyl sulfate (SDS), 1mM EDTA, 18% [w/v] dextran sulfate, 5x Denhardt's reagent (Denhardt, 1966). After hybridization, membranes were washed twice in 2x SSC (1x SSC is 0.15M NaCl, 15mM sodium citrate), 0.1% SDS and twice in 0.1x SSC, 0.1% SDS at 42°C. The filters were exposed to Kodak MS films for 30min to 48h at -80°C using a Transcreen HE enhancer (Kodak).

cDNA walking

For cDNA walking we also took advantage of the conserved 5' region in trypanosomatids. We chose the strategy shown in figure 1 to obtain full length cDNA sequences. mRNA was reverse transcribed by M-MuLV Reverse Transcriptase, RNase H^- (Finnzymes) using the RT-primer (table 2). RNA was removed by RNase A and H digestion. For RT-PCR, the cDNA was diluted 1:50 in TE and amplified using insert specific and the RT-primers to obtain the 3' end, or insert specific and the SL-primers (table 2) to obtain the 5' end. 40 cycles (30s 94°C, 30s 56°C, 2min 72°C) were performed in Taq buffer in presence or absence of 10% [v/v] DMSO using Taq and Pfu (Promega) DNA polymerases in a 10:1 proportion. PCR products were cloned and sequenced. If no products were obtained, a nested PCR was performed to obtain the 5' end. For primary PCRs, outer insert primers and the pSL-primer (table 2) were used, for nested PCRs, inner insert primers and the nSL-primer (table 2) were used and 2 x 25 cycles were performed. Cycle conditions and the concentrations of DMSO (0-10%), Tween20 (0-0.5% [v/v]) and BSA (0-0.5g/l) were optimized for each PCR. The primary PCR was performed with Taq/Pfu polymerase (10:1), the nested with Taq polymerase alone. The PCR products were cloned into pGEM-T (Promega) vector and sequenced.

Sequence analysis

The basic local alignment search tools (Blast) of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/, Blastx, Altschul et al., 1990; Gish and States, 1993) and EBI (Washington University Blast Version 2.0 Parasites, http://www.ebi.ac.uk/blast2/parasites.html) were used, transmembrane domains were predicted at CBS with TMHMM (Krogh et al., 2001, http://www.cbs.dtu.dk/services/TMHMM-2.0/) and motif scans were performed at

EBI with InterProScan (Mulder et al., 2003, http://www.ebi.ac.uk/interpro/scan.html).

Protein expression in E. coli

Proteins were expressed as N-terminal GST fusion proteins with a C-terminal 6 x His tag in pGEX-His vector. This vector was obtained by ligation of a His-adaptor made by the two oligo-nucleotides His-fw and His-rev (table 2) into *Sal*I- and *Not*I-digested (all restriction enzymes obtained from New England Biolabs) pGEX-6p-1 (Amersham). The following fragments were PCR amplified with primers containing a *Bam*HI and *Sal*I site, respectively: the C-terminal part of the putative steroid dehydrogenase (clone1, table 3) with primers 1-fw and 1-rev, and a fragment between two transmembrane domains of the glucose transporter (clone6) with the primers 6-fw and 6-rev (table 2). Prior to ligation, PCR products and the pGEX-His vector were cut with *Bam*HI and *Sal*I and purified by phenol extraction and PEG precipitation. Proteins were expressed in BL21 cells (Amersham) which were grown in Terrific Broth (Tartoff and Hobbs, 1987). When cultures reached an optical density of 1, protein expression was induced with 0.5mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 4 hours. All three proteins were insoluble in PBS/Triton X-100. Thus, after purification of the inclusion bodies, proteins 1 and 6 were purified with Ni-NTA Agarose (Qiagen) under denaturing conditions (protein 1 was not soluble in 8M urea, and urea was replaced with 6M guanidine chloride in all solutions).

Immunization

For each protein, two NMRI mice's were immunized with 1 and 5µg purified protein using CpG DNA as adjuvant (ImmunEasy™ Mouse Adjuvant, Qiagen). Two booster injections followed 2 and 4 weeks after the first immunization using the same conditions. 6 week after immunization, mice were sacrificed and polyclonal sera were collected.

Western blots

Proteins were run on 12.5% polyacrylamide gels in SDS/glycine buffer and blotted semi dry on nitrocellulose (Amersham) in 10mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 10% [v/v] methanol, pH10.5. In order to reduce

background and binding of anti-GST antibodies, an extract of induced BL21 cells containing the empty pGEX-6p-1 vector was used to adsorb the mouse sera.

Immuno fluorescent assays

500µl of a 10 day old *Leishmania in vitro* culture were spun and the pellet was resuspended in 20µl Fetal Calf Serum (FCS). 2.5µl each were used to prepare several smears on glass slides. Smears were dried, fixed in 1:1 methanol/acetone for 10min at -20°C. The slides were partitioned with a hydrophobic pen. *E. coli* adsorbed sera were used at a concentration of 1:20 in PBS, 1% BSA. For competition with clone 6, 0.1µg/µl recombinant protein was added, which lead to an urea concentration of 0.8M. Therefore, the positive control in this competition experiment was done in PBS, 1% BSA, 0.8M urea. Cy3 labeled goat anti-mouse IGg antibody (Jackson) was used as secondary antibody 1:200 in PBS, 1% BSA and 1ng/µl 4',6-diamidino-2-phenylindole (DAPI) as DNA stain.

Results & Discussion

Strain subtraction

In order to identify *Leishmania* virulence factors, we subtracted in a preliminary experiment cDNA of a strain of low infectivity (LEM 287) from a highly infective strain (LEM 768). We used PCR amplified spliced leader cDNA as tester and driver for the SSH (figure 2). Northern blot analysis showed that many of the genes selected were more strongly expressed by the highly virulent strain than by the low virulent strain (figure 3) and that SSH can be used when double stranded cDNA is replaced by PCR amplified spliced leader cDNA (obtained from total RNA). This is of importance in situations where starting material is limited (for instance for *in vivo* studies).

Sequence analysis of the genes identified with this first approach made it difficult to understand their involvement in virulence as it is unclear why genes such as the Histone H1 gene or genes involved in translation elongation should be associated with virulence. An explanation for this apparent experimental failure might lay in the large difference between both strains, not only seen in infectivity, but also possibly in growth rate and other aspects which might be reflected in virulence.

Nevertheless, the histone H1 locus of *L. major* was also examined by Belli et al. (2003) and found to be stage specifically expressed as sense and antisense RNA.

Stage subtraction

Figure 4 shows the outcome of the SSH. It clearly shows, that the band patterns differed, when different dilutions were amplified. This was not suggested in the kit's manual. We also introduced a hybridization control (figure 4) in which the same amount of DNA was added prior to PCR, but hybridization was eliminated by denaturation (figure 4: - gel). The high background of the undiluted negative control was probably caused by amplification of the most abundant tester cDNAs since PCR cycling allows hybridization of tester cDNA, which leads to amplification of the tester cDNA. Therefore, the background of the undiluted SSH is much higher because hybridization is more efficient in concentrated DNA solutions.

We selected 8 differentially expressed genes from the stage subtraction for which the results are shown in table 3. We selected genes which putatively coded for proteins involved in transmembrane transport (2 genes), in redox reactions (2 genes), in protein-protein interactions (1 gene), and 3 genes encoding proteins of unknown functions. Recently, extensive work has been published using microarray analysis (Saxena et al., 2003) and genes were identified which are expressed at higher levels in metacyclics of *L. major*. These were genes encoding proteins involved in cell division, metabolism, membrane transport, protein synthesis and other functions. Surprisingly, despite MAT-1 none of the genes described here were identified using the microarray technique.

In order to test whether the identified genes were indeed differentially expressed genes, northern blots of selected clones were performed (figure 5). Since double stranded DNA was used as probes for northern blots, we cannot determine whether we detected sense or antisense RNA. As expected, most of the selected genes were up regulated in stat phase cultures, but there was a background of constitutively expressed genes, and one clone was even down regulated. This was probably due to primer binding sites located within this gene: the adaptors provided by the SSH kit are composed of very GC rich sequences, which is also the case for the *Leishmania* genome. Therefore, the background probably increases when using the SSH kit for subtractions within GC rich genomes.

Using Blast searches we found that the inserts of clone 2 and 5 were located within

open reading frames (ORFs).
Clone 5 was identified as a fragment of the previously published metacylogenesis associated transcript 1 (MAT-1) which was found several previously by Brodin et al. (1992).
Blast searches with the insert of clone 2, which was down regulated in stat phase cultures, showed homologies to CN hydrolases (E-value= 2e-16) and ABC transporters (E-value= 2e-11), suggesting its involvement in amino acid (AA) transport and/or modification.
Blast searches to identify the other selected genes initially failed, because of the small average size of the inserts (~250bp) and because of their location in the 3' untranslated region (UTR). Therefore we used the strategy depicted in figure 1 to obtain the full length sequences.
A cDNA walk revealed that clone 1 was a sequence fragment of a gene encoding a protein involved in redox reactions namely a putative short chain dehydrogenase (motifscan: E-value=4.9e-29).
Clone 3 was identified as a fragment of a gene coding for a putative AA transporter (Blastx: AA permease AAP15LD *L. donovani*, E-value=4e-67), which is up regulated in stat-phase cultures. Its sequence differed substantially from the one found in *L. major* metacyclics by Saxena et al. (2003).
We further identified clone 4 as a gene fragment of a gene containing leucine rich repeats, which usually are implicated in protein-protein interactions. This protein is homologue to GU1 of *T. brucei* (Liniger et al., 2001, blastx: E-value=2e-31).
Clone 6 was found to be a gene fragment coding for a putative sugar permease (motif scan: E-value=3.1e-88). There are several glucose transporters known in *L. mexicana* (Burchmore and Landfear, 1998). LmGT1 and 3 are constantly expressed during the life cycle, whereas LmGT2 is up regulated in amastigote stage parasites. Here we show that expression levels of glucose transporters in the different promastigote stages of *L. infantum* also vary. Mouse sera elicited against the selected and recombinamtly produced sugar transporter gave a membrane staining in IFAs (figure 6).
Clone 7 was a fragment of a cytochrome b5 (CYTB5) like gene (motif scan: E-value=1.2e-15). One could speculate that this protein might be needed for the detoxification of oxygen and NO radicals which are present in abundance in activated macrophages.

After cDNA walking we obtained for clone 8 a 335 AA ORF but no significant homology to any known or hypothetical protein or putative domains could be detected therefore the gene function remains unknown.

In order to generate antisera in mice, fragments of the putative short chain dehydrogenase (clone 1) and the putative glucose permease (clone 6) were expressed in *E. coli* as GST fusion proteins containing a 6 x His tag. Both proteins were found in inclusion bodies, and different expression conditions (change of IPTG concentration, expression temperature, or addition of ethanol to induce chaperons) did not overcome this.problem. Sera which we obtained for the putative short chain dehydrogenase (clone 1) did not recognize the recombinant fragment and was excluded from further analysis. Sera against recombinant proteins derived from clones 6 showed multiple bands in western blots, despite absorption of sera on *E. coli* extract (data not shown). It might be possible that multiple bands are obtained because the different glucose transporter isoforms are highly conserved in *Leishmania* (Burchmore and Landfear, 1998), and therefore might allow cross-reactivity.
Immuno fluorescent assays (IFAs) with sera against clone 6 (glucose permease) showed a membrane staining, which decreased significantly after competition with the recombinant protein (figure 6).

Several genes we found contained transmembrane domains (TM, clones 1, 3 and 6) and might be localized at the parasite's surface as integral membrane proteins. Such localization for stage specific regulated genes is hardly surprising, since the environmental change from the digestive conditions in the insect gut to the harsh conditions in phagolysosomes of macrophages certainly requires extensive surface changes. And most probably not all membrane proteins will be functional in both environments, e.g. pH differences might causes protonization of functional amino acids, and thus reduce functional activity. Furthermore, the different environments certainly differ in the nutrient supply and these characteristics together may explains stage specific gene regulation in genes involved in AA transport, which we identified.
Whether the 3 surface antigens we selected are capable of inducing an immunological response, and whether this response has a protective value or

would even enhance infectivity needs to be determined in further studies. In human blood, about 90% of the metacyclic forms are killed right after transmission (Dominguez et al., 2002). This might indicate that only a small number of parasites needs to be eliminated to obtain complete protection. A vaccine might just provide that effect and clearance of all metacyclic stage parasites injected by the bite of the insect vector might be eliminated.

SSH

On a technical side it is interesting to note, that inserts derived from the subtraction library of different strains and generated by using spliced leader cDNA were usually located in the coding region. This facilitated the easy identification of the selected genes. In contrast, the stage specific subtraction library contained inserts predominantly derived from the 3'UTR and thus required cDNA walking for gene identification. For the stage subtraction we used cDNA which was generated by reverse transcription with Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) provided with the subtraction kit. Due to its powerful RNase H activity, AMV-RT can cleave the template near the 3' terminus (Kotewicz et al., 1988) resulting in cDNA fragments of restricted length. Consequently, most of the inserts in the subtraction library were 3' UTR. Further, many of the genes contained long 3'UTRs compared to the ORF: 0.1kb 5'UTR, 0.3kb ORF and 1.2kb 3'UTR in case of the CYTB5 like gene. Since most of the transcripts are polycistronic in trypanosomatids (Graham, 1995), the UTRs are probably strongly involved in regulation of the RNA stability hence in stage specific gene regulation.

During the SSH it proved useful to amplify different dilutions of the hybridization mix, since some genes were exclusively found in a subtraction library constructed by amplification of a distinct dilution, e.g. MAT-1.

Conclusions

As expected, SSH enriched differentially expressed genes in both subtraction experiments. It was furthermore possible to replace double stranded cDNA by PCR amplified cDNA, and this expands the use of SSH to experiments, where only limited amounts of RNA are available. In one subtraction library (stage comparison), most of the inserts were 3'UTR due to the use of a reverse

transcriptase with a powerful RNase H activity. This is not necessarily a drawback when working with completely sequenced genomes, but in the case of *L. infantum*, cDNA walking for gene identification was necessary and proved to be difficult and time consuming. This could be avoided by replacing AMV-RT by a reverse transcriptase lacking RNase H activity, or by using PCR amplified cDNA as starting material for SSH.

From the stage specific subtracted library, we selected 8 differentially expressed genes with interesting properties. But in most cases determination of RNA level and simple sequence analysis alone were not sufficient to address questions of gene function. In order to assign a role of the genes found up regulated in stat phase culture, these genes have to be examined *in vivo* by knock out or RNA interference experiments. Furthermore, the biochemical pathways in which the two selected enzymes (clones 1 and 7) are implicated have to be determined and the suitability of the 3 identified membrane proteins as vaccine candidates has to be tested. But in general, SSH is a technique which rapidly allows the identification and cloning of stage specifically regulated genes, even for organisms where yet micro array expression profiling experiments cannot be done, or micro arrays yet are incomplete. While microarray analysis of the *L. major* transcriptome has been performed, none of the genes we selected despite MAT-1 was described in these analyses. This might have occurred by chance but might show that microarray analyses can overlook other expressed genes. Therefore, classical methods such as subtractions still have their value.

Acknowledgement

The authors are grateful to the staff of the protozoology unit for providing *Leishmania* cultures and support. Financial support is acknowledged from the European Community/Bundesamt für Bildung und Wissenschaft under the grant number FAIR-CT-98-4104.

References

Abe, K., S. Kimura, R. Kizawa, F.K. Anan, and Y. Sugita. 1985. Amino acid sequences of cytochrome b5 from human, porcine, and bovine erythrocytes and comparison with liver microsomal cytochrome b5. The Journal of Biochemistry (Tokyo) 97, 1659-68.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. The Journal of Molecular Biology 215, 403-10.

Belli, S.I., S. Monnerat, C. Schaff, S. Masina, T. Noll, P.J. Myler, K. Stuart, and N. Fasel. 2003. Sense and antisense transcripts in the histone H1 (HIS-1) locus of *Leishmania major*. International Journal for Parasitology 33, 965-75.

Benyajati, C., A.R. Place, D.A. Powers, and W. Sofer. 1981. Alcohol dehydrogenase gene of Drosophila melanogaster: relationship of intervening sequences to functional domains in the protein. Proceedings of the National Academy of Sciences U S A 78, 2717-21.

Boothroyd, J.C., and G.A. Cross. 1982. Transcripts coding for variant surface glycoproteins of *Trypanosoma brucei* have a short, identical exon at their 5' end. Gene 20, 281-9.

Bork, P., and E.V. Koonin. 1994. A new family of carbon-nitrogen hydrolases. Protein Science 3, 1344-6.

Brittingham, A., C.J. Morrison, W.R. McMaster, B.S. McGwire, K.P. Chang, and D.M. Mosser. 1995. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. The Journal of Immunology 155, 3102-11.

Brodin, T.N., S. Heath, and D.L. Sacks. 1992. Genes selectively expressed in the infectious (metacyclic) stage of *Leishmania major* promastigotes encode a potential basic-zipper structural motif. Molecular and Biochemical Parasitology 52, 241-50.

Brun, R., and Schonenberger. 1979. Cultivation and *in vitro* cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Tropica 36, 289-92.

Burchmore, R.J., and S.M. Landfear. 1998. Differential regulation of multiple glucose transporter genes in *Leishmania mexicana*. The Journal of Biological Chemistry 273, 29118-26.

Burnett, W.V. 1997. Northern blotting of RNA denatured in glyoxal without buffer recirculation. Biotechniques 22, 668-71.

Coulson, R.M., and D.F. Smith. 1990. Isolation of genes showing increased or unique expression in the infective promastigotes of Leishmania major. Molecular and Biochemical Parasitology 40, 63-75.

Coulson, R.M., V. Connor, and J.W. Ajioka. 1997. Using 3' untranslated sequences to identify differentially expressed genes in Leishmania. Gene 196, 159-64.

Cunningham, I. 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. The Journal of Protozoology 24, 325-9.

Denhardt, D.T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochemical and Biophysical Research Communications 23, 641-6.

Descoteaux, A., and S.J. Turco. 2002. Functional aspects of the *Leishmania donovani* lipophosphoglycan during macrophage infection. Microbes and Infection 4, 975-81.

Diatchenko, L., Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, and P.D. Siebert. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and

libraries. Proceedings of the National Academy of Sciences U S A 93, 6025-30.

Dominguez, M., I. Moreno, M. Lopez-Trascasa, and A. Torano. 2002. Complement interaction with trypanosomatid promastigotes in normal human serum. The Journal of Experimental Medicine 195, 451-9.

Ghosh, M., and S. Bandyopadhyay. 2003. Present status of antileishmanial vaccines. Molecular and Cellular Biochemistry 253, 199-205.

Gish, W., and D.J. States. 1993. Identification of protein coding regions by database similarity search. Nature Genetics 3, 266-72.

Graham, S.V. 1995. Mechanisms of stage-regulated gene expression in kinetoplastida. Parasitology Today 11, 217-23.

Grimm, F., R. Brun, and L. Jenni. 1991. Promastigote infectivity in *Leishmania infantum*. Parasitology Research 77, 185-91.

Hartley, J., and H. Bowen. 1996. PEG precipitation for selective removal of small DNA fragments. Focus 18, 27.

Jornvall, H., B. Persson, M. Krook, S. Atrian, R. Gonzalez-Duarte, J. Jeffery, and D. Ghosh. 1995. Short-chain dehydrogenases/reductases (SDR). Biochemistry 34, 6003-13.

Killick-Kendrick, R. 1990. The life-cycle of Leishmania in the sandfly with special reference to the form infective to the vertebrate host. Ann Parasitol Hum Comp 65 Suppl 1, 37-42.

Knuepfer, E., Y.D. Stierhof, P.G. McKean, and D.F. Smith. 2001. Characterization of a differentially expressed protein that shows an unusual localization to intracellular membranes in Leishmania major. Biochemical Journal 356, 335-44.

Kobe, B., and J. Deisenhofer. 1994. The leucine-rich repeat: a versatile binding motif. Trends in Biochemical Sciences 19, 415-21.

Kotewicz, M.L., C.M. Sampson, J.M. D'Alessio, and G.F. Gerard. 1988. Isolation of cloned *Moloney murine leukemia* virus reverse transcriptase lacking ribonuclease H activity. Nucleic Acids Research 16, 265-77.

Krogh, A., B. Larsson, G. von Heijne, and E.L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of Molecular Biology 305, 567-80.

Liniger, M., K. Bodenmuller, E. Pays, S. Gallati, and I. Roditi. 2001. Overlapping sense and antisense transcription units in *Trypanosoma brucei*. Molecular Microbiology 40, 869-78.

Marin, M., C. Muskus, J.R. Ramirez, L.F. Arbelaez, J.F. Alzate, and C. Berberich. 2000. The gene encoding the metacyclogenesis-associated transcript Mat-1 is conserved in the genus *Leishmania* and shows a tendency to form dimers upon protein expression. Parasitology Research 86, 431-5.

McConville, M.J., S.J. Turco, M.A. Ferguson, and D.L. Sacks. 1992. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. The EMBO Journal 11, 3593-600.

Mueckler, M., C. Caruso, S.A. Baldwin, M. Panico, I. Blench, H.R. Morris, W.J. Allard, G.E. Lienhard, and H.F. Lodish. 1985. Sequence and structure of a human glucose transporter. Science 229, 941-5.

Mulder, N.J., R. Apweiler, T.K. Attwood, A. Bairoch, D. Barrell, A. Bateman, D. Binns, M. Biswas, P. Bradley, P. Bork, P. Bucher, R.R. Copley, E. Courcelle, U. Das, R. Durbin, L. Falquet, W. Fleischmann, S. Griffiths-Jones, D. Haft, N. Harte, N. Hulo, D. Kahn, A. Kanapin, M. Krestyaninova, R. Lopez, I. Letunic, D. Lonsdale, V. Silventoinen, S.E. Orchard, M. Pagni, D. Peyruc, C.P. Ponting, J.D. Selengut, F. Servant, C.J. Sigrist, R. Vaughan, and E.M. Zdobnov. 2003. The InterPro Database, 2003 brings increased coverage and new features. Nucleic Acids Research 31, 315-8.

Ozols, J. 1989. Structure of cytochrome b5 and its topology in the microsomal membrane. Biochimica et Biophysica Acta 997, 121-30.

Rossmann, M.G., A. Liljas, C.I. Brändén, and L.J. Banaszak. 1975. Evolutionary and structural relationships among dehydrogenases. In: P. D. Boyer, ed. The Enzymes, Vol. 11, Academic Press, New York, pp. 61–102

Saxena, A., E.A. Worthey, S. Yan, A. Leland, K.D. Stuart, and P.J. Myler. 2003. Evaluation of differential gene expression in *Leishmania major* Friedlin procyclics and metacyclics using DNA microarray analysis. Molecular and Biochemical Parasitology 129, 103-14.

Spath, G.F., L.A. Garraway, S.J. Turco, and S.M. Beverley. 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of Leishmania major infections in mammalian hosts. Proceedings of the National Academy of Sciences U S A 100, 9536-41.

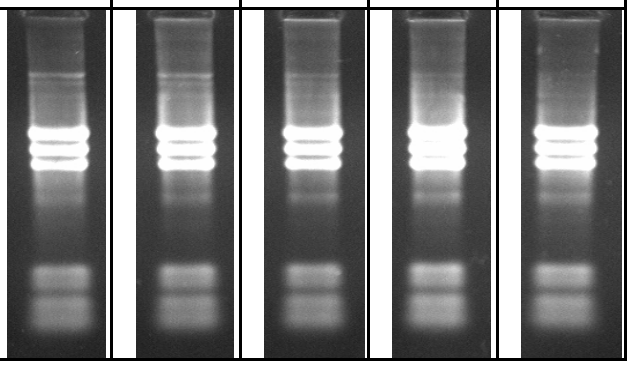
Tartoff, K.D., and C.A. Hobbs. 1987. Improved media for growing plasmid and cosmid clones. Focus 9, 12.

Weber, E., M.R. Chevallier, and R. Jund. 1988. Evolutionary relationship and secondary structure predictions in four transport proteins of Saccharomyces cerevisiae. Journal of Molecular Evolution 27, 341-50.

Yao, C., J.E. Donelson, and M.E. Wilson. 2003. The major surface protease (MSP or GP63) of *Leishmania sp*. Biosynthesis, regulation of expression, and function. Molecular and Biochemical Parasitology 132, 1-16.

Zhang, W.W., and G. Matlashewski. 1997. Loss of virulence in *Leishmania donovani* deficient in an amastigote-specific protein, A2. Proceedings of the National Academy of Sciences U S A 94, 8807-11.

Table 1: parasite densities and parasite volumes at different time points

Days	**0**	**1**	**1.7**	**2**	**3**	**5**	**7**
Density [counts/ml]	5x 10^5	3.8x 10^6	2.8x 10^7	5.2x 10^7	9.1x 10^7	9.9x 10^7	9.9x 10^7
Mean volume [fl]	24.3	31.5	27.1	21.9	18.9	15.3	13.6
Volume (max)[a] [fl]	17.1	26.3	16.3	13.4	12.1	8.1	7.7
Total RNA	Not isolated	Not isolated					

[a)] The most frequent cell volume.

Table 2: primers (all obtained from Qiagen). Restriction sites are ***highlighted***.

Name	**Sequence**	**Restriction site**
RT-primer	5'-ACGACTGTACGT***GAATTC***GC$(T)_{20}$	EcoRI
SL-primer	5'-GCTATATA***GAATTC***AGTTTCTGTACTTTAT	EcoRI
pSL-primer	5'-AACTAACGCTATATAAGTATC	
nSL-primer	5'-GTATCAGTTTCTGTACTTTATTGAGT	
His-fw	5'- TCGACATCACCATCACCATCACTAGC	
His-rev	5'-GGCCGCTAGTGATGGTGATGGTGATG	
1-fw	5'-AAGATC***GGATCC***CTGACATCCCTTAACATG	BamHI
1-rev	5'-ATGGCAC***GTCGAC***GCTTCGCCTCCATC	SalI
6-fw	5'-ATG***GGATCC***ACGCTGTTTGGCTACAG	BamHI
6-rev	5'-CGGCGA***GTCGAC***CGCTTTCCGACG	SalI

Table 3: differential expressed clones.

Clone	Accession number	Expression pattern[a]	Sequence used for Blastx and motif scan	Most homologous protein (Blastx, (Gish and States, 1993))	Motifs (Krogh et al., 2001; Mulder et al., 2003)
1		+	1.7kb cDNA including complete ORF	hypothetical protein C56G2.6 *Caenorhabditis elegans* E=3e-31	2 TM domains, NAD(P) binding Rossmann-fold domains E=3e-40 (Rossmann et al., 1975), short chain dehydrogenase (Benyajati et al., 1981; Jornvall et al., 1995) E=4.9e-29, glucose/ribitol dehydrogenase E=2.5e-15
2		---	510bp insert of SSH library	putative amidohydrolase *Neurospora crassa* E=2e-17	Nitrilase/N-carbamoyl-D-AA amidohydrolase E=2.4e-20 (Bork and Koonin, 1994), P-loop containing nucleotide triphosphate hydrolase E=4.6e-12, carbon-nitrogen hydrolase E=1.8e-5
3		++	1.8kb 5'end of cDNA	AA permease AAP15LD *L. donovani* E=4e-67	8 TM domains, TM AA transporter protein E=1.2e-6, aromatic AA permease II (Weber et al., 1988)
4		++	777bp 5' and 754bp 3'end of cDNA, assembled with *L. major* Friedlin chromosome 31 (AL499621.2) P=1.8e-133 (5') and P=9.5e-105 (3')	GU1 *Trypanosoma brucei* (Liniger et al., 2001) E=2e-31	leucine rich repeats (Kobe and Deisenhofer, 1994)
5		+++	534bp cDNA including complete ORF	Mat-1 protein *L. infantum* (Marin et al., 2000) (Knuepfer et al., 2001) E=2e-41	none
6		+	845bp 5' and 950bp 3' end of cDNA, assembled with *L. major* Friedlin chromosome 36 (AL499624.2) P=4.1e-142 (5') and P=5.9e-181 (3')	LmGT2 or 3 *L. mexicana* (Burchmore and Landfear, 1998) for both E=0.0	11-12 TM domains, general substrate transporter E=1.5e-105, sugar transporter E=3.1e-88 (Mueckler et al., 1985)
7		++	975bp 5'end of cDNA including complete ORF	CG2140-PB (CYTB5) *Drosophila melanogaster* E=8e-8	CYTB5 family E=1.2e-15 (Abe et al., 1985; Ozols, 1989), heme/steroid binding domain E=2e-6.
8		++	1.9kb cDNA including complete ORF	none	none

[a] +: up regulated in stat phase cultures, -: down regulated.

Figures

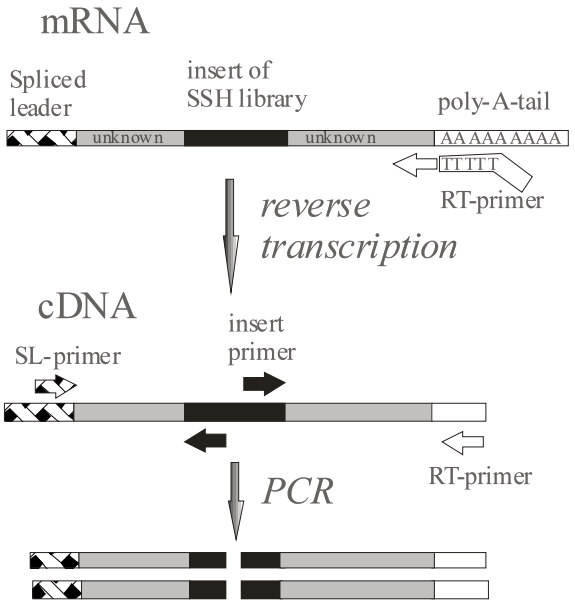


Figure 1: Most mRNAs in *Leishmania* contain a conserved spliced leader (SL) at the 5' end. Therefore, PCR was used for obtaining the full length cDNA sequence.

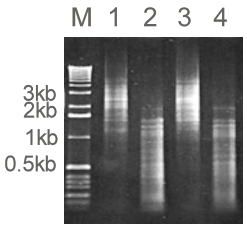


Figure 2: The starting material used for the strain subtraction. M: DNA marker; 1-4: SL-cDNA; 1,2: strain LEM 278; 3,4: strain LEM 768; 1,3: uncut; 2,4 Rsa I cut.

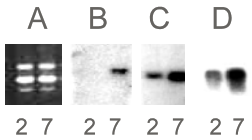


Figure 3: Northern blot analysis of 3 clones of the strain subtraction library. A: RNA confirming equal loading; B: zinc finger (DHHC type) family protein (BLAST: E=3e-7); C: not identified sequence; D: translation elongation factor α; 2: RNA of strain LEM 278, 7: RNA of strain LEM 768.

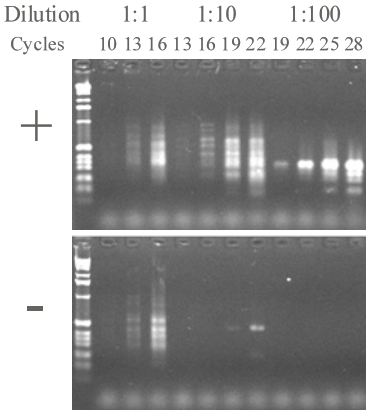


Figure 4: Effect of dilution and number of nested cycles to the outcome of the suppression subtractive hybridization. In the lower gel (-), the hybridization mix was denatured prior to the PCR in contrast to the upper (+) gel.

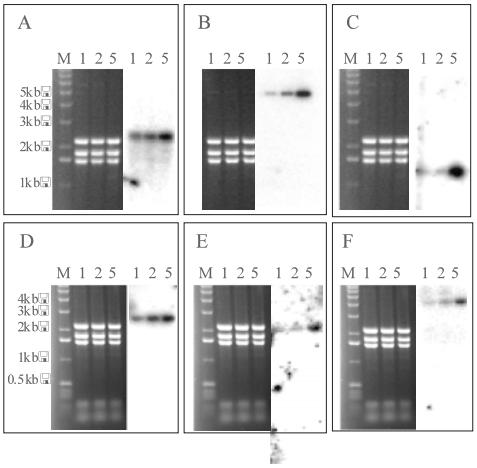


Figure 5: Northern blot analysis of 6 different clones. Left image: RNA gel confirming equal loading (with the 3 ribosomal bands typical for trypanosomatides); right image: northern blot. A: clone 1 (dehydrogenase); B: clone 3 (amino acid permease); C: clone 5 (MAT-1); D: clone 6 (GT 2?); E: clone 7 (cyt b5?); F: clone 4; M: 1kb DNA ladder (Invitrogen, DNA and RNA run +/- equal when treated with DMSO/glyoxal); 1: RNA of day 1.7; 2: RNA of day 2; 5: RNA of day 5 cultures.

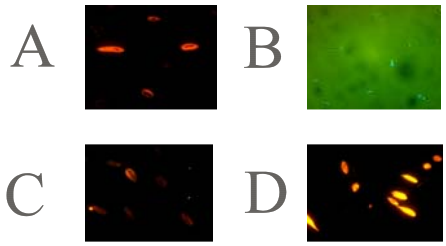


Figure 6: IFAs using sera against a putative glucose transporter (clone 6). A: serum mediated membrane staining, B: DAPI and bright field of the same region, C: competition with 0.1µg/µl recombinant protein, D same exposure time as C without recombinant protein.

4.4. Additional Methods and Results

The low quality cDNA obtained after 1st and 2nd strand synthesis when the enzymes provided with the subtraction kit were used is shown in figure 7, where it is compared to ds cDNA obtained after reverse transcription using M-MLV RT, RNase H and 2nd strand synthesis following a standard protocol (using RNase H, *E. coli* DNA polymerase I and *E. coli* DNA ligase at the same time, described in Sambrook and Russel, 2001)

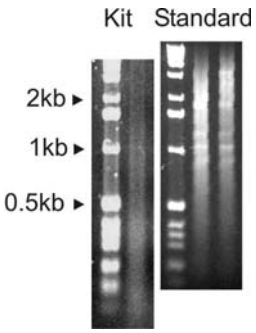


Figure 7: comparison of different ds cDNA preparations

Figure 8 shows glyoxal Northern blots using clone 2 and 8, which are not shown in the publication above. Furthermore, for some clones virtual Northern blots were performed in order to save RNA. For that, RNA was replaced by amplified SL cDNA (see appendix 2) and blotted after Southern blot standard procedures. Figure 9 shows a comparison of virtual Northern, formaldehyde Northern, and glyoxal Northern blots of clone 3. It is not surprising, that the cDNA size indicated by the band on the virtual Northern is much smaller, since cDNA walking revealed the ability of this particular cDNA to recombine during PCR amplification (figure 10). The walk had to be performed in a total of 3 steps and after sequencing it was clear, that most of the bands indeed derived from the expected DNA but they obtained central deletions of different sizes (amplification of the cDNA belonging to clone 4 resulted in similar observations).

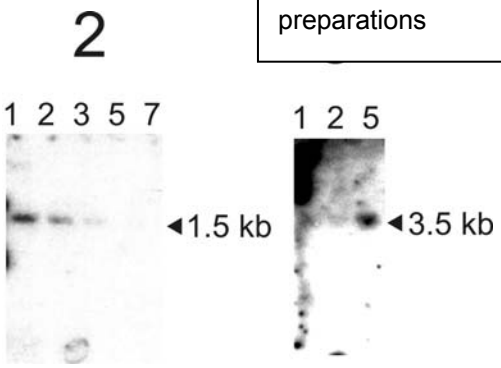


Figure 8: Northern blot analysis of the down regulation of clone 2 and up regulation of clone 8. The number above the lanes represent the age of the in vitro culture.

Figure 9: formaldehyde, glyoxal and virtual Northern of clone 3.

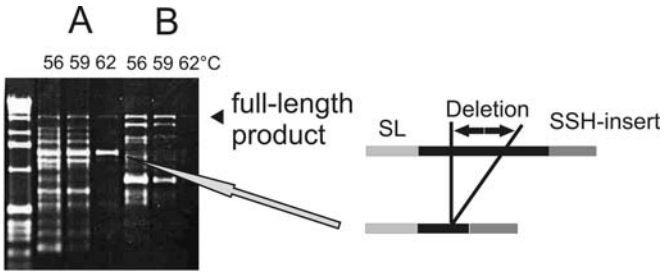


Figure 10: PCR products obtained by cDNA walking of clone 3 using different primers (A, B) and annealing temperatures. To the right: a deletion in the most prominent band when using primer A. It resulted from recombination during PCR.

Nevertheless, the expression pattern shown in the virtual Northern blot once more proved the quality of the amplified SL cDNA for many applications.

Three proteins were recombinantly expressed in *E. coli*: clone 1 encoding a putative short chain dehydrogenase, clone 5 representing MAT-1 and clone 6 encoding a putative glucose transporter. Figure 11 shows total cell lysates of the expression clones and purified proteins.

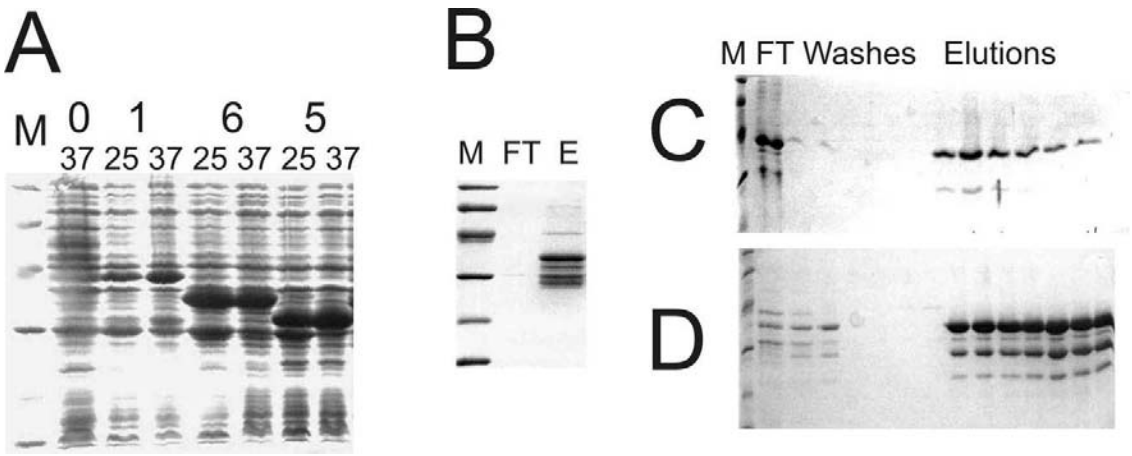


Figure 11: Expression of 3 *Leishmania* proteins in *E. coli*. A: E. coli lysate; M: marker, 37, 25: expression temperature; 0: no induction; 1, 6, 5: clone 1, 6, 5. B: purified protein of clone 5; FT: flow through, E: elution. C,D: purified protein of clone 1 and 6.

MAT-1 (clone 5) encodes the SHER protein, which was described by Knuepfer et al. (2001). In order to access the localization of this protein, the protein was expressed in *E. coli* in order to perform IFAs.

cDNA encoding the N-terminal part was amplified using the primers 5'-TTGGGATCCATGATTATCCGCTATATGTC and 5'-GAGAGAGTCGACGAAAAGGGAGAGAAGAG, cut with Bam HI and Sal I and ligated into the pGEX-His vector (appendix 6.A10). As described by Marin et al. (2000), the protein showed to be toxic for *E. coli* leading to frame shifts and

preliminary stop codons, which resulted in products of restricted length. Consequently, the C-terminal His-tag was deleted in the recombinant protein. While expressed at high levels, the protein showed to be highly insoluble, and it was finally purified at mild denaturing conditions using sarkosyl and glutathione sepharose after the method of Frangioni and Neel (1993), appendix 6.A12) with low yield. Previously, several different renaturation protocols were tested without success for proteins in inclusion bodies which were dissolved in urea or guanidine chloride. Polyclonal sera against the protein recognized several bands in Western blots using total protein of *Leishmania* promastigotes. However, there was a cytoplasmatic structure stained in IFAs (figure 12). Using light microscopes of higher resolution and TEM, Knuepfer et al. (2001) showed that this protein is associated with the ER.

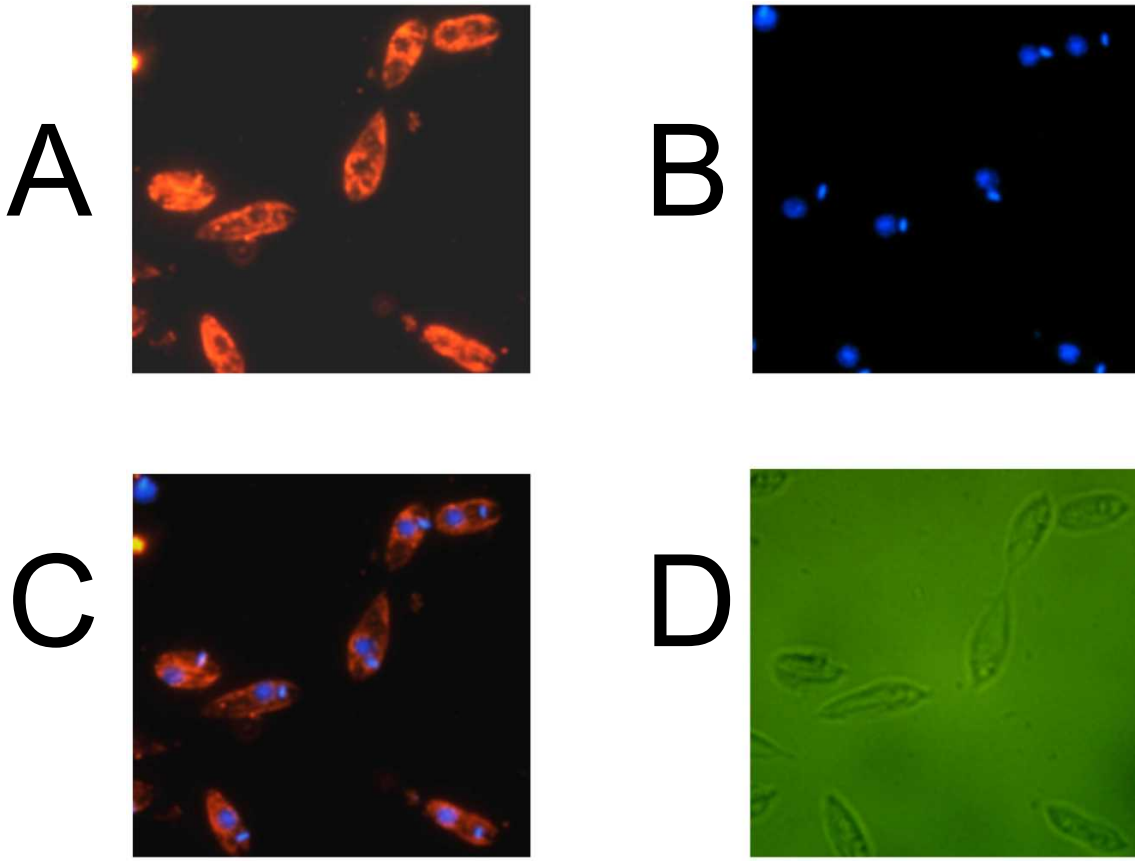


Figure 12: A: IFAs using a polyclonal serum against recombinant SHERP. B, D: DAPI stain and bright field of the same region. C: merge of A and B.

4.5. Discussion

4.5.1. Choice of the approach

We tried to identify potential virulence factors using cDNA subtraction. In our first experiment, we subtracted cDNA of an avirulent from a virulent strain. The selected genes would therefore have a direct link to virulence. Unfortunately, the difference of two strains was not only drawn by virulence, but also by other aspects. Therefore, equation of the genes selected by the subtraction with potential virulence factors was not possible.
Thus, we then subtracted stages of the same strain differing in virulence. The latter approach had the disadvantage that there usually are many stage specific expressed genes, but only a few of them are involved in virulence. Therefore, a direct connection between selected genes and virulence lacked.
We selected 3 differentially expressed genes which most probably are involved in nutrient transport. Without further experiments, it was not distinguishable whether these genes are stage specific regulated or induced/repressed by changes in nutrient concentration. *In vitro* cultivation was done without changing the medium; therefore, the nutrient concentration differed substantial with the age of the culture. However, many researchers believe that genes are regulated rather by fixed programs in, what they call lower eukaryotes as by changes in the environment.
Nevertheless, due to the fact that MAT-1, a gene known to be metacyclic specific, was selected by the subtraction proved the selectivity of the technique, considers our approach as successful. However, we cannot claim to have identified only virulence associated genes

4.5.2. Choice of the technique

Several methods exist for detection of differentially expressed genes: many subtraction techniques, differential display or DNA microarray analysis. The latter approach is only possible, when the genome of the organism of interest is (almost) sequenced, and usually yields huge amount of data. Since at the beginning of this project the sequencing of *L. majors*' genome was in its infancy the application of this method was not feasible. However, it is

interesting to note that, beside MAT-1, none of the genes we selected using SSH was found by micro analysis by Saxena et al. (2003). Whether this is due to chance, or whether it shows that, despite a large amount of generated data, microarray analyses miss a lot of differentially regulated genes is unclear. Furthermore, microarrays will fail to detect RNA editing and alternative splicing, which has been described for more and more genes (however as known so far, introns are very rare in trypanosomatids).
Compared to conventional subtraction methods, SSH is an advanced technique, because it incorporates normalization during the first round of subtraction. The method therefore yields fragments of both, abundant and low abundant, differentially expressed RNA. Thus, SSH was the method of choice in our experiments.
However, a RNA species becoming more and more important will be missed by all techniques mentioned above: micro RNAs (miRNAs). These usually about 22nt long miRNAs are missed during reverse transcription (e.g. using oligo-dT derived primers) and further, in microarray analysis, because of their restricted length yielding in low melting points (the spotted fragments in micro array usually are 50nt to several 100nt long). Nevertheless, miRNA are highly abundant in both, prokaryotes and eukaryotes, and are involved in as important features as gene repression, regulation of RNA stability and both, translational repression and activation (reviewed by Bartel, 2004).

The objective was to identify potential virulence factors, drug targets and vaccine candidates. Whether the genes we have selected by cDNA subtraction fulfil this objective cannot yet be determined. However, some of the selected genes show interesting properties: the gene encoding domains involved in protein-protein interactions (a homologue protein with unknown function has been described in the pathogen *T. brucei brucei*) and the gene encoding a protein lacking any domains and homologies to known or hypothetical proteins as examples.
In our subtraction, the slowest step surely was the identification of the selected genes. Therefore, many more genes might have been selected and identified if the subtraction was repeated using a reverse transcriptase lacking RNase H activity or when we would have managed to get to work the modifications of SSH described chapter 4.2.
In order to perform localization experiments three of the selected genes were expressed recombinantly in *E. coli*. Immunization with clone 1 did not result in useful sera and the localization of MAT-1 encoding SHERP was previously described. In IFAs, antibodies against clone 6 stained the parasite's surface;

this confirmed the sequence analysis, which showed that this clone encoded a glucose transporter.

Alternatively, instead of immunization experiments, gene overexpression or knock outs could have been performed in transformation experiments. Since there was no experience in our group on *Leishmania* transformation, this would have been a high risk project. Whether such an approach would be able to define the effect of this particular gene on virulence remains to be shown. All together it seems that the chosen methods were appropriate within reasonable costs, but modification might yield more results.

5. General Discussion

Although the three projects can be merged only loosely under the term leishmaniasis, there is a molecular link between them: the mini-exon gene encoding the spliced leader. The gene itself was used as template for the diagnostic PCR, spliced leader cDNA was used to construct expression and subtraction libraries and it enabled cDNA walking for identification of genes selected in subtractions.
Histones represent another tie between the subtraction experiment and the serum screen: core histones showed to be of diagnostic value, and histone H1 was higher expressed in metacyclics of the more virulent *Leishmania* strain. The interesting properties of *Leishmania* histones (unusual high diversity and differential expression in strains differing in infectivity) might indicate the presence of a goldmine for understanding these parasites.
The three projects were performed in order to develop tools for diagnosis of human and canine leishmaniasis, and for identification of vaccine candidates and potential virulence factors.

5.1. Diagnosis

A PCR assay allowing diagnosis and species differentiation of *Leishmania* species causing human leishmaniasis was not yet available. Therefore, we developed a mini-exon PCR which exactly fits into this missing field. While the PCR and RFLP assay now is routine use at the STI showing satisfying results, distinguishing between *Viannia* species still remains a challenge. Since the required treatment of species belonging to this subgenus differs substantially, this is a major draw back. However, the assay might be able to be improved: restriction digests show only different patterns, if mutations are present within the recognition sequences of the selected enzymes. This problem could be overcome with chip technology, which then could facilitate the differentiation of *Viannia* species, or by using an additional genetic marker.

While many different diagnostic tools are available for canine leishmaniasis, a diagnostic test capable of detection of early stages of infection is still lacking. We selected a few promising antigens suitable for an early diagnostic test for canine leishmaniasis. However, *Leishmania* histones have been previously

described as antigens, inducing strong humoral responses in both, infected dogs and humans. Whether the epitopes we selected incorporated better properties has to be tested using sera of many more naturally and artificially infected dogs. Since we used cDNA derived from a highly infective and virulent strain for the serum screen, the epitopes we identified might be good candidates. Collaboration with a laboratory in Spain will provide this information.

However, the goal of an early diagnostic test for canine leishmaniasis was to allow mass diagnosis and treatment street dogs as a control tool for leishmaniasis in Southern Europe. Whether this dodgy approach will succeed, has to be shown in future. It surely will be intensive in costs and work and will not protect treated dogs from relapses and re-infection. Furthermore, the consequences in respect to drug resistance might be fatal. Therefore, it seems that it would be more feasible to concentrate all means on the development of a vaccine for both, humans and dogs, and for development of new drugs. Nevertheless, an early diagnostic test for canine leishmaniasis would be an improvement of veterinarian praxis.

5.2. Vaccine candidates

By enriching metacyclic stage specific genes, we also enriched for potential transmission blocking vaccine candidates. Several integral membrane proteins were selected, two transporters and a dehydrogenase. Whether these proteins are suitable as vaccine candidates has to be shown in future studies. Here fore, they must be able to induce a long lasting protective T_H1 type immune answer, and must be stable and produced cheaply to allow mass treatment. Although the probability whether these conditions are fulfilled by one of the three proteins we selected is rather low, the immense value of a vaccine as a powerful tool for control of leishmaniasis justifies a slight hope. Nevertheless, the enrichment of potential vaccine candidates was only a byproduct of a subtraction designed for the selection of potential virulence factors.

An alternative to designing a vaccine based on single or several parasites' molecules is to develop live or killed vaccines. However, costs for *Leishmania* cultivation currently are much too high. By lowering these costs, (e.g. achieving serum-free cultivation in a cheap medium), the use of attenuated parasites would be a true alternative. Furthermore, cultivated parasites are

not only worthwhile as vaccine but are also of excellent diagnostic value.

5.3. Virulence factors

Classically, virulence factors have been defined as factors causing or enhancing the severity of a disease. However, the distinction of virulence factors (causes for pathology) from factors involved in pathogenicity appears to be artificial. Thus, all parasite factors enabling/enhancing the parasite's infectivity, survival within the host and factors defining the severity of the disease could be handled as virulence factors. Understanding the mode of action of these factors is crucial for understanding leishmaniasis.
We performed two experiments in order to enrich potential virulence factors. However, whether the selected genes are involved in virulence has to be shown using *in vivo* studies. Due to the complexity of the matter, assigning the role of virulence factors as well as their mode of action is a challenge, as the example of LPG shows. Although LPG was suspected to be a virulence factor many years ago, its impact was unclear or misunderstood. Since initial findings showed that LPG enhances the metacyclic's resistance to the complement and is involved in the transient inhibition of phagolysosomal fusion, these properties were believed to be the mode of action. However, using lpg$^-$ mutants, Spath et al. (2003) showed that at least in mice, complement mediated lysis is an ineffective way of controlling the disease. Lpg$^-$ metacyclics were opsonised by complement factors and entered macrophages. They also showed that inhibition of phagolysosomal fusion is not essential for the parasite's survival within phagocytes. Nevertheless, lpg$^-$ mutants showed a significant higher sensitivity to oxidative stress, decreasing the parasite's ability to resist the oxidative burst generated by activated macrophages. The different observations and conclusions on LPG's impact were most probably derived from using different approaches: the initial experiments were performed *in vitro* and using biochemical methods (LPG shares domains with many other *Leishmania* derived molecules). The example of LPG clearly shows, that defining a gene as virulence factor is intricate: although recent studies confirm that LPG is involved in virulence, this could in principle have been disproved just as well.
Furthermore, the results of *in vivo* studies using mice cannot be directly assigned to humans, due to significant differences in the immune system.

Therefore, much care has to be taken when defining virulence factors, since the outcome of experiments depends on the approach, technique and system used.
These facts clearly show that identification of virulence factors would be far beyond the reach of this PhD project. Nevertheless, our experiments were designed to select for *potential* virulence factors. The selected genes were expressed at higher levels in the more infective strain or in the infective parasite stage respectively, which is a first indication, that they *could* be involved in virulence.

5.4. Drug targets

Gene products expressed in metacyclic and amastigote parasites are potential drug targets for prophylaxis and/or treatment, if they are essential for the parasite and differ in the tertiary structure from homologues expressed in the host. Therefore, we enriched also potential drug targets by the cDNA subtraction. However, since expression in amastigotes was not yet shown, the use of these genes might be of limited value as drug targets. Nevertheless, some of the genes, such as the GU1 like gene, have no homologies at all to mammalian proteins known so far (at least in the primary structure).
There are some newer antileishmanial drugs in use and in the pipeline, but their number is restricted, and most of them are no oral agents. In order to avoid progression of drug resistance (which already has spread widely to antimonials), alternative drugs are highly welcome. By using several different drugs at different locations and time points, progression of resistance might be successfully suppressed and even reversed. However, in addition to evolution-driven selection of resistant phenotypes, *Leishmania* parasites can react unspecifically to drug pressure by gene amplification yielding in extrachromosomal plasmids and mini chromosomes. Nevertheless, some chemical and physical properties of a drug can aggravate the development of resistances:

1. A rapid mechanism of action, as observed by the antimalarial artemether, prevents unspecific adaptations of the parasite such as gene amplification. However, drug resistance can still progress by selection of resistant phenotypes formed by chance in the whole parasite population.
2. It is more difficult for the parasite to develop resistance to drugs which

don’t need parasite’s transporter molecules to reach the point of action. These are either drugs damaging the parasite surface or hydrophobic molecules which are able to diffuse through biological membranes. However, against the latter drugs, an effective countermeasure is active transport out of the parasites cytosol.

Development of antileishmanials surely is a challenge: *Leishmania* is endemic in poor regions of the world, therefore production should be of low costs and oral application should be possible (in order to lower treatment costs). Furthermore, the drug should exhibit low rates of adverse reactions. The fact that toxic antimonials still are widely used clearly shows the deficits of today’s antileishmanials. The property of *Leishmania* parasites to multiply within acidic compartments might be utilized by the drug development: hydrophobic molecules, which are uncharged at neutral pH but protonated within phagolysosomes are able to diffuse through membranes when they are uncharged. Once protonated, membrane diffusion is disabled. This leads to accumulation of high drug concentrations within phagolysosomes, which might enhances the efficiency of the drug concurrent with reduction of adverse reactions.

However, it is more likely that new drugs are identified by screening of thousands of compounds than by specific design upon potential drug targets. The selected drugs then could be chemically modified using the criteria mentioned above. Nevertheless, some parasite specific proteins without mammal homologues might ask for direct design of drugs. Whether the GU1 homologue we identified or other genes selected by cDNA subtraction qualify for this purpose cannot yet be answered.

However, the cDNA subtraction experiments proved their ability to select genes exhibiting interesting properties not only in respect to potential virulence factors, but also to potential transmission blocking vaccine candidates and to potential drug targets. However, it has to be straightened out that these preliminary results are far from practical application, they only exhibit indications that further research on some genes we identified might yield in powerful tools for control of *Leishmania*.

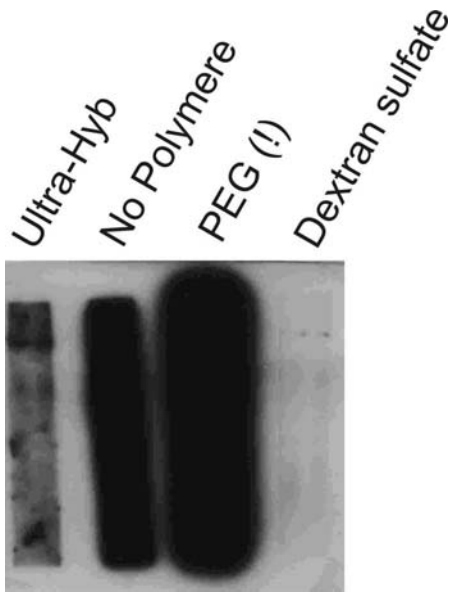
6. Appendices

6.A1. Hybridization buffers for Northern blot analysis

	Buffer A	Buffer B
SSPE pH 7.2	5x	-
MOPS pH 7.0	-	50mM
NaCl	-	1M
Form amide	50% [w/v]	50% [w/v]
SDS	0.5% [w/v]	0.5% [w/v]
Dextran sulfate	8% [w/v]	15% [w/v]
Denhardt's reagent	5x	5x

Hybridization temperature: 42°C for both buffers

Buffer A was more stable but less sensitive than buffer B. This was most probably due to the fact, that it contained less dextran sulfate yielding in lower viscosity (the higher the viscosity, the higher the sensitivity especially when using double stranded probes). Addition of more dextran sulfate resulted in an insoluble precipitate most probably mediated by the phosphate buffer (many phosphates are insoluble). Therefore, the phosphate buffer was replaced by MOPS in buffer B.



Ingredients

- Dextran sulfate was clearly the better choice for raising the viscosity compared to PEG (figure).
- The Na^+ concentration of approximately 1M of both buffers is optimal for hybridizations
- Form amide reduces the melting point of nucleic acids and discriminates DNA-DNA base pairs compared to RNA-DNA base pairs.
- SDS lowers the surface tension.
- Denhardt's reagent is used to block the membrane.

6.A2. Construction of spliced leader cDNA

Primers: ***Eco* RI**

1str Eco primer 5'-CGACTGTACGT**GAATTC**GC$(T)_{20}$

SL Eco primer 5'- GCTATATAA**GAATTC**AGTTTCTGTACTTTATTG

Reverse Transcription

1 To 1.5µg total or mRNA add 14µl 10µM 1str Eco primer and TE to 23.5µl final volume.

2 Incubate 5min @ 70°C, snap cool, quick spin.

3 Add 3µl 10x 1st strand buffer,
1.5µl 10mM dNTPs,
0.5µl RNasin (Invitrogen)
1.5µl M-MLV RT, RNase H^- (Finnzymes)

4 Incubate 1hour @ 37°C.

When total RNA was used:

5 Heat inactivate RT for 2min @ 95°C.

6 Quickspin, add 1µl RNase A (1g/l) and incubate 15min @ 37°C. Store @ -20°C.

When mRNA was used:

5 Add 22.7µl H_2O
6µl 10x RNase H buffer
1µl RNase A (1g/l)
0.3µl RNase H (5U/µl)

6 Incubate 20min @ 37°C. Store @ -20°C and use a 1/10 dilution in TE as PCR template.

PCR

1 Prepare a mastermix containing 1x Taq buffer (1.5mM MgCl2), 10% DMSO, 0.2mM dNTPs, 0.5µM SL Eco and 1str Eco primer, and use Taq:Pfu DNA polymerase in a 8:1 proportion.

2 Add 1-2µl 1st strand cDNA for a 50µl reaction.

3 Perform 20-35 cycles at the following conditions
30sec 94°C
30sec 54°C
2min 72°C

6.A3. Isolation of genomic *Leishmania* DNA

Cells are lysed in SDS, proteins are digested by proteinase K and extracted with phenol

From in vitro cultures

1 Centrifuge culture 5min @ 1'500g.
2 Wash the cells in PBS.

From biopsy

1 Store specimen in 0.9% NaCl @ -20°C.
2 Thaw material quickly.

3 Resuspend in proteinase K buffer (10mM TrisCl pH8, 5mM EDTA, 0.5% SDS) culture: 10^8 cells/ml, biopsy: depends on the size of specimen, ~500µl.
4 Add proteinase K to a final concentration of 0.5g/l.
5 Incubate 3 hours to overnight @ 56°C.
6 Extract the aqueous phase once with 1 volume phenol (pH 8), once with 1 volume phenol/chlorophorme, and once with 1 volume chlorophorme.
7 Ethanol precipitate the DNA using 1/10 volume of 3M NaAcetate (pH 5.2) and 2-3 volumes of EtOH abs.
8 Incubate 30min @ RT.
9 Spin 30min @ full speed.
10 Wash the pellet in 75% EtOH.
11 Resuspend the DNA in 50-100µl TE (10mM TrisCl, pH 8, 0.5mM EDTA).
12 Optional: add RNase A to a final concentration of 10mg/l.
13 Store @ -20°C.

6.A4. Selective DNA precipitation using PEG

Modified from Hartley and Bowen (1996)

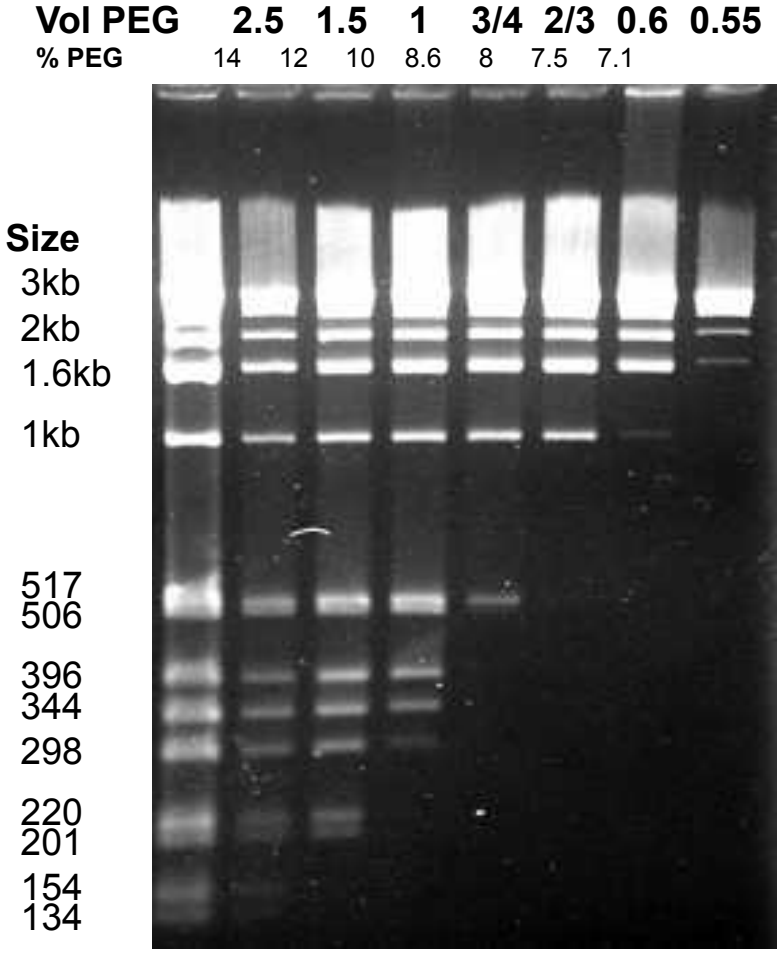


Figure: 0.5µg 1kb DNA ladder (2.5µg/ml in TE) was precipitated by addition of 0.55 to 2.5 volumes PEG 8000. The samples were incubated 10minn @ RT and spun 30min @ 16'000g and RT. The pellets were washed in 75% EtOH and resuspended in TE.

Protocol

1. Add 0.5 to 2.5 volumes[1, 2)] of PEG stock (20% PEG 8000, 1M NaCl) to 1 volume DNA solution.
2. Incubate 10min @ RT
3. Spin 30min @ 15'000g[3)] and RT.
4. Wash pellet in 75% EtOH.
5. Resuspend in whatever you need it.

1) For purification of PCR products: 1vol PEG (>400bp), 1.5vol PEG (200-400bp).
2) PEG precipitation is DNA concentration dependent: try different amounts if you need it exactly.
3) No significant loss was observed by spinning a 96 well plate 60min @ 4'000g.

6.A5. Sequencing of GC-rich DNA

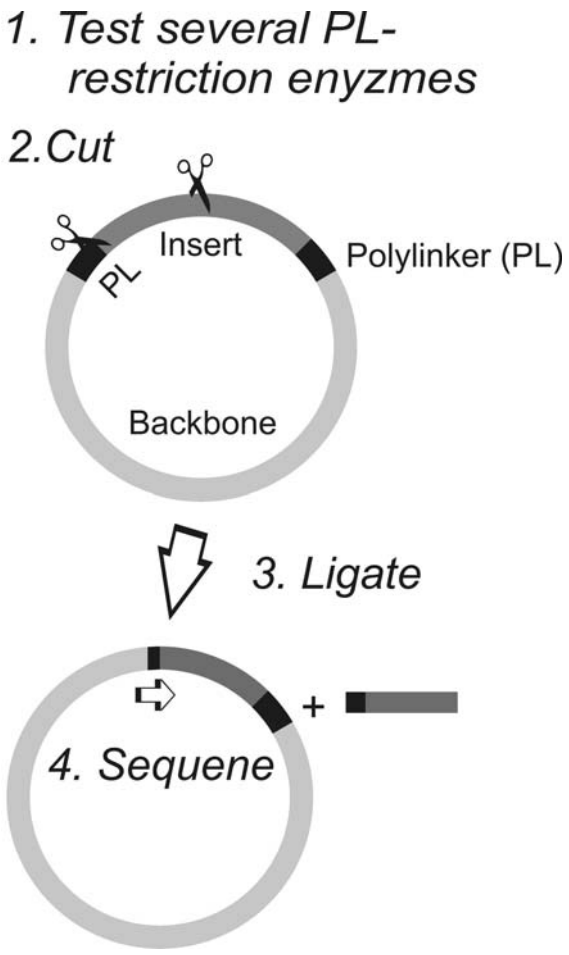
1. Denature the template dissolved in H_2O for 5min at 95°C
2. Add the sequencing primer and TRR (Perkin Elmer).
3. Perform 30 cycles at the following conditions:
   5sec 98°C
   90sec 60°C
   90sec 50°C
4. Follow the standard protocol

6.A6. Sequencing of large inserts

Find one or several polylinker restriction enzyme cutting within the insert sequence.

After cutting and religation, the plasmid obtains a deletion. Sequencing using vector primers results in a sequence starting centrally within the primary insert.

Sequence assembly reveals the complete insert sequence.



6.A7. Cycle restriction ligation (CRL)

After Pusch C, Schmitt H, Blin N. Increased cloning efficiency by cycle restriction-ligation (CRL) Technical Tips Online, 1: T40071

Here, ligation is performed in the presence of a polylinker restriction enzyme: whenever the vector is recirculated without insert, the restriction enzyme will cut it again. When an insert is ligated into the vector, the restriction site is destroyed. This protocol uses *Sma* I (recognition sequence CCC▼GGG, blunt end cutter). Addition of K^+ in this protocol is required for activity of *Sma* I. If you use a different restriction enzyme (e.g presence of a *Sma* I site within your insert, or your insert starts with sequence 5'-CCC, etc), make sure that it is active in the ligation buffer.

Prepare on ice:

- 1µl 10x T4 ligation buffer containing ATP
- 1ul 50% PEG8000
- 0.5µl 0.1M KCl
- 0.5µl 0.1M DTT
- x µl vector DNA (uncut)
- y µl insert DNA
- 1µl 10u/ml *Sma* I
- 1µl T4 DNA ligase
- dH_2O to 10µl

Place in a thermocycler and run the following program:

20min	25°C		}
			}
1min	10°C	}	}
30sec	22°C	} 6x	} 20x
			}
30sec	4°C		}
30sec	30°C		}
1h	25°C		
15min	65°C		

6.A8. Small scale preparation of electro-competent cells

1. Thaw a glycerol stocks of any overnight culture, which was not overgrown too heavily.
2. Spin the culture and discard the supernatant.
3. Wash the pellet 2 times in 1ml ice-cold dH_2O.
4. Resuspend the pellet(s) in 50µl ice-cold dH_2O.

The cells are now ready for electroporation. Do NOT freeze them, only for immediate use!

6.A9. *E. coli* transformation

Based on Inoue et al. (1990)

Media and buffers

SOB medium:

20g/l bacto tryptone
5g/l yeast extract
0.5g/l NaCl
0.19g/l KCl

Autoclave.

Just before use add $MgCl_2$ to 10mM end concentration (SOB agar plates: 20mM) from an autoclaved 2M stock.

SOC medium:

Add 2% filter sterilized 1M glucose to SOB (20mM end concentration).

ITB (Inoue transformation buffer)

1. Dissolve
   10mM HEPES or PIPES,
   15mM $CaCl_2$,
   250mM KCl
   in dH_2O.
2. Adjust pH to 6.7 with KOH.
3. Add $MnCl_2$ to 55mM.
4. Sterilize with a 0.45µm filter and store @ 4°C.

Preparation of competent cells

1. Inoculate 200ml SOB with about 10 large colonies.
    - For best results pick colonies from a freshly grown plate. Use different amounts for inoculation to have one culture at the desired density the next day.
2. Incubate for about 20 hours at RT (best: 18-22°C) with vigorous shaking (200-250rpm) until OD_{600}=0.45
    - OD=0.3-0.55 works well.
3. Place the culture on ice for 10min.
4. Spin 10min @ 2'500g and 4°C.
5. Resuspend the pellet in 64ml ice cold ITB, incubate 10min on ice and spin again.
6. Resuspend the cells in 16ml ice cold ITB.
7. Slowly add 1.2ml DMSO while gently swirling and incubate 10min on ice.
8. Dispense the suspension into 0.2–1ml aliquots, immediately freeze by immersion into liquid N_2. Store @ -80°C.
9. Thaw an aliquot for testing your cells (pos AND neg!).
    - Expect 10^8–10^9 transformants/µg supercoiled plasmid in DH5α.

Transformation

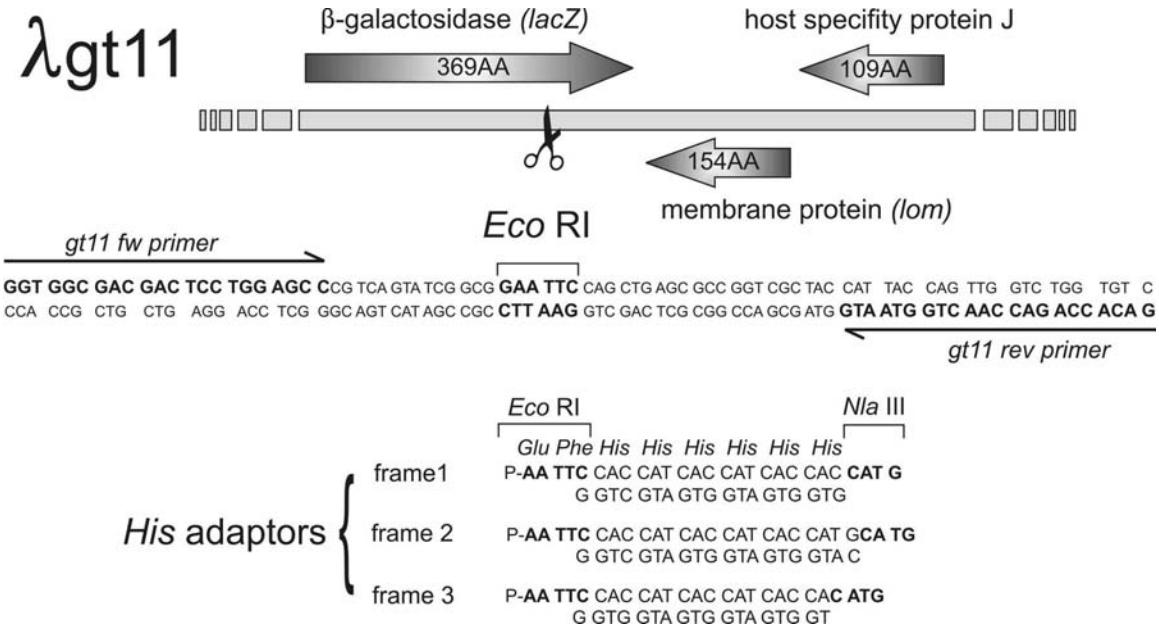
1. Thaw cells @ RT and immediately transfer on ice.
2. Add 50-100µl cells to your DNA (the volume of the DNA should not exceed 5%).
   It is not necessary to precipitate a ligation mix prior to transformation.
3. Incubate on ice for 15min.
4. Heat shock 1min @ 42°C in a water bath, cool in ice water.
    - Alternatively, you can plate the transformation mix directly on prewarmed plates.
5. Add 900ml SOC, incubate 30min @ 37°C and plate the bacteria.

6.A10. Modified expression vectors

λgt11-His

Modification
Addition of 6xHis adaptors in 3 reading frames into the *Eco* RI site of λgt11.

Use
Cnstruction of cDNA expression libraries. The His tag allows recognition of the expressed lacZ fusion proteins by anti-His antibodies and facilitates the protein purification after the inserts are subcloned into an expression plasmid.



Construction of the vector
The adaptors containing a 5' phosphorylated *Eco* RI and a 3' *Nla* III overhang (figure) were ligated to *Eco* RI cut and dephosphorylated λgt11 DNA (Pharmacia). All three adaptors at a concentration of 1µM each were ligated to 0.5µg λ arms in 10µl reaction volume. The DNA was purified by phenol extraction, ethanol and PEG precipitation.

Sequencing primers
See figure.

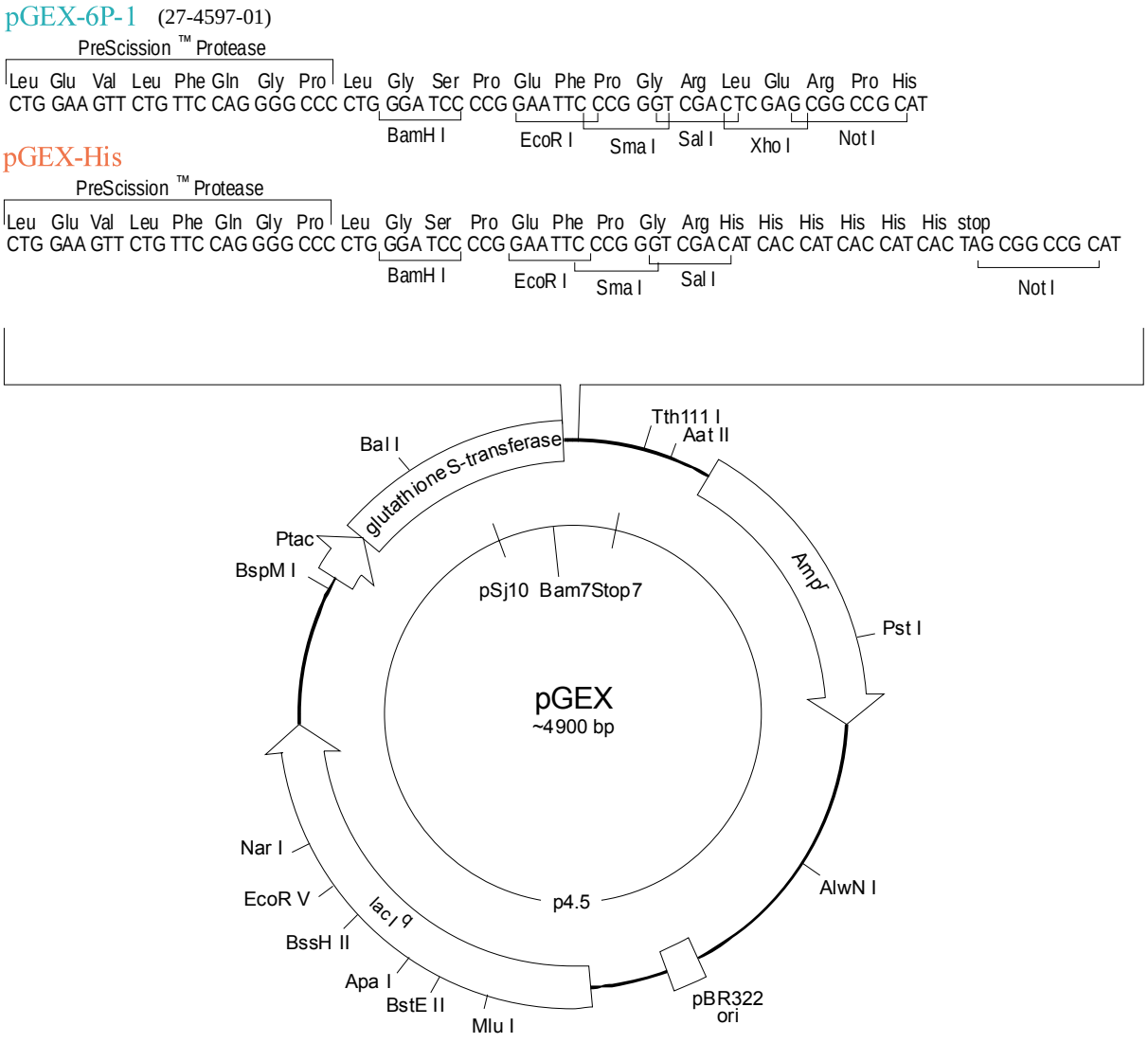
pGEX-His

Modification

Addition of a 6xHis tag at the C-terminus of the GST fusion protein.

Use

Expression and purification of full length fusion proteins containing an N-terminal GST and a C-terminal 6xHis tag.



Construction of the vector

The adaptor obtained by annealing the two oligos 5'-AATTCCCGGGTCGACATCACCATCACCATCACTAGC and 5'-GGCCGCTAGTGATGGTGATGGTGATGTCGACCCGGG was ligated into the polylinker of pGEX-6P-1 (Pharmacia). 100ng of pGEX-6P-1 was digested by *Sal* I and *Not* I, phenol extracted, ethanol and PEG precipitated. 1µM adaptor was ligated to 50ng of the cut vector in 10µl reaction volume. The ligation mix was used to transform chemo competent Sure cells. 5 colonies were picked to prepare mini preps, which were *Xho* I digested in order to check for adaptor insertion. 3 minipreps containing the adaptor were sequenced.

Sequencing primers

pGEX fw: 5'-GGC TGG CAA GCC ACG TTT GG

pGEX rev: 5'-GAA ACG CGC GAG GCA GAT CG

6.A11. Purification of active eukaryotic proteins from inclusion bodies in *E. coli*

Biotechniques: Vol XI, No. 6, December 1991, pp748-752

The essence of this method is to purify the inclusion bodies followed by selective solubilization of the expressed protein.

This protocol was designed for a 1L culture. However, one can scale the volumes up or down, depending on the type of experimentation performed. Both 100ml cultures and 500 ml cultures were prepared for this protocol (the results section specifies further).

BUFFER A	BUFFER P	Buffer W
20mM Tris-HCl pH 7.5	PBS w/o Ca, Mg	PBS w/o Ca, Mg
20% Sucrose	5mM EDTA	25% Sucrose
1mM EDTA	1X PIN, PMSF,--added fresh	5mM EDTA
		1% Triton

BUFFER R	BUFFER D	BUFFER U1
50Mm Tris-HCl pH 8.0	50mM Tris-Hcl pH 8.0	50mM Tris-HCl pH 8.0
1mM DTT	5M Guanidium HCl	4M Urea
20% Glycerol	5mM EDTA	

BUFFER U2
50mM Tris HCl pH 8.0
8M Urea

1. The cells from the 1L culture are transformed into two 500ml tubes and are pelleted by centrifugation at 5000rpm/min at 4 degrees C. To prevent contaminating the inclusion bodies by proteins from the outer membrane, the outer membrane is removed by resuspending the cell pellet from 1L culture in 50ml BUFFER A. (If 100ml culture was prepared, only 5 ml Buffer A would be required. This down -scaling of volumes is required whenever a volume is indicated).
2. Incubate on ice for 10 minutes. Pellet the cells by centrifugation at 6000rpm/5min at 4 degrees C. Resuspend in 50ml of ice cold water. Leave on ice for 10 min. Centrifuge at 8200 rpm/5 min at 4 degrees C.
3. Resuspend the pellet in 10ml Buffer P containing protease inhibitors.
4. Sonicate the resuspended mixture 3x (50W) each with a 30sec. pulse with a 30 sec. pause in between pulses. RNase T1 (1.3x103 U/10ml) + DNase I (400 ug/10ml) are added to the sonicated cell suspension and incubated at room temp for 10 min.
5. The suspension is further diluted by addition of 40 ml of Washing Buffer P and the crude inclusion bodies are pelleted by centrifugation at 11,000 rpm for 30 min at 4 degrees C. Keep aliquot and assay the supernatant for proteins.
6. The pellet (inclusion bodies) is suspended in 40 ml of Buffer W. Incubate on ice for 10 min and centrifuge at 15,000 rpm/5min at 4 degrees C. for 10 min. Keep aliquot of supernatant. Repeat this washing step and save the supernatant.
7. Resuspend the pellet in 10ml of Buffer D. A brief sonication with a 5 sec. pulse (50W) facilitates the solubilization of the aggregated proteins (NOTE: the pellet may also be resuspended in 4M or 8M urea in addition to BUFFER D).
8. The resultant suspension is incubated on ice for an additional 1 hour.
9. Centrifuge at 10,500 rpm for 30 min at 4 degrees C. Add the supernatant to 100ml of Renaturation Buffer R, and stir gently at 4 degrees C. over night to renature the proteins.
10. The next day, the supernatant is clarified by centrifugation at 12,000 rpm for 30 min at 4 degrees C. The supernatant contains highly purified expressed proteins.

6.A12. Purification of GST Fused Proteins

After Frangioni and Neel (1993), written by Chia Jin Ngee

Many people have vented out frustration over insoluble GST-fused proteins. This is a protocol that for enzymatically active soluble GST-fused proteins. All GST-fused proteins are rendered soluble with this technique though enzyme activitiy can range > from 30-90%.

Materials/Reagents

- STE Buffer

10 mM Tris-HCl, pH 8.0
1 mM EDTA
150 mM NaCl

- Lysozyme solution

10 mg/ml in water (make fresh)

- PBS
- Elution Buffer

50 mM Tris.Cl, pH 9.0
20 mM GSH

- 10% Sarkosyl in STE Buffer
- 10% Triton X-100 in STE Buffer
- 1 M DTT
- 100 mM IPTG

Method

Day 1

1. 1. Set up an overnight culture in 50 ml 2XTY with 150 mg/ml of ampicillin.

Day 2

2. Seed 5 ml of overnight culture to 500 ml 2XTY with 150 mg/ml of ampicillin.
3. Grow at 37^{o}C to an A_{600} of 0.6 to 0.8.
4. Induce with 0.1 mM to 2 mM of IPTG. Grow for 3 hr at 37^{o}C or grow overnight at room temperature.
   Lower IPTG concentrations and lower growing temperatures tend to produce greater solubility at the expense of yield.
5. Pellet cells by centrifuging at 3000 *g*, 4^{o}C for 10 min. Decant media and resuspend cells in 30 ml ice-cold PBS to wash. Transfer to a 40-ml Oak Ridge tube and centrifuge at 3000 *g*, 4^{o}C for 10 min. Decant PBS.
6. This is a convenient point to stop and to store pellets at -80^{o}C. Else continue to lyse cells.
7. Thaw pellet on ice if cells are frozen else proceed to the next step.
8. Resuspend pellet in 10 ml of ice cold STE Buffer.
9. Add 100 µl of freshly prepared lyozyme solution, incubate on ice for 15 min. Just before sonication, add 100 µl of 1 M DTT and 1.4 ml of 10% Sarkosyl. Mix thoroughly by inversion and sonicate for a total time of 1 min.
10. Centrifuge 16,000 rpm for 20 min on the SS34 rotor to pellet debris. Transfer supernatant to a 50-ml conical tube and discard the pellet. Add 4 ml of 10% Triton X-100 and top up with STE Buffer to 20 ml. The effective concentration of Sarkosyl and Triton X-100 will be 0.7% and 2% respectively. Incubate at room temperature for 30 min.
11. Pour the lysate to 1 ml bed of prepared Glutathione Sepharose in PBS. Incubate at room temperature for 30 min to 1 hr with agitation.
    To prepare the 50% slurry, shake up the media and pipette 2 ml to a 50 ml tube. Fill to 50 ml with PBS, invert tube a few times. Centrifuge to 2000 rpm on a swing bucket centrifuge then switch off. Carefully suck off PBS and resuspend beads with 1 ml of PBS.
12. Wash the beads with 3 X 50 ml of PBS. Finally resuspend in 5 ml of PBS. Pour to a dispo-column. Wash the 50-ml conical tube with an additional 5 ml of PBS. Pool with the first 5 ml in the dispo-column.
    To wash, use the same centrifugation technique for preparing the beads. When transferring beads to column, do not pipette but pour. The beads tend to stick to pipette tips.
13. If desired, elute with 10 x 1 ml fractions of Elution Buffer. Determine desired fractions with SDS PAGE.

7. Acknowledgments

First of all I'd like to thank Hans-Peter Beck for enabling and supervising this PhD project. I appreciate the freedom I had in choosing the way to go during this thesis. Thanks also for always having time for answering my questions.

I thank Prof. N. Weiss and Prof M. Tanner for being part of my thesis committee and for their support.

Many thanks to Jutta Marfurt for adding that many efforts in the diagnostic test project (chapter 2): she did the industrious work (screening the cDNA libraries), whereas I picked the interesting part of the work (constructing the libraries). Thanks also for accepting me and my moods as coworker in the same lab!
I also want to thank Sylvia Steiger, who did incredible amounts of SDS-PAGEs for this project.

For the mini-exon project (chapter 3), I thank again Jutta Marfurt who did the main job, Divine Makia who elaborated the RFLP scheme among other things, and Ingrid Felger for supervising (but of course also for her spontaneous comments)!

Many thanks to the whole group of Hans-Peter Beck for the great working atmosphere, and their help on scientific topics, as an example: Tobias Spielmann had the great idea of adding His adaptors to λgt11 phages (appendix 6.A10).

I'd like to thank Reto Brun and his group for making the parasite stabilates available and for introducing me into the field of *Leishmania* cultivation.

I also want to thank the group of Gerd Pluschke for helping out when antibodies and other ingredients for IFAs were used up and for loading the sequencer.

Many thanks to Hans-Peter Beck and Ingrid Felger for their great parties and (this was really incredible) the excellent dinners!

Thanks a lot to Till Voss, Tobi Spielmann, Roland Degen, Mirjam Kästli, Christian Flück, Cornelia Spycher, Christian Scherer, Sebastrian Rusch, Selina Bopp, Alexandra Lüscher, Diana Diaz, Shinji Okitsu, Attila Regös, Matthias Rottmann, Eric Huber, Michi Oberle, Sonja Bernhard, Martin Nägeli, Erwin Kump, André Tiaden (wow, what a list!)
and all the others for the brilliant garden parties, ping pong matches, billiard-, Döggele-, Jass-, poker-evenings and for the Aare-raft! Also thanks for loosing sometimes, but no thanks for winning…

I want to thank Till and Tobi for providing bed and breakfast (and more important: beer) in Australia! Thanks also to Cornelia for securing my 85kg live (fat) weight during rock climbing.

8. References

Alexander, J., A.R. Satoskar, and D.G. Russell. 1999. *Leishmania* species: models of intracellular parasitism. J Cell Sci 112 Pt 18, 2993-3002.

Anbar, M., and P. Neta. 1967. A compilation of specific bimolecular rate constants for the reaction of hydrated electrons, hydrogen atoms and hydroxyl radical with inorganic and organic compounds in aqueous solution. Int J Appl Radiation Isotopes 18, 493-523.

Anderson, S., J.R. David, and D. McMahon-Pratt. 1983. In vivo protection against *Leishmania mexicana* mediated by monoclonal antibodies. J Immunol 131, 1616-8.

Angarano, G., P. Maggi, M.A. Rollo, A.M. Larocca, M. Quarto, A. Scalone, and L. Gradoni. 1998. Diffuse necrotic hepatic lesions due to visceral leishmaniasis in AIDS. J Infect 36, 167-9.

Balestieri, F.M., A.R. Queiroz, C. Scavone, V.M. Costa, M. Barral-Netto, and A. Abrahamsohn Ide. 2002. *Leishmania (L.) amazonensis*-induced inhibition of nitric oxide synthesis in host macrophages. Microbes Infect 4, 23-9.

Baneth, G., and S.E. Shaw. 2002. Chemotherapy of canine leishmaniosis. Vet Parasitol 106, 315-24.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-97.

Beckman, J.S., T.W. Beckman, J. Chen, P.A. Marshall, and B.A. Freeman. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 87, 1620-4.

Benne, R., J. Van den Burg, J.P. Brakenhoff, P. Sloof, J.H. Van Boom, and M.C. Tromp. 1986. Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. Cell 46, 819-26.

Berman, J. 2003. Current treatment approaches to leishmaniasis. Curr Opin Infect Dis 16, 397-401.

Brittingham, A., C.J. Morrison, W.R. McMaster, B.S. McGwire, K.P. Chang, and D.M. Mosser. 1995. Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J Immunol 155, 3102-11.

Campbell, D.A., S. Thomas, and N.R. Sturm. 2003. Transcription in kinetoplastid protozoa: why be normal? Microbes Infect 5, 1231-40.

Carrera, L., R.T. Gazzinelli, R. Badolato, S. Hieny, W. Muller, R. Kuhn, and D.L. Sacks. 1996. *Leishmania* promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J Exp Med 183, 515-26.

Chang, K.P., S.G. Reed, B.S. McGwire, and L. Soong. 2003. Leishmania model for microbial virulence: the relevance of parasite multiplication and pathoantigenicity. Acta Trop 85, 375-90.

Chiplunkar, S., G. De Libero, and S.H. Kaufmann. 1986. *Mycobacterium leprae*-specific Lyt-2+ T lymphocytes with cytolytic activity. Infect Immun 54, 793-7.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156-9.

Clayton, C.E. 2002. Life without transcriptional control? From fly to man and back again. Embo J 21, 1881-8.

Croft, S.L., and G.H. Coombs. 2003. Leishmaniasis - current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 19, 502-8.

De Souza Leao, S., T. Lang, E. Prina, R. Hellio, and J.C. Antoine. 1995. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci 108 (Pt 10), 3219-31.

Deniau, M., C. Canavate, F. Faraut-Gambarelli, and P. Marty. 2003. The biological diagnosis of leishmaniasis in HIV-infected patients. Ann Trop Med Parasitol 97 Suppl 1, 115-33.

Diatchenko, L., Y.F. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov, and P.D. Siebert. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93, 6025-30.

Frangioni, J.V., and B.G. Neel. 1993. Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem 210, 179-87.

Ghosh, M., and S. Bandyopadhyay. 2003. Present status of antileishmanial vaccines. Mol Cell Biochem 253, 199-205.

Gibson, W., and J. Stevens. 1999. Genetic exchange in the trypanosomatidae. Adv Parasitol 43, 1-46.
Guerin, P.J., P. Olliaro, S. Sundar, M. Boelaert, S.L. Croft, P. Desjeux, M.K. Wasunna, and A.D. Bryceson. 2002. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. Lancet Infect Dis 2, 494-501.
Hartley, J., and H. Bowen. 1996. PEG precipitation for selective removal of small DNA fragments. Focus 18, 27.
Hermoso, T., Z. Fishelson, S.I. Becker, K. Hirschberg, and C.L. Jaffe. 1991. Leishmanial protein kinases phosphorylate components of the complement system. Embo J 10, 4061-7.
Herwaldt, B.L. 1999. Leishmaniasis. Lancet 354, 1191-9.
Hindle, E. 1931. The development of various strains of *Leishmania* in Chinese sandflies. Proc Roy Soc (Series B) 108, 366-83.
Inoue, H., H. Nojima, and H. Okayama. 1990. High efficiency transformation of Escherichia coli with plasmids. Gene 96, 23-8.
Kirkpatrick, C.E., and J.P. Farrell. 1982. Leishmaniasis in beige mice. Infect Immun 38, 1208-16.
Kirkpatrick, C.E., and J.P. Farrell. 1984. Splenic natural killer-cell activity in mice infected with *Leishmania donovani*. Cell Immunol 85, 201-14.
Knuepfer, E., Y.D. Stierhof, P.G. McKean, and D.F. Smith. 2001. Characterization of a differentially expressed protein that shows an unusual localization to intracellular membranes in Leishmania major. Biochem J 356, 335-44.
Launois, P., F. Tacchini-Cottier, C. Parra-Lopez, and J.A. Louis. 1998. Cytokines in parasitic diseases: the example of cutaneous leishmaniasis. Int Rev Immunol 17, 157-80.
Liew, F.Y., and C.A. O'Donnell. 1993. Immunology of leishmaniasis. Adv Parasitol 32, 161-259.
Marin, M., C. Muskus, J.R. Ramirez, L.F. Arbelaez, J.F. Alzate, and C. Berberich. 2000. The gene encoding the metacyclogenesis-associated transcript Mat-1 is conserved in the genus *Leishmania* and shows a tendency to form dimers upon protein expression. Parasitol Res 86, 431-5.
McConville, M.J., S.J. Turco, M.A. Ferguson, and D.L. Sacks. 1992. Developmental modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. Embo J 11, 3593-600.
Modabber, F. 1989. Experiences with vaccines against cutaneous leishmaniasis: of men and mice. Parasitology 98 Suppl, S49-60.
Moll, H. 2003. Dendritic cells as a tool to combat infectious diseases. Immunol Lett 85, 153-7.
Moll, H., S. Flohe, and M. Rollinghoff. 1995. Dendritic cells in *Leishmania major*-immune mice harbor persistent parasites and mediate an antigen-specific T cell immune response. Eur J Immunol 25, 693-9.
Montalban, C., J.L. Calleja, A. Erice, F. Laguna, B. Clotet, D. Podzamczer, J. Cobo, J. Mallolas, M. Yebra, and A. Gallego. 1990. Visceral leishmaniasis in patients infected with human immunodeficiency virus. Co-operative Group for the Study of Leishmaniasis in AIDS. J Infect 21, 261-70.
Moore, K.J., and G. Matlashewski. 1994. Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. J Immunol 152, 2930-7.
Myler, P.J., L. Audleman, T. deVos, G. Hixson, P. Kiser, C. Lemley, C. Magness, E. Rickel, E. Sisk, S. Sunkin, S. Swartzell, T. Westlake, P. Bastien, G. Fu, A. Ivens, and K. Stuart. 1999. *Leishmania major* Friedlin chromosome 1 has an unusual distribution of protein-coding genes. Proc Natl Acad Sci U S A 96, 2902-6.
Nadim, A., E. Javadian, G. Tahvildar-Bidruni, and M. Ghorbani. 1983. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. Bull Soc Pathol Exot Filiales 76, 377-83.
Olmo, A., R. Arrebola, V. Bernier, D. Gonzalez-Pacanowska, and L.M. Ruiz-Perez. 1995. Co-existence of circular and multiple linear amplicons in methotrexate-resistant *Leishmania*. Nucleic Acids Res 23, 2856-64.
Puentes, S.M., R.P. Da Silva, D.L. Sacks, C.H. Hammer, and K.A. Joiner. 1990. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. J Immunol 145, 4311-6.
Reid, S.D., G. Penna, and L. Adorini. 2000. The control of T cell responses by dendritic cell subsets. Curr Opin Immunol 12, 114-21.
Reiner, N.E., W. Ng, and W.R. McMaster. 1987. Parasite-accessory cell interactions in

murine leishmaniasis. II. *Leishmania donovani* suppresses macrophage expression of class I and class II major histocompatibility complex gene products. J Immunol 138, 1926-32.
Reithinger, R., and C.R. Davies. 2002. Canine leishmaniasis: novel strategies for control. Trends Parasitol 18, 289-90.
Rittig, M.G., and C. Bogdan. 2000. *Leishmania*-host-cell interaction: complexities and alternative views. Parasitol Today 16, 292-7.
Rivas, L., J. Moreno, C. Canavate, and J. Alvar. 2004. Virulence and disease in leishmaniasis: what is relevant for the patient? Trends Parasitol 20, 297-301.
Rosenthal, E., and P. Marty. 2003. Recent understanding in the treatment of visceral leishmaniasis. J Postgrad Med 49, 61-8.
Sacks, D., and S. Kamhawi. 2001. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol 55, 453-83.
Sah, J.F., H. Ito, B.K. Kolli, D.A. Peterson, S. Sassa, and K.P. Chang. 2002. Genetic rescue of Leishmania deficiency in porphyrin biosynthesis creates mutants suitable for analysis of cellular events in uroporphyria and for photodynamic therapy. J Biol Chem 277, 14902-9.
Saha, A., G. Chakrabarti, S. Sen, and S. Bandyopadhyay. 1999. Leishmania donovani parasites interact with gamma/delta+ human peripheral blood T cells and induce susceptibility to NK cell-mediated lysis. Scand J Immunol 50, 588-95.
Sambrook, J., and D.W. Russel. 2001. Molecular Cloning. 3rd ed. Cold Spring Harbor Laboratory Press, New York.
Saxena, A., E.A. Worthey, S. Yan, A. Leland, K.D. Stuart, and P.J. Myler. 2003. Evaluation of differential gene expression in *Leishmania major* Friedlin procyclics and metacyclics using DNA microarray analysis. Mol Biochem Parasitol 129, 103-14.
Schlein, Y., R.L. Jacobson, and G. Messer. 1992. *Leishmania* infections damage the feeding mechanism of the sandfly vector and implement parasite transmission by bite. Proc Natl Acad Sci U S A 89, 9944-8.
Singh, S., and R. Sivakumar. 2003. Recent advances in the diagnosis of leishmaniasis. J Postgrad Med 49, 55-60.
Singla, N., and V.K. Vinayak. 1994. Leishmania donovani flagellum-specific epitopes mediating host-parasite interactions. FEMS Immunol Med Microbiol 8, 175-81.
Spath, G.F., L.A. Garraway, S.J. Turco, and S.M. Beverley. 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. Proc Natl Acad Sci U S A 100, 9536-41.
Sundar, S. 2001. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health 6, 849-54.
Titus, R.G., F.J. Gueiros-Filho, L.A. de Freitas, and S.M. Beverley. 1995. Development of a safe live *Leishmania* vaccine line by gene replacement. Proc Natl Acad Sci U S A 92, 10267-71.
Ullu, E., C. Tschudi, and A. Gruenzl. 1996. Trans-splicing in trypanosomatid protozoa. In: M. Parsons, ed. Molecular Biology of parasitic protozoa, Vol. Oxford University Press Inc., New York, pp. 115-33
Wilson, S.M. 1995. DNA-based methods in the detection of *Leishmania* parasites: field applications and practicalities. Ann Trop Med Parasitol 89 Suppl 1, 95-100.
Yan, S., P.J. Myler, and K. Stuart. 2001. Tetracycline regulated gene expression in *Leishmania donovani*. Mol Biochem Parasitol 112, 61-9.
Zambrano-Villa, S., D. Rosales-Borjas, J.C. Carrero, and L. Ortiz-Ortiz. 2002. How protozoan parasites evade the immune response. Trends Parasitol 18, 272-8.

Curriculum Vitae

Igor Niederwieser

Date/ place of birth 1.11.1971, Fällanden (Schweiz)

Education

1989 Matura Typus B (Hohe Promenade, Zürich)

1992-1995 Basic studies in biology at the University of Zürich.

1996 6 months practical course at La Roche (Basel)

1997-1999 Main course in biology II at the Biocenter (Basel) and diploma thesis "analysis of the expression and diversity of gametocyte specific genes in *Plasmodium falciparum*" at the Swiss Tropical Institute (STI, Basel). Research advisors: Dr. Hans-Peter Beck (STI) and Prof. Peter Philippsen (Biocenter).

1999-2004 PhD thesis: „Molecular analysis of *Leishmania infantum*" at the STI. Research advisors: Dr. Hans-Peter Beck, Prof. Niklaus Weiss, and Prof. Marcel Tanner.

Publications

First autor: Niederwieser I, Felger I, Beck HP.
Plasmodium falciparum: expression of gametocyte-specific genes in monolayer cultures and malaria-positive blood samples.
Exp Parasitol. 2000 Jul;95(3):163-9.

Niederwieser I, Felger I, Beck HP.
Limited polymorphism in *Plasmodium falciparum* sexual-stage antigens. Am J Trop Med Hyg. 2001 Jan-Feb;64(1-2):9-11.

Others: Marfurt J, Niederwieser I, Makia ND, Beck HP, Felger I.
Diagnostic genotyping of Old and New World *Leishmania* species by PCR-RFLP. Diagn Microbiol Infect Dis. 2003 Jun;46(2):115-24.

Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I.
Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis.
J Clin Microbiol. 2003 Jul;41(7):3147-53.

Talks

Niederwieser I, Beck HP
Enrichment of potential *Leishmania infantum* virulence factors by cDNA subtraction. Parasitology Meeting for PhD Students, Schloss Münchenwiler, 2001.

Niederwieser I, Beck HP.
Differntially expressed genes in *Leishmania infantum* vector stages
Swiss Trypanosomatid Meeting, Leysin, 2004.

Posters

Niederwieser I, Beck HP.
Differentially expressed genes in *Leishmania infantum*.
Joint Meeting Swiss Society for Microbiology/Infectious Diseases/ Parasitology, Basel, 2003.

During my studies I attended lectures and courses of the following lecturers

H.R. Oswald, H. Fischer, H.R. Bosshard, G. Wagnière, H.H. Storrer, M. Noll, R. Wehner, J. Robinson, H.R. Hohl, C.D.K. Cook, H. Rieber, W. Schaffner, A. Dübendorfer, P. Matile, R. Bachofen, D. Rast, V. Ziswiler, H. Weber, B. Wechsler, P. Borner, H. Kummer, R.D. Martin, P. Tardent, C. Thompson, P. Phillipsen, U. Jenal, P. Jenö, M. Tanner, H.P. Beck, W. Keller, W.J. Gehring, M. Rüegg, G.R. Cornelis, T. Bickle, H. Reichert, M. Spiess, H. Riezmann, M.N. Hall, T. Schirmer, A. Engel, J. Seelig, M. Affolter, U.A. Meyer.