Development of affordable molecular techniques for the diagnosis of leishmaniasis in Yemen

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ZUSAMMENFASSUNG

Die Leishmaniose ist eine durch einzellige Parasiten der Gattung *Leishmania* verursachte Infektionskrankheit, die durch den Stich von Sandmücken übertragen wird. Als erstes klinisches Symptom bildet sich eine nicht abheilende ulceröse Wunde von bis zu mehreren cm Durchmesser um die Einstichstelle herum. Nach mehreren Monaten kann die Wunde unter Hinterlassung einer Narbe spontan abheilen. Falls es sich um eine Infektion mit einer weniger virulenten Form des Parasiten gehandelt hat, ist der Patient geheilt und für den Rest des Lebens resistent gegen diese Form. Andere Stämme des Parasiten lösen aber die mucocutane Form der Krankheit aus, die zu schmerzhaften destruktiven Läsionen der Lippen- und Nasenschleimhäute führt, oder die viszerale Form der Leishmaniose, die innere Organe angreift und unbehandelt tödlich verläuft. In der Republik Jemen sind die cutane und die viszerale Leishmaniose endemisch, werden aber aus Mangel an Geld in der Bevölkerung weder hinreichend diagnostiziert noch behandelt.

Aufzeichnungen über epidemiologische Daten gibt es nur für wenige staatliche Krankenhäuser, die Zahl der unregistrierten Fälle ist wahrscheinlich sehr hoch. Um in Zukunft zu Kontrollmaßnahmen gegen die Krankheit beitragen zu können, wurde der Versuch unternommen, neue molekulare Diagnoseverfahren zu entwickeln, die einerseits sehr empfindlich und spezifisch sind, andererseits aber nicht zu kompliziert und teuer, so dass sie unter den limitierten Voraussetzungen des Landes realisiert werden können.

Um eine Einblick in das wahre Ausmaß der Krankheit im Jemen zu erhalten, wurden die in den staatlichen Krankenhäusern in den letzten Jahren gesammelten Daten ausgewertet. Außerdem wurden in verschiedenen Teilen des Landes mehr als 200 Proben von Patienten mit dem Verdacht auf cutane oder viscerale Leishmaniose gesammelt. Dies Proben wurden zunächst im Jemen mikroskopisch auf cutane Leishmaniose und mit Hilfe einer sehr unspezifischen Formol-Präzipitation-Methode auf viscerale Leishmaniose untersucht. Für einige Proben stand ein kommerzieller ELISA zur Verfügung. Von allen gesammelten Blutproben wurde die Plasmafraktion und aus angereicherten Leukozyten die DNA gewonnen und für nähere Analysen zur Universität Giessen gebracht.

Von den mittlerweile verfügbaren Sequenzdaten für die Leischmania-Stämme *L. infantum* (viszerale Leishmaniose) und *L. major* (cutane Leishmaniose) wurden

spezifische PCR-Primer für die Entwicklung neuer Diagnoseverfahren abgeleitet. Die neuen Verfahren wurden anhand von gereinigter DNA dieser beiden im Labor kultivierten Stämme getestet. Zur Entwicklung neuer PCR-Verfahren wurden fünf verschiedene Zielsequenzen von hoch-repetitiven Regionen der Genome ausgewählt. Zwei Genus-spezifische Tests und ein Test, der die Unterscheidung von cutaner und viszeraler Leishmaniose zulässt, wurden erfolgreich entwickelt.

Die Anwendung dieser Tests für die im Jemen gesammelten Patientenproben ergab, dass die PCR-Diagnose wesentlich empfindlicher und spezifischer ist, als die traditionell eingesetzten Methoden. Freilich konnte im Rahmen dieser Arbeit weder ein vollständiges Bild über die Epidemiologie der Leishmaniose im Jemen noch ein endgültig perfektes Diagnoseprotokoll entwickelt werden. Dies wird Aufgabe für die folgenden Jahre sein. Immerhin weisen die ermittelten Daten einerseits darauf hin, dass die Lage der Leishmaniose im Jemen sehr viel schwerwiegender ist, als offiziell zugegeben. Andererseits wurden diagnostische Verfahren entwickelt, die ausreichend einfach und so kostengünstig sind, dass sie im Land selbst eingeführt werden können. In optimierter Form könnten diese Verfahren zu einem besseren Stand der Kenntnis über diese Krankheit im Land führen und auf Dauer zu einer Verbesserung der gesundheitlichen Situation der Bevölkerung beitragen

SUMMARY

Leishmaniasis is an infectious disease caused by different species of unicellular *Leishmania* parasites which are transmitted by the bite of sandflies. As first clinical symptome, a non-healing ulcerous wound of up to several centimeters diameter forms around the byte of the insect. After several months, the wound may heal spontaneously leaving a scar. If the infection was due to a less virulent species of the parasite, the patient is healed and resistant against this form for the rest of life. However, other strains of the parasite lead to mucocutaneous leishmaniasis which lead to painfull destructions of the mucosa of lipps and nose, or to visceral leishmaniasis which affects inner organs and is lethal without treatment. Cutaneous as well as visceral leishmaniasis are endemic in the Republic of Yemen, but due to limiting financial resources, the disease is neither appropriatelytly diagnosed nor treated in the population.

Recorded epidemiological data exist only for some governmental hospitals, but the number of unregisterd cases may be huge. In order to shed more light in the real extent of the disease in Yemen and to contribute to future control measures, it was attempted to design new molecular procedures for the diagnosis, which are, at the one hand, highly sensitive and specific, at the other hand not too complicated and inexpensive, so that they could be performed under the limiting conditions of the country.

To obtain some insight in the sitation of leishmaniasis, the data registered in governmental hospitals during the past years were collected and evaluated. In addition, samples from more than 200 patients with suspected cutaneous and visceral leishmaniasis were collected in different parts of the country. These samples were analysed first in Yemen by microscopy for cutaneous leishmaniasis and by a highly unspecific procedure called formol gel precipitation for visceral leishmaniasis Some of the samples were also analysed by a commercial ELISA. From all collected blood samples the plasma fraction and DNA from enriched leucocytes were prepared and transported to the University of Giessen for further analysis.

By using the available sequence data of the *Leishmania* strains *L. infantum* (visceral leishmaniasis) and *L. major* (cutaneous leishmaniasis), specific PCR primers were developed for the design of new diagnostic procedures. The assays were tested by means of purifed DNA from these two strains which had been

cultivated in the laboratory. Five different target sites from highly repeated sequences of the genomes were selected to develop new diagnostic PCR assays. Two assays specific for the genus *Leishmania*, and one assay allowing differentiation of the two strains were constucted allowing to discriminate between cutaneous and visceral leishmaniasis.

Applying these tests on the patient samples collected in Yemen revealed that PCR diagnosis is more sensitive and specific than the traditional methods used. Of course, neither a comprehensive epidemiology of Yemen nor a ultimative perfect diagnostic protocol could be obtained during this work. This has to be established during the following years. However, the results reveal at the one hand that the situation of leishmaniasis in Yemen is more severe than officially stated. At the other hand, diagnostic procedures were developed which are simple and inexpensive enough to be introduced in the country. When optimized, these techniques may lead to more awareness of this disease in the country and to an improvement of the health condition of the suffering population.

CONTENT

1. INTRODUCTION

1.1 Epidemiology of leishmaniasis

Leishmaniasis is a protozoan parasitic disease which is prevalent in many parts of the tropics, subtropics, and Southern Europe. The disease is caused by infection with different species of *Leishmania*, which are transmitted by the bite of phlebotomine sand flies. The most common forms of leishmaniasis in the Old World are *cutaneous leishmaniasis* (CL), causing skin sores (review: Reithinger et al., 2007), and *visceral leishmaniasis* (VL), affecting internal organs of the body such as spleen, liver, and bone marrow (review: Berman, 2006). A third form of the disease, *mucocutaneous leishmaniasis* (MCL) leading to severe destruction of mucosal areas of the mouth, nose and pharynx occurs exclusively in Latin America (reviewed by Myler and Fasel, 2008). There is an estimated prevalence of approximately 12 million infected people worldwide with 1.0 - 1.5 million new cases of cutaneous leishmania and 400,000 - 600,000 new cases of visceral leishmania per year. Leishmaniasis is endemic in 88 countries and It has been estimated that 350 million people are at threat. Confirmed cases of VL have been reported from 66 countries, 90% of the world's VL occurs in five countries (India, Bangladesh, Nepal, Sudan and Brazil), and 90% of all CL cases are concentrated in 7 countries (Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria) showing that Middle East is a focus for cutaneous leishmania (Kumar et al., 2007). At present 90% of all MCL occurs in Bolivia, Peru and Brazil. It is the most feared form of leishmaniasis because of its disfiguring lesions of the face.

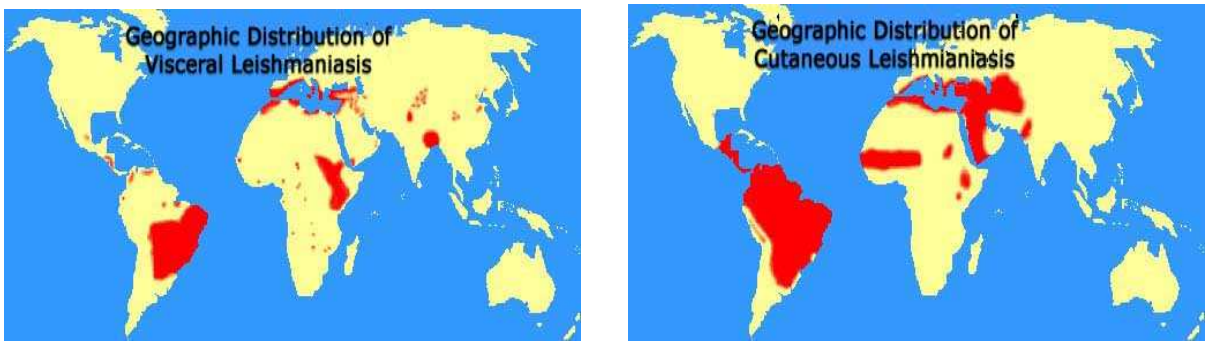

Figure 1: World distribution of visceral and coutaneous leishmaniasis (source: WHO)

1.2 The Parasite

Leishmania is a protozoon belonging to the order of *Kinetoplastida* and to the family of *Trypanosomatidae*. The genus *Leishmania* includes more than 20 different species. The parasites exist in two morphological forms: the non-motile intracellular amastigote (3-5 micrometer in diameter) living in macrophages of the mammalian host, and the motile extracellular promastigote (15-30 micrometer in length, plus the flagellum) living in the intestinal tract of the sandfly vector. The amastigotes are able to survive inside the macrophages and to multiply within the acidic phagolysosomes of these host cells (reviewed by Alexander et al., 1999).

After infection by a bite of a sandfly, promastigotes enter macrophages, transform into amastigotes within 12-24 h and continue multiplication until the host cell dies. The released amastigotes infect other macrophages and the infection spreads. The parasite contains two striking organelles, the nucleus and the kinetoplast. The kinetoplast is found in all protozoa of the order kinetoplastidae (*Leishmania, Trypanosoma, Plastocrithidia, Crithidia*, *Endotrypanum, Herpetomonas, Leptomonas, Phytomonas, and Wallaceina*). It is a rod-shaped mitochondrial structure consisting of a DNA network with two types of DNA: about 10,000 minicircles of approximately 2 kilobasepairs (kb) and 25-250 maxicircles of approximately 30 kb each. These together constitute the mitochondrial genome (reviewed by Singh 2006).

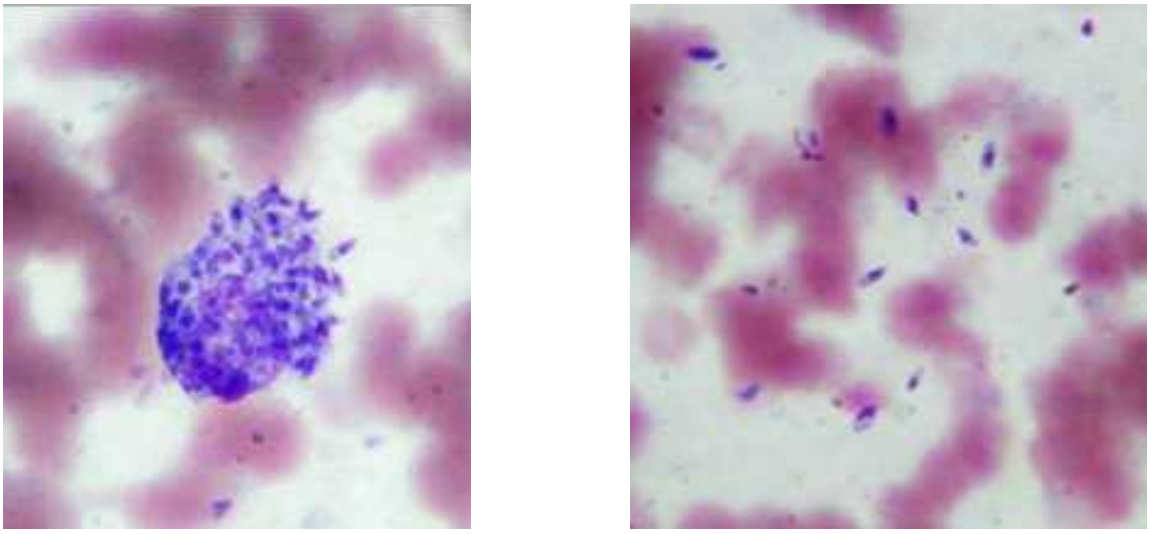

Figure 2: Intracellular and extracellular leishmanial amastigotes in a Giemsa-stained smear made from scrapings of cutaneous lesions (bright-field microscopy, x 1000; pictures from Al-Jawabreh, 2005).

The life cycle of *Leishmania*:

During its life cycle, Leishmania is alternating between two major forms: as extracellular promastigotes in the gut of the sand fly and as amastigotes inside the macrophages of the mammalian host. When the sand fly feeds, promastigotes are injected into the skin and are engulfed by host mononuclear phagocytes, where they transform into amastigotes and multiply within the phagolysosomal compartment until the phagocytic cells are destroyed, releasing the parasites to enter further cells and repeat the cycle.The life cycle is shown in Figure 3.

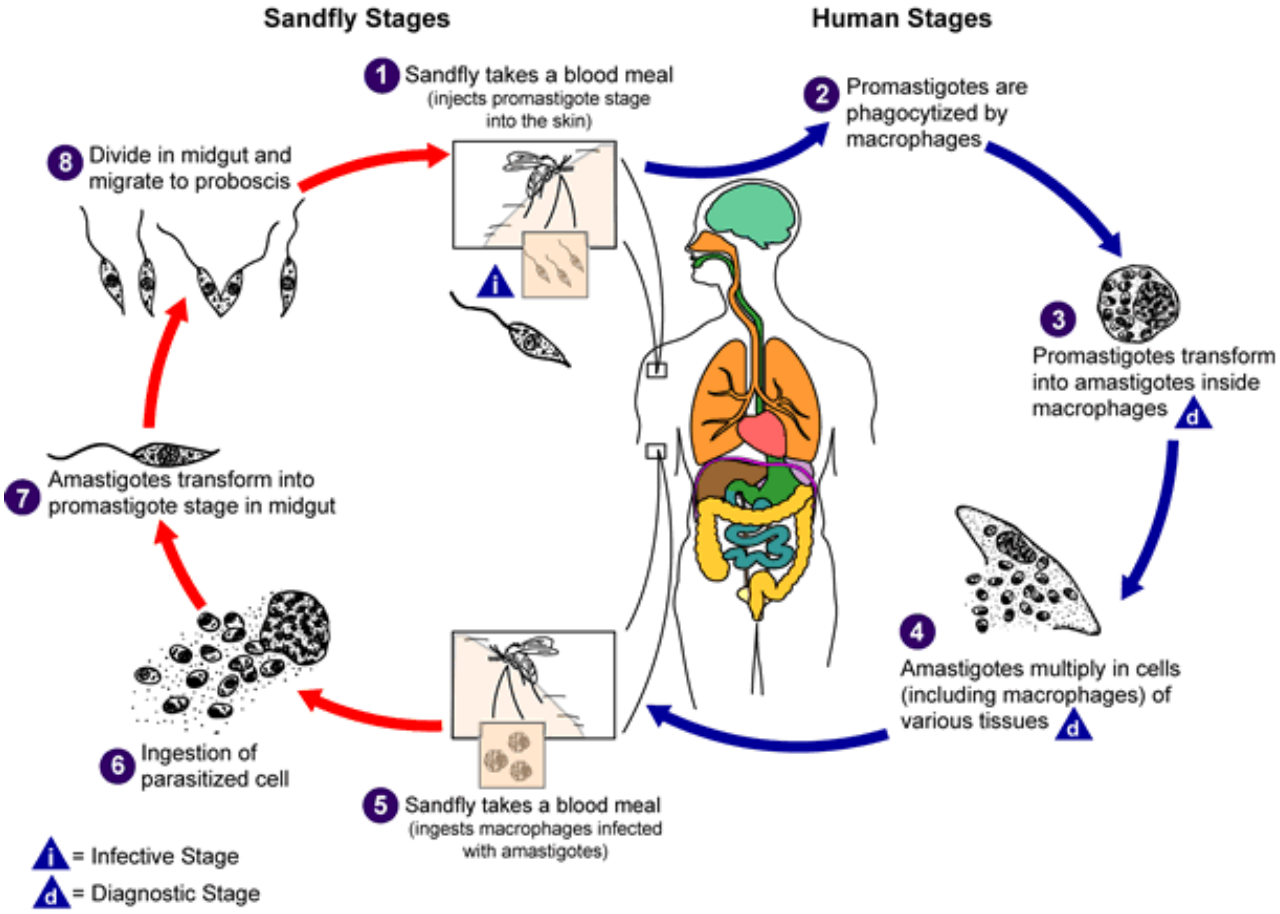

Figure 3: Scheme of the life cycle of Leishmania (picture from Rey, 2007)

Clinical symptoms of leishmaniasis

The three different clinical forms of leishmaniasis, cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL) and their

causative agents are summarised in Tables 1 and 2. There is a clear correlation between *Leishmania* species and the different clinical manifestations.

The clinical presentation of cutaneous leishmaniasis begins as an erythematous papule which transforms first in a nodule and later on to an ulcerative lesion. The lesion heals usually within several months leaving a more or less visible scar. This holds for all types of leishmaniasis. However, in case of MCL, lesions occure months or years later in the mucosa of the mouth and nose becomming gradually painful and eroding underlying tissue and cartilage. In case of VL, leasons occure after months in inner organs.

Table 1. Human pathogenic species of *Leishmania* in Old World, their clinical manifestation and geographical distribution.

Complex	***Species***	***Clinical manifestation***	***Geographical distribution***	***Reservoir***	***Vector***
	L. major	CL	Western and Central Asia Middle East, Africa, India	rodents: Fat sand rat (Psammomys.obesus), gerbils (Meriones, Rhombomys)	Phlebotomus paptasi
	L. tropica	CL, L. recidi-vans	Western and Central Asia Middle East, North Africa, Sub-Saharan Savanna, India	humans, dog infections reported, hyraxes suspected	P. sergenti
L.donovani	L. donovani	VL, PKDL	India, Sudan, Kenya	humans	P. argentipes
L.donovani	L. infantum	VL, (CL), CVL	Mediterranean basin Middle East, China	dogs, wild canids (foxes, jackals)	P. perniciosus, P. ariasi
	L. aethiopica	CL, DCL	Highlands of Ethiopia and Kenya, Sudan	hyraxes	P. longipes, P. pedifer

Table 2. Human pathogenic species of *Leishmania* in New World , their clinical manifestation and geographical distribution.

Complex	***Species***	***Clinical manifestation***	***Geographical distribution***	***Reservoir***	***Vector***
L. braziliensis	L. braziliensis	CL, MCL	Central and South America	forest rodents	Lutzomyia sp.
L. braziliensis	L. panamanensis	CL	Central America, Columbia	sloths	Lutzomyia sp.
L. braziliensis	L. guyanensis	CL	Guyana, Brazil	sloths	Lutzomyia sp.
L. braziliensis	L. peruviana	CL	Peru, Argentine	dogs, humans?	Lutzomyia sp.
L. mexicana	L. mexicana	CL, Chiclero's ulcer	Central and South America, Texas	forest rodents	Lutzomyia sp.
L. mexicana	L. amazonensis	CL, DCL	Brazil, Venezuela, mostly north of the Amazon	forest rodents, Opossums	Lutzomyia sp.
L. donovani	L. chagasi	VL, atypical CL, CVL	Central and South America CL in Honduras, Nicaragua	dogs, wild canids	Lutzomyia longipalpis

Mucocutaneous leishmaniasis is manifested solely in South America, especially in Brazil, Paraguay, Ecaudor, Bolivia, Peru, Colombia, and Venezuela. The highest percentage of the cases occur in Brazil, Bolivia, and Peru. Twenty percent of all leishmaniasis patients in Brazil develop MCL. It is a chronic and very serious condition, developing years after self-cure of cutaneous lesions. Mucosal lesions can progress to involve the entire nasal mucosa and the hard and soft palates. Without treatment, the entire nasal mucosa and palates become deformed with ulceration and erosion of the nasal septum, lips, and palate. The disease attacks cartilaginous areas, but usually spares bony structures, and it can leave extreme disfigurement. It can be lethal, mostly by aspiration pneumonia (Myler and Fasel, 2008).

Visceral leishmaniasis has many different symptoms as the disease developes. At the beginning, headache and fever occurre. Lateron, sweating with cough, diarrhea, vomiting, bleeding of the gums and weight loss are observed. In the late stage of disease, the clinical symptoms transforme to hepatomegally, splenomegally, anemia with leucopenia and lymphadenopathy. The mortality ranges from one year in acute cases up to 2-3 years in chronic cases. (Garg and Dube 2006).

1.3 Immunology of Leishmania infections

Within the mammalian host, *Leishmania* is able to survive as an amastigote in phagocytic cells such as macrophages, dendritic cells and neutrophils. The entering promastigote forms of the parasites are attacked by the complement system, however, some of them survive and enter the macrophages via the C3 receptor (Figure 4).

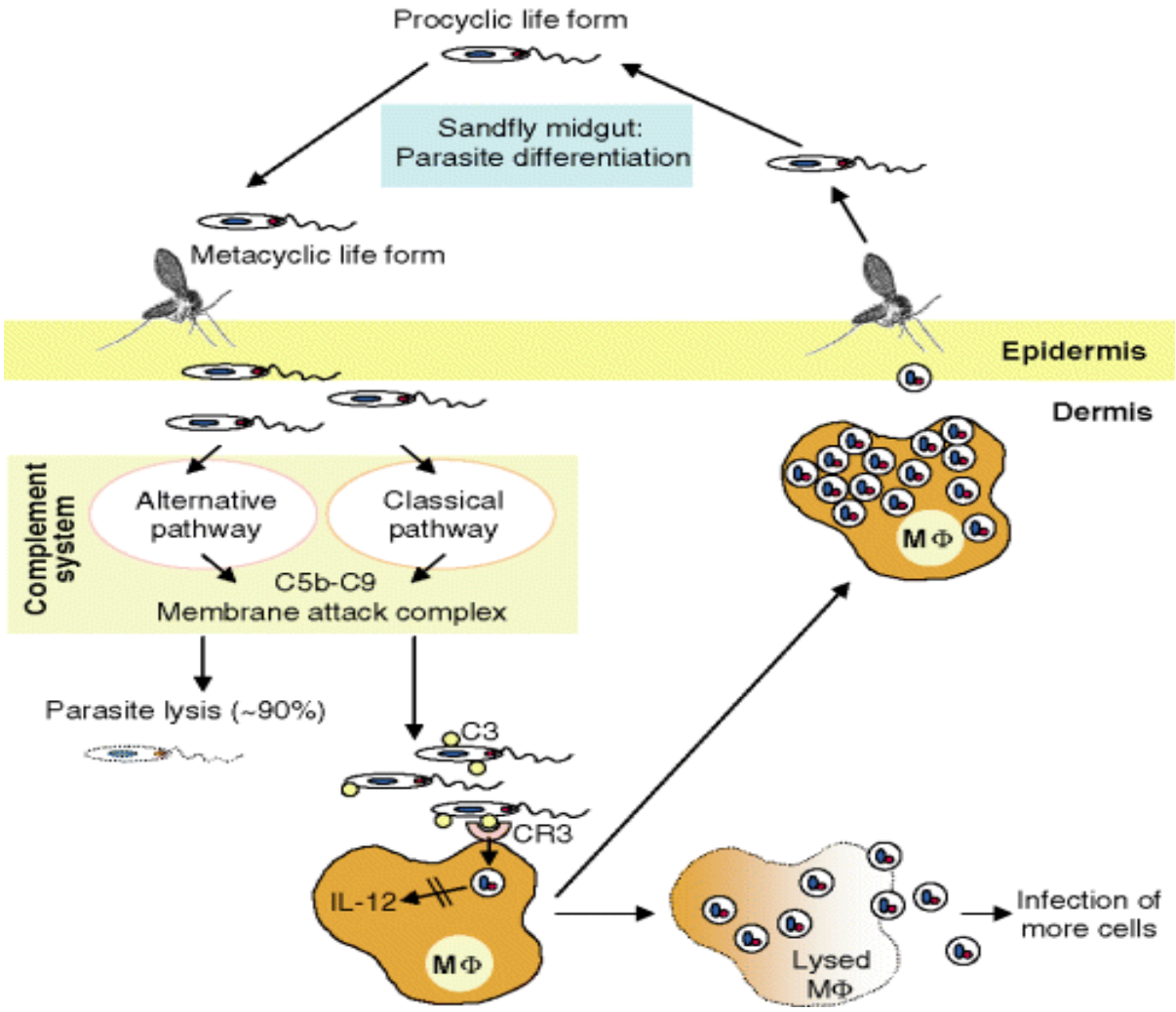

Figure 4: Life cycle of *L. major*. After inoculation of metacyclic promastigotes of *L. major* into the skin by the bite of a sand fly, parasites activate complement and are partially lysed. Surviving parasites utilize C3 to invade macrophage (MΦ) host cells via CR3, and – within these cells – they transform into obligate intracellular amastigotes. MΦ infection does not lead to cell activation (e.g. IL-12 release). Inside of MΦ, parasites replicate and more amastigotes are released into the tissue. The life cycle of *L. major* is completed upon uptake of parasites by another bite of a sand fly. (Reproduced from von Stebut, 2007)

Because the organisms are located intracellularly, an effective immune response includes mainly cell-mediated mechanisms for resistance to the parasite. Cytokines produced by T cells, natural killer (NK) cells, and antigen-presenting cells (APC) play an essential role in this process. (Roberts, 2006). The outcome of infection is determined by the parasite species and the host's immunological response. The $CD4^+$ T helper cell is critical with animal models demonstrating that cure is associated with strong IFN-gamma, interleukin (IL)-2 and IL-12 responses in the absence of classical Th2 cytokines or IL-10.

The outcome of the infection appears also to be influenced by the saliva of the transmitting sand fly. The saliva contains a repertoire of pharmacologically active molecules that hampers the host's haemostatic, inflammatory and immune responses. This interferes with the early interactions between Leishmania and the host's immune response. There are concrete indications that the host response against sand fly saliva influences disease outcome in leishmaniasis (Andrade et al., 2007, Figure 5).

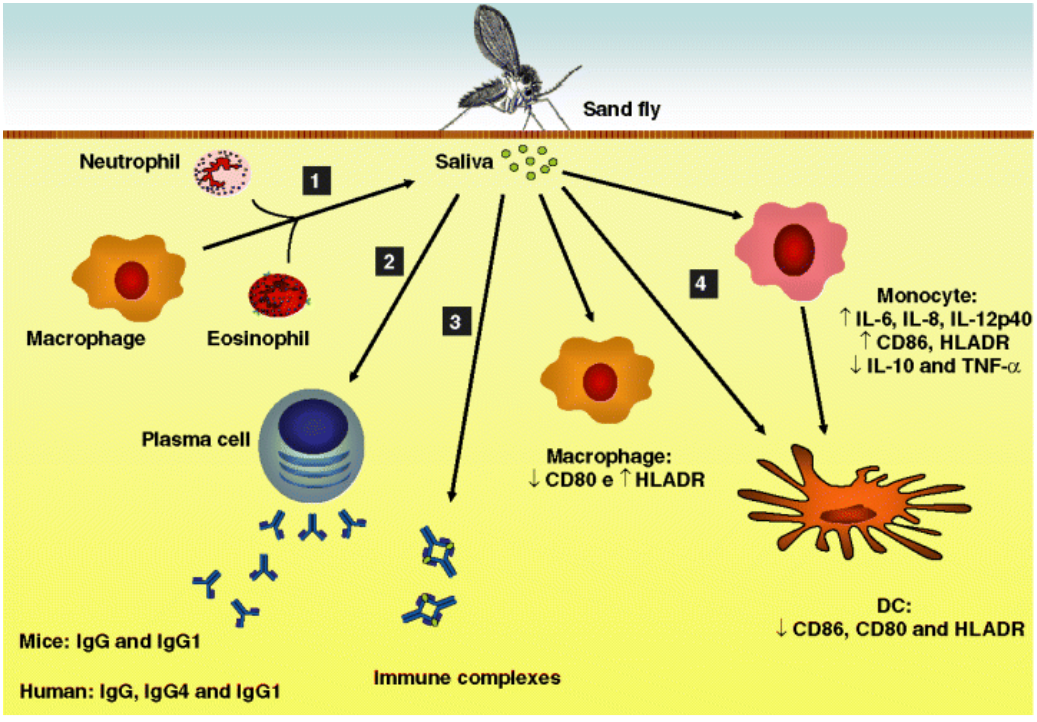

Figure 5: Effects of *Lutzomyia longipalpis* saliva on vertebrate host. When the sand fly saliva is injected into host skin, it induces an inflammatory cell infiltration (1), and antibody production (2). In this scenario, immune complexes are formed [20] at early phases of exposure. Moreover, sand fly saliva also modulates co-stimulatory molecules and cytokine release by antigen presenting cells. (Reproduced from Andrade et al., 2007)

Cutaneous leishmaniasis (CL) is worldwide by far the most frequent consequence of the disease. In the Old World, CL is also known as Oriental Sore, Delhi boil, Baghdad boil and Quetta Sore (Myint et al., 2008) and is usually caused by *L. major, L, tropica* and *L. aethiopica.* In Latin America, CL is caused by other *Leishmania* strains (see Table 2). This form of disease heals spontaneously after few months leading to life-long immunity against re-infection and is normally not treated with drugs. Healing is characterized by induction of IFN-gamma releasing T-cells (Kurtzhals et al., 1994), whereas failure to cure is associated with elevated levels of IL-4 with low IFN-gamma responses from *Leishmania*-specific T-cells (Ajdary e al., 2000). As all forms of leishmaniasis begin with a cutaneous lesion at the site of entry of the parasites, clear diagnostic differentiation at this stage would be needed to discriminate CL from the severe forms MCL and VL.

MCL is mostly caused by *Leishmania* species belonging to the *L. braziliensis* complex. However, it can also be caused by *L. panamanensis* (Barral et al., 1992). The serious hazard of developing MCL after cure of CL is estimated to be up to 40%. The immunology of MCL has not yet been studied in detail.

Visceral Leishmaniasis (VL) or Kala azar is usually caused by members of the *L. donovani* complex (Singh 2006). *L. donovani* is found to be the causative agent in endemic areas of Bangladesh, India and Sudan, *L. d. infantum* is widly distributed in Middel East and in Mediterranean countries and *L. d. chagasi* is the only causative agent in the New World. VL is characterized by suppression of T cell activity in acute disease due to increased expression of IL-10 (Ghalib et al., 1993). Increased expression of classical Th2 cytokines has been reported in VL with elevated levels of IL-4 particularly associated with treatment faillure (Sundar et al., 1997). Elevated levels of IL-13 have been observed in active disease that returned to normal after successful treatment (Babaloo et al., 2001).

1.4 Leishmaniasis and HIV-coinfection

Leishmaniasis has been identified as a frequent opportunistic infection in patients infected with human immunodeficiency virus type-1 (HIV-1) which amplifies in cells of the immune system thus reducing the ability to resist other infections. It has

been observed that the visceral form of leishmaniasis accelerates the course of HIV-1 disease progression and shortens the life expectancy of persons in areas where both diseases are endemic (Pourahmad 2009).

The greatest prevalence of co-infection with HIV and Leishmania have been observed in the Mediterranean basin with more than 2,000 cases reported from 35 countries to the World Health Organization. About 90 per cent of all cases come from Spain, Italy, France and Portugal. In Africa the increase of co-infection with HIV has been reported in Ethiopia and Sudan which is mainly caused by social troubles such as war or increase of population in urban and suburban areas in regions where both vector and reservoir are abundant. In South America, the majority of co-infections has been reported in Brazil.(Cruz et al., 2005)

1.5 Treatment of leishmaniasis

Pentavalent antimony-containing compounds are the main drugs used to treat leishmaniasis. These drugs need to be delivered by intramuscular or intravenous routes daily for 4-6 weeks and are quite toxic leading to severe side effects including serious heart arrhythmias, pancreatitis and and hepatic dysfunction. They cannot be used for pregnant women. In rich countries the fungizide amphotericin B in liposomal form in has become the treatment of choice. Since short time, a new oral agent, Miltefosine, has been licensed in India, Germany and Colombia for use in adults and children. Both drugs have lower side effects and show high cure rates. However, the cost of these agents restrict a more widespread use (Roberts, 2006).

By these reasons, less virulent cutaneous leishmaniasis is normally left to self-healing. Treatment is only important for the severe forms MCL and VL and to reduce scarring when cosmetically sites are involved. Plastic surgery may be needed to correct disfigurement by destructive facial lesions caused by cutaneous leishmaniasis. The most common drugs used for treatment of leishmaniasis, their application and side effects are listed in Table 3.

Table 3 Treatments for cutaneous, muco-scutaneous and visceral leishmaniasis (modified according to Roberts 2006)

Drug	Regimen	Comments/side effects
Cutaneous		
Antimonials	Observation alone	60–70% cure rate
Antimonials	IM/IV 20 mg/kg daily for 10 days	
Paromomycin	IL 1 ml/lesion weekly for 8 weeks	Pain/erythema
Miltefosine	Topical bd daily for 2 weeks	Pain/erythema
Fluconazole	PO 2.5 mg/kg daily for 28 days	
Pentamidine	PO 200 mg daily for 6 weeks	Nausea/vomiting
Imiquimod	IM 8 mg/kg daily for 7 days	Adjunctive therapy
Muco-cutaneous		
Antimonials	IM/IV 20 mg/kg daily for 20 days	
Liposomal amphotericin	IV 3 mg/kg daily for 20 days	Renal failure/low K+
Visceral		
Antimonials	IM/IV 20 mg/kg daily for 20 days	
Miltefosine	PO 2.5 mg/kg daily for 28 days	
Amphotericin B	IV 1 mg/kg daily for 5 days	Renal failure/low K+
Liposomal amphotericin	IV 3 mg/kg daily for 5 days	Renal failure/low K+
Paromomycin	IM 20 mg/kg daily for 21 days	

1.6 Transmission of leishmaniasis

The transmission of leishmaniasis depends on many different ecological and biological factors. Leishmania can only be transmitted, if suitable conditions are present for both the reservoir and the vectors. Only if the host animal and the sandfly species live close enough together, transmission of the parasite is possible.

Cutaneous leishmaniasis occurs either as a zoonotic or as an anthroponotic infection. The transmission of *Leishmania major* causing Old World cutaneous leishmaniasis in humans is a rural zoonotic disease with rodents serving as the reservoir. In zoonotic leishmaniasis, humans are only accidental hosts and usually not directly involved in the transmission cycle (Al-Jawabreh et al., 2008). New endemic foci of CL may occur, when environmental changes take place such as rise of population under poor housing conditions (slums) or specific forms of agriculture. In the Middle East, the fat sand rat (*Psammomys obesus*) is the main reservoir animal. In addition, jirds (*Meriones crassus*) have been found to host the parasite (Jaffe et al., 2004). In the arid regions of central Asia, Iran and Afghanistan, the great gerbil (*Rhombomys opimus*) is main reservoir of *L. major* (Wasserberg et al., 2002).

The main insect vector for transmission of *L. major* is the sand fly species *Phlebotomus papatasi* (EL- Badry et al., 2008). In Kenya, *L. tropica* has been isolated from hyraxes (procavia ssp. German: Klippschliefer) (Sang et al., 1993). Hyraxes are also the main reservoir of *L. aethiopica* in Ethiopia (Ashford et al., 1973).

In some urban centers of Middle East and Asia exist completely anthroponotic life cycles of the parasites, i.e. human beings are the main or only reservoir host. In such places cutaneous leishmaniasis caused by *L. tropica* can be highly endemic, but no animal reservoir is to be recognized. In Central and South Western Morocco, the transmission of *L. tropica* is anthroponotically as well. The parasite is mainly transmitted by sandflies of the species *Phlebotomus sergenti* (Ramaoui et al., 2008).

Prevention of infection

The best way to prevent leishmaniasis is by personal protection from sandfly bites, which includes for example long sleeve clothing in evening hours. The use of insecticides for house and space spraying reduce sandfly populations, and fine-weave pyrethroid-impregnated bednets have successfully been used in Burkina Faso, Sudan, and Columbia (Markle and Makhoul, 2004).

1.7 Introduction to Yemen

The Republic of Yemen – once ruled by the Queen of Sheba, and still in our days full of historical tresures from ancient times - is situated in the southwestern area of the Arabian peninsula. The country which covers about 460,000 km^2 is bordered by Saudi Arabia in the north, by the Arabian Sea and Indian Ocean the south, by the Red Sea in the west and by the Sultanate of Oman in the east (Figure 6)

Yemen is characterized by varied landscape, diversified terrain and climate. Overlooking the coastal plains are ranges of low and high altitude mountains, many of which are terraced. Throughout, Yemen maintains plateaus, hills and plains. These can be wide in some areas or narrow in others. Green wadis (or valleys) are

riverbeds for the rainwater runoff during the two rainy seasons in December to February and May to June. Desert extends eastward and northward. This natural terrain is broken down into the coastal region, the plateaus, the mountainous highlands and desert of the Empty Quarter.

Figure 6: Map of the Republic of Yemen

(source: http://www.maparchive.org/details.php?image_id=415

The most populated coastal area includes the lower plains overlooking the Red Sea, the Gulf of Aden and the Arabian Sea. The plains are interconnected to form a coastal strip starting with the Tehama (meaning hot land), which extends from Gizan in the North to Bad Al-Mandab Strait southwards. This region also includes a range of mountains, with an altitude ranging between 200 to 900 meters above sea level. It also encompasses the coastal plains tapering into the Gulf of Aden. The total length of this coastline reaches approximately 2,000 km and covers approximately 3000 km^2. Administratively, this area is divided in the governates Aden, parts of Hadhramaut and Al-Mahara.

The Plateaus Region in the East is reaching elevations as hihg as 1,000 meters is weakly populated and extends towards the Empty Quarter desert, with water flowing down from the mountains to wadis like famous Wadi Hadhramaut, which has been an essential centre of Arabian culture since the second millennium b.C. The peripheries of the Plateaus Region intertwine from the east with the rough terrain of Ramlat Al-Sabein and desert of Saada, Al-jawf, Shabwa, Hadhramaut and Al-Mahara.

The Mountain Highlands Region was formed as a result of the African split from Asia, which led to the cleavage forming the Red Sea and the Gulf of Aden. The altitude of Yemen's mountain range reaches between 1,000 to 3,600 meters above sea level. The summit of Jabal (Mount) Al-Nabi Shu'aib with an altitude of 3,666 meters and a snowy cap during winter time is the highest elevation in the Arabian Peninsula. At the foot of the highland are different flat basins and many wadis with land for agriculture and water reservoirs. Wadis flowing into the Gulf of Aden and the Arabian Sea include Wadi Tiban, Wadi Bana, Wadi Ahwar and Wadi Hadhramaut. Other wadis slope eastward towards the desert (Wadi Khabb, Wadi Al-Jawf and Wadi Adhanah) or towards the North and Northeast (Wadi Harib, Wadi Marakhah, Wadi Jirdan, Wadi Aiwah Al-Sai'ar, Wadi Rammah and Wadi Sha'at). Administratively, the Mountain Highlands Region comprises the Governorates of Raimah, Sana'a, Hajjah, Al-Mahweet, Sa'ada, Dhamar, Ibb, Ta'ez, AI-Beidha and Al-Dhali'a.

The almost unpopulated Empty Quarter represents the desert region of Yemen. It is intertwined with wild vegetation, especially in the peripheries that run into the Plateau Region through wadis and sand beds. Vegetative life and water get scarce inward of the Empty Quarter, whereas shifting sand dunes increase. These

dunes bury archeological sites and ruins. Former names of the Empty Quarter region were Rijraj Sea, the Safi Sea, the Great Desert and Al-Ahqaf Desert.

Climate

Yemen is a country with a high level of solar radiation all around the year. Diversity in topography provides varied climate. In the coastal areas, the climate is characterized by high temperatures and humidity during summer and moderate temperatures during winter. Moderate climate prevails in the western slopes, plateaus and flat land. Temperatures reach 10-30° C entigrade and may fall below 0° C in winter. Humidity can reach 80%. The annual average rainfall is 300 to 1,000 mm. The climate in the eastern parts of Yemen differs considerably with temperatures exceeding 40° C during the summer fall ing to 10°-15° C in the winter. Annual rainfall does not exceed 50-100 mm, especially in the periphery of the Empty Quarter. The transitory area is found between these two climatic regions. extending from north and east of Sana'a to the western parts of Marib. The entire area of Yemen is affected by monsoon winds blowing from the east due to low air pressures in the west. The monsoons are usually accompanied by rain in the summer and lesser rain in winter.

Population

The population of Yemen is 19.7 million people according to the population census of 2004. The annual population growth rate has declined from 3.7 % in 1994 to 3.0 %. This is seen as a major success in curbing population growth.

About 27 % of the population lives in major cities and 73 % live in rural areas. The movement of people is a decisive factor in the re-distribution of population among the different regions of Yemen. The rate of urban growth reaches 9 % in the Capital Secretariat (City of Sana'a). Sana’a includes about 28 % of the total urban population of the country followed by Hodeida and Aden with 16 % and 14 %.

With a per capita-income of only 450 Euro per year (in 2008), Yemen is by far the poorest country in Near and Middle East. More than two thirds of the inhabitants live from less than two US-Dollars per day. The health system of the country is underdeveloped, children and mother mortality are high, and life expectancy is low.

Medical installations and qualified personell are limited and the population, especially women and children, suffer from deficiencies of medical care. Under these circumstances, there exist no much information on specific diseases such as leishmaniasis.

1.8 Leishmaniasis in Yemen

There are very few reports on leishmaniasis in Yemen in the international literature. Even though it is not well documented, the disease seems to be endemic in the country, and is primarily widespread in arid and semiarid areas. It is also endemic in in the plateau and mountainous areas of Hajjah and Amran governorates. Cutaneous leishmaniasis has first been reported in Sana'a as early as 1933 (Sarnelli 1933). In 1986, *Leishmania tropica* was identified in 18 patients in Naghdi Dhamran and Wadi Dhamran (Rioux 1986). Another study revealed 42 cases of cutaneous leishmaniasis in the Hajjah governorate and neighboring regions (reviewed by Khatri et al., 2006). *Leishmania major* as well as *Leishmania tropica* account for the majority of infections in Yemen. Pratlong et al. (1995) reported from a patient who developed localized cutaneous lesions due to *Leishmania donovani sensu stricto*, following a stay in Yemen. The patient lived in southern France and visited Yemen for 2 weeks during November 1992 when he was bitten by sandflies. He had not traveled elsewhere in the previous 3 years. Studies of Leishmania strains in Yemen and in countries close to Yemen suggest that coutaneous leishmaniasis is mainly caused by *L. major* and *L. tropica,* whereas the agent of visceral leishmaniasis is *L. infantum* (Boyer, 1993; Piarroux et al., 1995; Khatri et al., 2006; Nasereddin et al., 2009)

1.9 Diagnosis of Leishmaniasis

Parasitological diagnosis is considered to be golden standard in diagnosis of leishmaniasis because of its high specificity. It includes microscopic examination of Gimsa- stained smears derived from biopsies of cutaneous lesions, or aspirates from lymph nodes, bone marrow, or spleen. Histopathological examination of fixed lesion biopsies or culture of biopsies is also performed. Microscopic examination is still a widespread method in endemic countries, because it is quite inexpensive and does not require sophisticated laboratory equipment. In contrast, culture in combination

with multilocus enzyme electrophoresis allowing identification of the parasite species and leading to definite diagnosis requires a wealth of technical training and takes long time to obtain results. Disadvantages of microscopy and culture are the low sensitivity, especially in the chronic stage of the disease when the number of parasites decreases (Reithinger, 2007).

Serological methods

Indirect diagnostical methods include the relatively sensitive, but not highly specific diagnostic procedure is the Leishmanin skin test (LST) also known as the Montenegro reaction (Montenegro, 1926). Leishmanial antigen (killed promastigotes), or disrupted promastigotes in pyrogen – free phenol saline - is applied intradermally and a delayed hypersensitivity reaction can be observed. A cross-reaction has been reported with tuberculosis. Leishmanin skin test is used as an indicator of the distribution of cutaneous and mucocutaneous leishmaniasis in both humans and animals, in visceral leishmaniasis usually only after treatment and cure (Sadeghian et al., 2006).

Most sensitive and specific test especially for the diagnosis of visceral leishmaniasis include serological methods such as the enzyme linked immunosorbent assay (ELISA). Since few years, a highly specific recombinant antigen, K39, which is part of a kinesin-related gene and contains a repetition of 39 amino acid residues has been developed and is being widely used for diagnosis meanwhile. K39-ELISA was found to be more sensitive and specific than immunoassays including crude perasite extracts such as the direct agglutination test (DAT) and soluble antigen (SA) ELISA used before. In India, recombinant K39 has been widely used for detecting visceral leishmaniasis even in immunocompromised patients with VL-HIV co-infection (Singh, 2006).

Western blots have been used as a diagnostic tool to identify by anti-leishmanial antibodies by *Leishmania* antigens. In the western blot technique several antigenic products can be distinguish by different antibodies in one assay. Before introduction of recombinant K39, the western blot technique proved to be the most sensitive and specific serological methods for diagnosis of leishmaniasis. The development of the rk39 dipstick test it is successfully employed in the diagnosis of

VL as will as PKDL in India. It was found to be 95-100 % sensitive when tested in VL Patients. (Salotra et al., 2003)

The direct agglutination test (DAT) is a comparatively simple and inexpensive test suitable for field work and for screening patient sera in the laboratory (Harith et al., 1986). The method is based on the agglutination of stained promastigotes available either as a suspension or in a freeze- dried form with *Leishmania*-specific antibodies. The freeze-dried form is more stable and improves the usefulness of the DAT in the field. The sensitivity of the test is almost 100 %, and the specificity for leishmaniasis is equally high except of countries with Chagas disease, where crossreactivity with *Trypanosoma cruzi* is being observed (El Harith et al., 1988).

Molecular methods

The polymerase chain reaction (PCR) has offered a new dimension in the diagnosis and characterization of various infectious agents, enabling to perform tests in few hours. It proved to be very useful for the diagnosis of leishmaniasis. In recent studies many different PCR assays have been developed for the diagnosis of *Leishmania* DNA in a variety of clinical samples such as peripheral blood, skin biopsies, bone marrow and lymph node aspirates. In several studies comparison of PCR was found to be more sensitive than microscopy and culture for diagnosis of VL, especially in immune- compromised patients. Sensitivities between 80 and 90 % have been reported which is markedly better than microscopy and culture (approximately 55 % sensitivity each). Different targets have been chosen for the PCR, multicopy sequence repeats such as genes for ribosomal RNAs and kinetoplast DNA are usually prefered (Schallig and Oskam, 2002). The genes of the small subunit ribosomal RNA (SSUrRNA) consist of both conserved and variable regions which are almost species-specific and can be used for the development of specific diagnostic approaches. The kinetoplast DNA (kDNA) contains the most highly repetitive sequences in *Leishmania*. It consists of approximately 10.000 copies of plasmid-like minicircles of different size, but all of them share a conserved region of 120-140 bp. This region contains two short highly conserved sequences which are perfectly homologous in all *Leishmania* species. This fact has been utilized for a genus-specific diagnostic PCR approach (Review: Henk et al., 2002).

1.10 Objective of the study

The objective of this study was to shed some light in the epidemiology of leishmaniasis in the Republic of Yemen and to develop simple molecular approaches for the detection of the disease under the limited economical conditions of the country. The official data on leishmaniasis provided by the government are incomplete and may only indicate the peak of an iceberg of under-reported cases.

Diagnosis of cutaneous leishmaniasis in Yemen is based solely on microscopic examination, which yields a good sensitivity in acute infection but is of limited use in the chronic phase of the disesase when the number of parasites in biopsy samples decreases. Diagnosis of visceral leishmaniasis by microscopic examination of spleen aspirates is rarely used because it is technically demanding and needs trained personell. Instead, a formol gel test is wildly used for the diagnosis of visceral leishmaniasis because it is very cheap, but the specificity reaches hardly 50 %. In order to get a more detailed aspect on the epidemiology of leishmaniasis in Yemen and to contribute to a more specific and sensitive diagnosis, the following issues were addressed:

- To collect information of incidence of leishmaniasis in different parts of Yemen.
- To collect information on control measures and treatment of leishmaniasis in Yemen.
- To take blood and tissue samples from patients suspected to have cutaneous or visceral leishmaniasis.
- To analyse the tissue samples of CL patients (Giemsa-stained smears from the wound) by microscopy in Yemen.
- To harvest lymphozytes from the blood samples and extract DNA for later PCR analysis.
- To prepare serum (or plasma) from the blood samples for later immunological analysis.
- To establish affordable procedures for molecular diagnosis of human leishmaniasis in the laboratory in Giessen by designing primers which lead to high specificity and sensitivity in the PCR assays. These procedures had to

bo be simple, easy to perform and inexpensive in order to make their use realistic in a poor country.

- To increase sensitivity and specificty of diagnostic tests by developping nested PCR assays.
- To transfer the new molecular technology to Yemen in order to improve diagnosis of the disease in the country.

2 MATERIALS and METHODS

2.1 Instruments

Gel electrophoresis systems

Horizontal minigel system (8 x 8 cm)	AGS, Heidelberg, Germany
Vertical minigel chamber (8 x 10 cm)	Keutz, Reiskirchen, Germany
Power supply EPS 500/400	Pharmacia, Freiburg, Germany

Shakers

Certomat R	Braun, Melsungen, Germany
Vortex Genie 2	Scientific Industries, Bohemia, NY, USA

Centrifuges

Cooling centrifuge Beckman J2-21,	Beckman Instruments, Summerset, USA
Microfuge: Biofuge Pico	Heraeus Instruments, Hanau, Germany
Multifuge 3	Heraeus Instruments, Hanau, Germany

Waterbath

GFL Wasserbad 1013	Gesellschaft für Labortechnik, Burgwedel, Germany

Thermocyclers

T1 Thermocycler 96	Biometra GmbH, Göttingen, Germany
Primus 96	MWG Biotech AG, Ebersberg, Germany

Photographic equipment

UV Transilluminator	Herolab GmbH, Wiesloch, Germany
Polaroid MP-4 Land Camera	Polaroid Corporation, Cambridge, MA, USA
Film**:** Polaroid 667 Professional	Polaroid Corporation, Cambridge, MA, USA

2.2 Chemicals

Acrylamid	Serva, Heidelberg, Germany
Agarose for gel electrophoresis	Sigma-Aldrich, Munich, Germany
Ammoniumpersulfate (APS)	Serva, Heidelberg, Germany
Ammoniumsulfate	Carl Roth GmbH, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich, Munich, Germany

Adenil Triphosphate (ATP)	Carl Roth GmbH, Karlsruhe, Germany
Biorex 70 resin	BIO-RAD Laboratories, Hercules, USA
Coomassie Brilliant Blue R250	Serva, Heidelberg, Germany
Dithiothreitol (DTT)	Biomol, Heidelberg, Germany
dNTP-Set (100 mM per dNTP)	Carl Roth, Karlsruhe, Germany
Ethidium bromide	Serva, Heidelberg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Serva, Heidelberg, Germany
Fetal calf serum	Sigma-Aldrich, München, Germany
Glutamine	Carl Roth, Karlsruhe, Germany
Guanidiniumhydrochlorid (GuHCl)	ICN Biomedicals, Germany
Heparin sepharose	Pharmacia-LKB, Upsala, Sweden
Medium 199	Sigma-Aldrich, München, Germany
ß-Mercaptoethanol	Serva, Heidelberg, Germany
MEM Earle's Medium	Gibco BRL, Eggenstein
N,N,N',N'-Tetramethylethylendiamine (TEMED)	Serva, Heidelberg, Germany
Oligonucleotides	MWG-Biotech, Ebersberg, Germany
Phenylmethylsulphonylfluoride (PMSF)	Carl Roth GmbH, Karlsruhe, Germany
Penicillin/Streptomycin	Carl Roth GmbH, Karlsruhe, Germany
Polyethyleneimine	Sigma-Aldrich, München, Germany
Tween-20	Serva, Heidelberg, Germany
Triton X-100	Merck, Darmstadt, Germany
Tris (hydroxymethyl) aminomethane	Carl Roth GmbH, Karlsruhe, Germany

2.3 Leishmania parasites

L. major and *L. infantum* were obtained from Prof. Bernhardt Fleischer, Bernhard-Nocht-Institut, Hamburg. They were cultivated in vitro using either medium 199 plus 10 % heat-inactivated fetal calf serum, or Earle's medium supplemented with

glutamine and 10 % heat-inactivated fetal calf serum at 30℃ in the presence of 5 % CO_2. A mixture of penicilline and streptomycin (from 100 x penicillin/streptomycin stock solution) was added to prevent bacterial contaminations. After 3-4 days, parasites were transfered in 3 volumes of fresh medium and further incubated. The parasites were harvested by centrifugation for 20 min at 2.000 x g and frozen.

2.4 Patient samples

Blood samples were collected from approximately 150 patients visiting hospitals in six different regions in north Yemen and the central blood bank in Sana'a with lesions suspected to be cutaneous leishmaniasis over a period of more than two years. Skin scrapings were taken from the borders of the wound and analysed microscopically. As this kind of diagnosis has a limited sensitivity (approximately 70 %), all blood samples were kept and processed as described below for further analysis by molecular methods lateron. Most of the examined patients came from endemic areas (urban as well as rural), had been travelling inside the country, or had been stationed as soldiers in the desert area. Blood samples from 26 patients with suspected visceral leishmaniasis were collected in several locations in the western part of Yemen.

3-5 ml of collected blood were processed immediately by sedimenting the blood cells using a manual centrifuge (see Figure 10 in the Result section). Plasma and buffy coat were collected as described below. Plasma was conserved by the addition of 50 % glycerol, and the DNA of the purified buffy coat was stabilized in 500 µl of guanidinium-hydrochloride lysis buffer. Whenever possible, the samples were kept at 4℃, however, they were transported for several days at ambient temperatures without loss of biological activity.

Microscopical analysis of skin scrapings

Small quantities of tissue obtained by skin scrapings were smeared on glass slides, air dried and fixed with methanol for a few seconds. Giemsa stain was filtered and diluted 1:20 with phosphate buffer (pH 7.2). After 20 minutes of staining the slides were washed with tap water and air dried. The stained smears were examined under the microscope with a 40 x lens and with a 100 x oil immersion lens. If at least one intra- or extra-cellular amastigote with a distinctive kinetoplast was found the

smear was declared positive. When no amastigotes were seen after 15 minutes of inspection, the smear was declared negative. Many of the patient smears were double checked, the observations were in concordance. All samples collected during visits six different region and central blood bank were stained and examined in Yemen.

2.5 DNA purification

GuHCl lysis buffer (DNA extraction buffer)

5 M Guanidinum hydrochloride (GuHCl)
40 mM Tris/HCl pH 7.0
20 mM EDTA
1 % Tween 20

GuHCl lysis buffer
24 g GuHCl
2 ml 1 M Tris/HCl pH 7.0
2 ml of 0.5 M EDTA
0.5 ml Tween 20

Ethanol washing buffer

70 % ethanol
10 mM TrisHCl pH 7.0

Ethanol washing buffer
70 ml ethanol
1 ml 1 M Tris/HCl pH 7.0
29 ml H_2O

Silica suspension for purification of nucleic acids

50 g of silica (SiO_2) powder (Sigma No. S 5631) were suspended in 250 ml of water, centrifuged for 3 min at 5000 x g, the milky supernatant was discarded, the sediment resuspended in another 250 ml of water and centrifuged again. The procedure was repeated 3-5 times more until supernatant became clear. The sediment was suspended in one volume of water and kept in 2 ml aliquots at -20℃.

Isolation of DNA using guanidinium-hydrochloride/silica

The following procedure is appropriate to isolate total DNA from cultivated *Leishmania* parasites (10 to 20 mg). 500 µl of guanidinum lysis buffer were added to 10-20 µl sedimented parasites, vortexed and incubated for 10 min at 65℃. Thereafter the solution was vortexed vigorously again to reduce viscosity. After 3 min centrifugation at 13.000 rpm in a minifuge, the supernatant was transferred to a new tube and 10-20 µl of silica suspension were added, mixed and incubated for 5 min at room temperature. The sample was centrifuged for 30 s at 13.000 rpm, the

pellet resuspended in 500 µl 70 % ethanol washing buffer, centrifuged for 30 s at 13.000 rpm and the supernatant removed. The pellet was washed in the same way three more times with ethanol washing buffer. The last pellet was centrifuged again and residual ethanol was removed. 10-20 µl of TE buffer (10 mM Tris/HCl pH 7.5, 0.1 mM EDTA) were added, incubated for 5 min at 55°C and centrifuged for 1 min at 13.000 rpm. The supernatant was collected and the pellet eluted with another 10-20 µl TE buffer. The supernatants were combined.

DNA of *Trypanosoma cruzi* was a kind gift of Prof. Bernhardt Fleischer, Bernhard-Nocht-Institut, Hamburg.

DNA extraction from buffy coat

2-3 ml of citrate or EDTA-blood were centrifuged for 15 minutes at 1000 x g. Then, supernatant plasma was collected and kept in the refrigerator. The yellow thin "buffy coat" layer on top of the sedimented erythrocytes was collected with a pipette (200 to 300 µl) and transferred to an Eppendorf tube. 1.2 ml of RBC lysis buffer (300 mM NH_4Cl, 30 mM NH_4HCO_3, 30 mM KCl. 0.1 mM EDTA) were added, mixed shortly by vortexing, incubated 10 minutes at room temperatures, inverting the tube several times. Then, the tube was centrifuged 2 minutes at 2000 x g, and the supernatant removed. A pellet of 10-30 µl was obtained and mixed with DNA extraction buffer. DNA was extracted as described above.

2.6 Purification of recombinant Taq DNA polymerase

Taq DNA polymerase was purified from a recombinant strain of *E. coli* (*E. coli* XL-1 Blue/pQE-Taq) expressing an exonuclease-free mutant of the enzyme. The strain was constructed previously in the laboratory of Prof. E. Beck, University of Giessen (unpublished results). The thermostable enzyme can also be purified without the cooling steps described in the following protocol.

1 litre of LB medium containing 25 µg/ml kanamycin and 50 µg/ml ampicillin was inoculated with 100 ml of an over night culture of XL-1 Blue pQE-Taq cells. The cells were grown at 37°C with optimal aeration for 1-2 hours. At an OD_{600} of 1.8, 1 ml of 1 M IPTG was added and then the culture vigorously shaked for another 4 hours. Cells were harvested by centrifugation (yield ~5 g) and frozen at -20°C. Freezing of

the cells can be omitted, but is recommended if the enzyme preparation is not performed immediately. The cells were resuspended in 15 ml TMN buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 20 mM $(NH_4)_2SO_4$) together with 8 mg of lysozyme (from 10 mg/ml stock solution) and incubated for 15 min at 20-25°C. After adding 200 µl of 10 % Triton X-100 and 200 µl of 10 % Tween 20, the culture was mixed and incubated at 80°C for 20 min in a 50 ml screw-cap Falcon tube. After the heating step, 250 µl of 100 mM PMSF were added to prevent proteolytic degradation.

DNA was sheared by 1 min sonification (it is also possible to do it by repeated pressing through a syringe first with a 1 mm diameter needle and then with smaller needles) and centrifuged 15 min at 20.000 x g at 4°C. The supernatant (~15 ml) was collected and 0.6 ml of 5 M NaCl (0.2 M final concentration) were added.

The DNA was precipitated with polyethyleneimine (PEI) by adding dropwise 500 µl of 5 % PEI solution, mixing and incubating in ice for 10 min. The sample was centrifuged at 10.000 x g for 5 min. 4 aliquots of 500 µl of supernatant were transferred to Eppendorf tubes each and mixed with increasing amounts (2-8 µl) of 5 % PEI solution. They were incubated 5 min on ice, centrifuged and the amount of pellet compared. The minimal amount of PEI to precipitate DNA quantitatively was determinated and added to the bulk extract (usually 100-200 µl; use 1/100 volume of 5 % PEI solution in excess). The suspension was left 20 min on ice and centrifuged at 20.000 x g for 20 min. The supernatant was collected and diluted 6 fold with KTA buffer (20 mM Tris/HCl pH 8.5, 10 mM beta-mercaptoethanol, 10 % (w/v) glycerol, 0.1 mM EDTA, 0.05 % Triton X 100, 0.05 % Tween 20).

The excess of PEI was removed by passing the extract through a 2 ml BioRex 70 column equilibrated in KTA buffer + 30 mM ammonium sulphate. A disposable 4 ml plastic column (International Sorbent Technology, Hengoed, Mid Glamorgan, UK) was used. The column was rinsed with 2 ml KTA buffer and the flow-through loaded on another plastic column containing 2 ml heparin sepharose equilibrated with KTA buffer, 30 mM ammonium sulphate. The column was washed first with 50 ml KTA buffer, 40 mM ammonium sulphate. Thereafter, the column was washed with 20 ml KTA buffer, 40 mM ammonium sulphate, 50 % glycerol. Taq DNA polymerase was eluted with KTA buffer, 150 mM ammonium sulphate, 50 % glycerol. 0.5 ml fractions were collected and 3 µl aliquots analysed on a 12.5 % SDS polyacrylamide gel. The enzyme was found in fractions 4 to 7. The enzyme was stored at -20°C. For long

term storage, Tween 20 to 1 % final concentration was added. The yield was approximately 30.000 units of enzyme at a concentration of 80 units/µl in the peak fraction.

2.7 Polymerase chain reaction (PCR)

For all PCR assays, the same Taq DNA polymerase buffer was used. It is prepared as a 10 x buffer and has the following composition:

1 x Taq polymerase buffer

40 mM Tricine-KOH (pH 8.7 at 25℃)
15 mM K acetate
3.5 mM acetate
0.1 % gelatine
0.05 % Tween 20

10 x Taq polymerase buffer (10 ml)
4 ml 1 M Tricine-KOH
1.5 ml 1 M K acetate
350 µl 1 M acetate
2.5 ml 4 % heated gelatine solution
500 µl Tween 20
1.15 ml H_2O

Standard PCR reaction assay

Using efficient standard thermocyclers such as T1 thermocycler 96 from Biometra, or Primus 96 from MWG Biotech which are appropriate for 200 µl reaction tubes, have a heated lid and heating/cooling rates of at least 2-3℃/s, highly reproducible results were obtained with assays as small as 10 µl. A standard PCR reaction mixture had the following composition:

1 µl 10 x Taq buffer
1 µl mixture of 4 dNTPs (2.5 mM each)
0.5 µl 10 µM forward primer
0.5 µl 10 µM reverse primer
1 µl template DNA
5.8 µl H_2O
0.2 µl Taq DNA polymerase (5 units/µl)
10.0 µl

The standard program of the thermocycler was set as follows:

step 1: 2 min at 94℃ (denaturing of DNA templat e)

step 2: 35 cycles of [94℃ for 20 s, 60℃ for 1 min, 72℃ for 1 min]

step 3: 5 min at 72℃ (optional, to fill-in the DNA ends completely)

The same conditions were applied for nested PCR. 1 µl of a 1:10 dilution (with H_2O) of the result of first PCR assay was used as DNA template.

Avoiding contamination

The sensitivity of PCR diagnosis is very high. Theoretically DNA from less than one parasite can be detected. This implied a high risk for contamination, which had to be strictly avoided. As a general rule, the extraction of DNA, the preparation of the PCR and electrophoresis of the PCR products should be strictly separated. It is also recommended to use different sets of pipettes for the different parts of the work. Due to limitations of space and technical equipment this standard could not always be met. Instead, extra precautions had to be taken.

- DNA extraction from clinical material and preparation of the PCR was performed in a hood, located in a small room separated from the rest of the laboratory. PCR-cycling and gel electrophoresis were performed on an extra bench.
- All solutions used for DNA extractions were divided in small aliquots from a stock solution and discarded in case of a suspected contamination
- An extra pipette (“dirty pipette”) was used exclusively for loading of the gels with the higly amplified PCR products.
- Filter tips were used for DNA extraction and for all pipetting steps for PCR.
- Gloves were changed during the procedures from time to time.
- The rotor of the micro-centrifuge was washed prior to DNA extractions since the tops of the 1.5 ml tubes came in contact with the upper margin of the cups holding the tubes. Since also highly amplified DNA

was centrifuged in the same centrifuge, these cups were assumed to be a source of contamination.

- Whenever possible 200 µl tips were used instead of 10 µl tips. Due to the length of the 200 µl tips the shaft of the pipette could not touch the 1.5 ml tubes from inside.

Negative controls

The extraction of DNA as well as the PCR had to be monitored strictly for possible cntamination. Two negative extraction controls were routinely used for every extraction series. The PCR itself was monitored by one reaction in every PCR, which contained only the reagents and no template.

2.8 DNA gel electrophoresis

Agarose gel electrophoresis buffer (E-buffer)

40 mM Tris/acetate (pH 8,0)
40 mM Na acetate
2.0 mM EDTA

20 x E-buffer
193.8 g Tris-base
131.2 g Na acetate
160 ml 0.5 M EDTA
adjust pH 8.3 with acetic acid
add H_2O to 2 litre

Acrylamide gel electrophoresis buffer (TBE-buffer)

90 mM Tris/borate pH 8.3
2.5 mM EDTA

10 x TBE-buffer
108 g Tris-OH
55 g boric acid
40 ml 0.5M EDTA
adjust pH 8.0 with acetic acid
add H_2O to 1 litre

Loading buffer

20 mM Tris/HCl pH 7.5

50 % glycerol

0.02 % bromphenol blue

0.02 % xylene cyanol blue

20 mM EDTA

Loading buffer
0.2 ml 1 M Tris/HCl pH 7.5
5 ml glycerol
0.2 ml 10 mg/ml bromphenol blue
0.2 ml 10 mg/ml xylene cyanol blue
0.4 ml 0.5 M EDTA

Ethidium bromide staining solution

1 µg ethidium bromide / 100 ml E-buffer

Staining solution
400 ml 1 x E-buffer
40 µl 10 mg/ml ethidium bromide solution

DNA size marker

:

Plasmid pSP 64 was cleaved a) with Hind III, b) with Dra I and c) with Hinf I. The individual cleavage assays were mixed in the relation 1:2:4 with respect to the amount of plasmid DNA, resulting in the fragment sizes indicated in the figure (in basepairs)

Agarose gels

Agarose gel electrophoresis was performed according to standard procedures (Sambrook et al. 1989). Plasmid DNA and PCR fragments were analysed on horizontal 1 % or 2 % agarose gels using a Tris/acetate electrophoresis buffer (E-buffer). To check migration of the DNA fragments, the samples were mixed with 20 % loading buffer containing 50 % glycerol and marker dyes. In 1 % agarose gels,

bromphenol blue migrates at the position of 200 bp, whereas xylenecyanol blue migrates at the position of 300 bp. The gels were stained for 20 min with ethidium bromide staining solution. Then they were rinsed with tape water, the DNA bands visualised on an UV transilluminator and photographed by a Polaroid camera. Alternatively, a simple digital camera in combination with a yellow filter was used.

Polyacrylamide gels

For the analysis of proteins, discontinuous polyacrylamide gels (10 x 8 x 0.1 cm) containing SDS (Lämmli, 1970) were prepared in different concentrations according to the following recipe:

	Stacking gel	**Separating gel**	
	6 %	12,5 %	15 %
40 % Acrylamide / 1.3 % bisacrylamide solution	1,5 ml	5,0 ml	6,0 ml
4 x buffer	2,5 ml	4,0 ml	4,0 ml
H_2O	6,0 ml	7,0 ml	6,0 ml
Ammonium persulphate	10 µg	10 µg	10 µg
TEMED	15 µl	20 µl	20 µl
	10 ml	**16 ml**	**16 ml**

Stacking gel buffer:

125 mM Tris-HCl (pH 6.8)

0.1 % SDS

4 x Stacking gel buffer
12.14g Tris-OH
8 ml 10% SDS
adjust pH 6.8 with HCl (~8 ml HCl 37%)
add H_2O to 200 ml

Separating gel buffer:

375 mM Tris-HCl (pH 8.8)

0.1 % SDS

4 x Separating gel buffer
45.43 g Tris-OH
10 ml 10% SDS
adjust pH 8.8 with HCl (~6 ml HCl 37 %)
add H_2O to 250 ml

3 RESULTS

3.1 Evaluation of epidemiological data on leishmaniasis in Yemen

Official data on the prevalence rate of leishmaniasis in Yemen have been obtained from the Ministry of Health and from the Office of Surveillance of Disease in the capital Sana'a. However, these data have to be regarded with care, as they include only infections reported from governmental hospitals, the two biggest ones located in Sana'a and Taizz. Much of the medical treatment for the population is managed in private clinics, or by non-governmental organisations which do not report to the central administration of the country. Furthermore, in the absence of any support for health care, poor people do not seek medical aid for small wounds such as the ulcers of cutaneous leishmaniasis. Therefore, the real number of Leishmania infections is most probably by far higher than officially documented. The data obtained from the Ministry of health for the years 2005 to 2008 are shown in Table 4.

Table 4 : Leishmaniasis in the diferent governates of Yemen in 2005-2008

2005 Governate	Rimah	Abyan	Ibb	Sana'a city	Almahra	Hadarmot	Aldhale	Aden	Amran	Alhudaidah	Albaidah	Algawf	Marib	Dhamar	Lahj	Taiz	Sadah	Shabwah	Almahweet	Sana'a	Hajjah	total
number of patients	58	0	15	247	0	279	5	1	62	0	18	90	19	12	4	53	13	16	10	17	72	991
male	40	0	12	201	0	240	5	1	45	0	14	75	14	8	4	45	10	12	7	12	60	805
female	18	0	3	46	0	39	0	0	7	0	4	15	5	4	0	8	3	4	3	5	12	176
cutaneous leishmaniasis	55	0	14	227	0	249	5	1	60	0	18	90	19	11	4	51	13	16	9	17	65	924
visceral leishmaniasis	3	0	1	20	0	0	0	0	2	0	0	0	0	1	0	2	0	0	1	0	7	37

2006 Governate	Rimah	Abyan	Ibb	Sana'a city	Almahra	Hadarmot	Aldhale	Aden	Amran	Alhudaidah	Albaidah	Algawf	Marib	Dhamar	Lahj	Taiz	Sadah	Shabwah	Almahweet	Sana'a	Hajjah	total
number of patients	642	0	81	230	0	153	12	5	76	44	5	242	91	57	85	130	43	3	0	17	54	1970
male	480	0	60	160	0	96	7	0	46	27	5	220	72	45	70	110	37	3	0	12	45	1495
female	162	0	21	70	0	57	5	5	30	17	0	22	19	12	15	20	6	0	0	5	9	475
cutaneous leishmaniasis	605	0	77	220	0	153	12	5	70	44	5	242	91	57	85	125	43	3	0	12	50	1899
visceral leishmaniasis	37	0	4	10	0	0	0	0	6	0	0	0	0	0	0	5	0	0	0	0	4	66

Table 4 continued

2007 Governate	Rimah	Abyan	Ibb	Sana'a city	Almahra	Hadarmot	Aldhale	Aden	Amran	Alhudaidah	Albaidah	Algawf	Marib	Dhamar	Lahj	Taiz	Sadah	Shabwah	Almahweet	Sana'a	Hajjah	total
number of patients	65	1	51	343	0	74	15	1	44	9	2	20	89	61	37	303	31	0	4	0	29	1179
male	55	1	47	320	0	65	12	1	35	6	2	16	81	55	33	370	28	0	4	0	25	1156
female	10	0	4	23	0	9	3	0	9	3	0	4	8	6	4	33	3	0	0	0	4	123
cutaneous leishmaniasis	58	1	48	327	0	74	15	1	44	9	2	20	89	58	37	293	31	0	4	0	26	1137
visceral leishmaniasis	7	0	3	16	0	0	0	0	0	0	0	0	0	3	0	10	0	0	0	0	3	42

2008 Governate	Rimah	Abyan	Ibb	Sana'a city	Almahra	Hadarmot	Aldhale	Aden	Amran	Alhudaidah	Albaidah	Algawf	Marib	Dhamar	Lahj	Taiz	Sadah	Shabwah	Almahweet	Sana'a	Hajjah	total
number of patients	70	0	6	367	2	120	13	7	32	0	7	25	89	40	110	105	0	5	85	32	45	1160
male	62	0	4	350	2	109	10	6	24	0	5	22	81	32	98	92	0	5	78	25	35	1040
female	8	0	2	17	0	11	3	1	8	0	2	3	8	8	12	13	0	0	7	7	10	120
cutaneous leishmaniasis	60	0	5	355	2	120	13	7	32	0	7	25	89	35	110	105	0	5	82	32	36	1120
visceral leishmaniasis	10	0	1	12	0	0	0	0	0	0	0	0	0	5	0	0	0	0	3	0	9	40

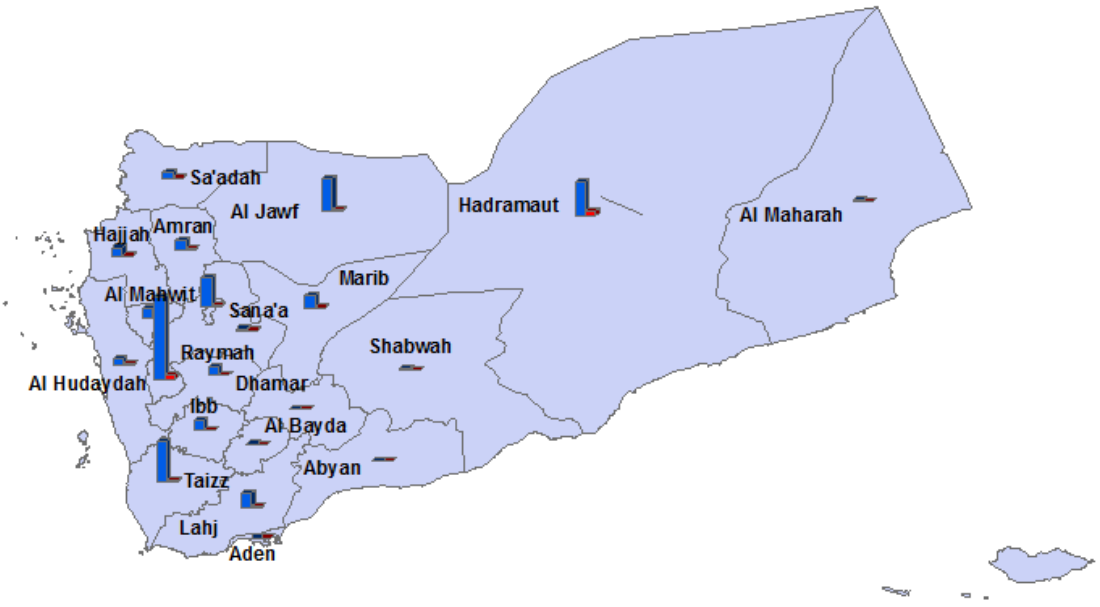

Figure 7: Distribution of Leishmaniasis in the different governates of Yemen

Visceral leishmaniasis Cutaneous leishmaniasis

The location of the different governates and the distribution of leishmaniasis as listed in Table 3 is shown in Figure 7. Even though the information on leishmaniasis is limited and the actual incidence of the disease is probably much higher, some intersting conclusions can be drawn from these data. One aspect is the relation between the cutaneous and the visceral form of the disease. Only 3.3 % of the infected patients suffer from visceral leishmaniasis whereas the majority of cases correspond to the cutaneous form of the disease as graphically shown in Figure 7.

The relation of female patients versus male patients is also striking and differs markedly form other endemic countries where there is no difference between the genders. This may be explained by the fact that traditionally women protect their bodies – including the face - completely with clothes when they are outside of doors, whereas men are less covered and can be easier be attacked by sand flies. The major sites of ulcers due to *Leishmania* are typically face and legs (see Figure 8). Another explanation is the shyness of women to visit male doctors, and there are very few female doctors in the country.

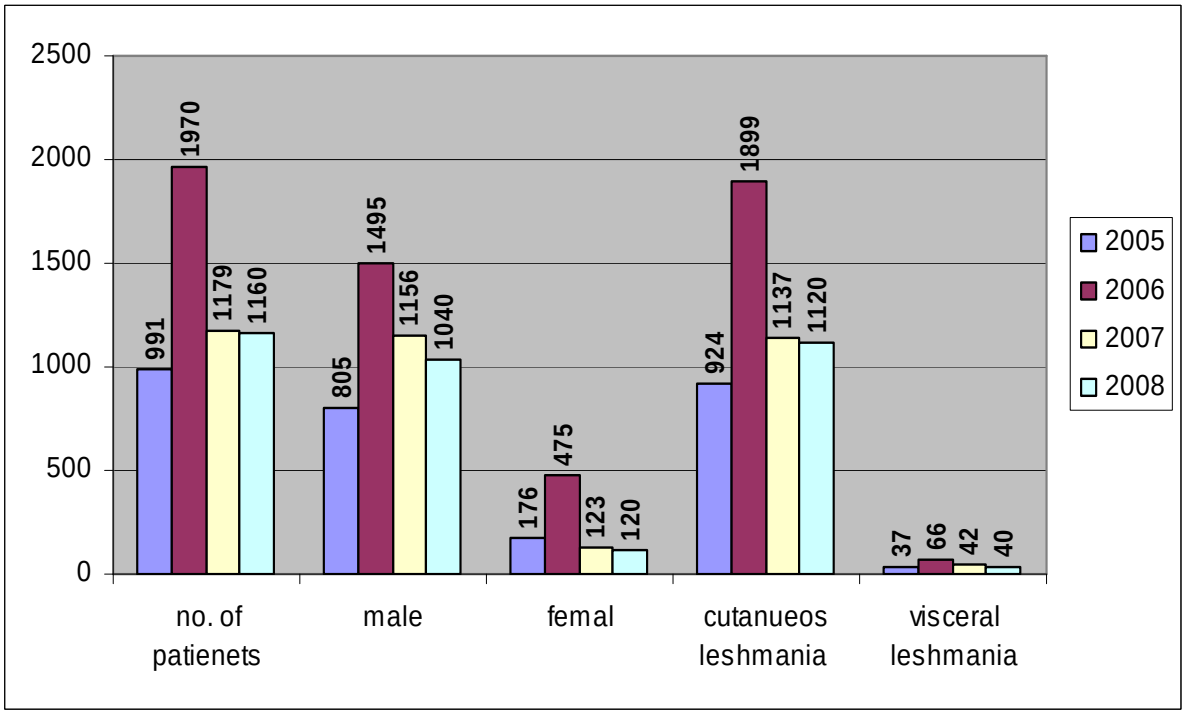

Figure 8: Frequency of leishmaniasis in Yemen during the years 2005 – 2008

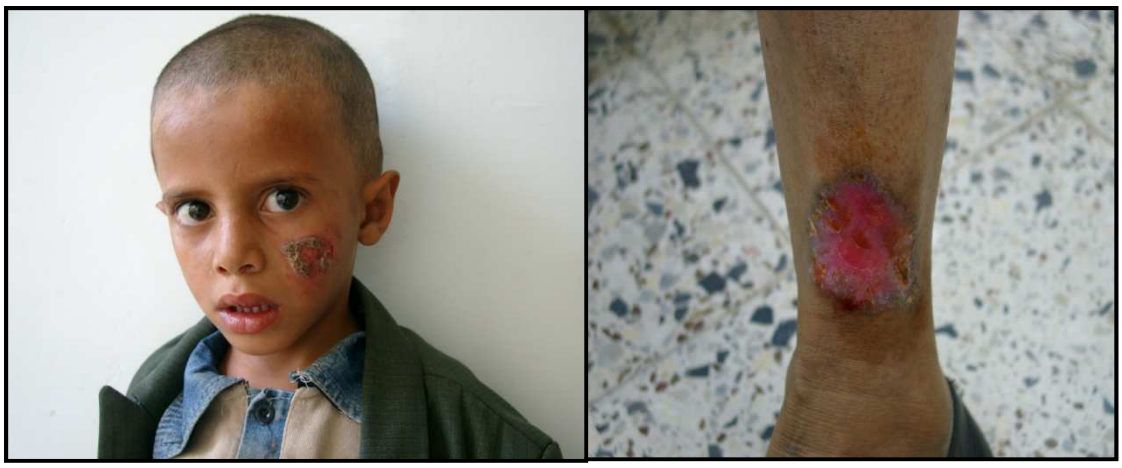

Figure 9: Cutaneous leishmaniasis. Typical sites of ulcers are face and legs which are usually most exposed to bite of sandflies.

Another interesting aspect is the number of cases of leishmaniasis during the different years. In 2006, there was a high increase of the disease as compared to the other years. This may be explained by the climate, more specifically by the amount of rainfall which is needed for breeding of the phlebotomine vectors. There are no measures to control sandflies in Yemen.

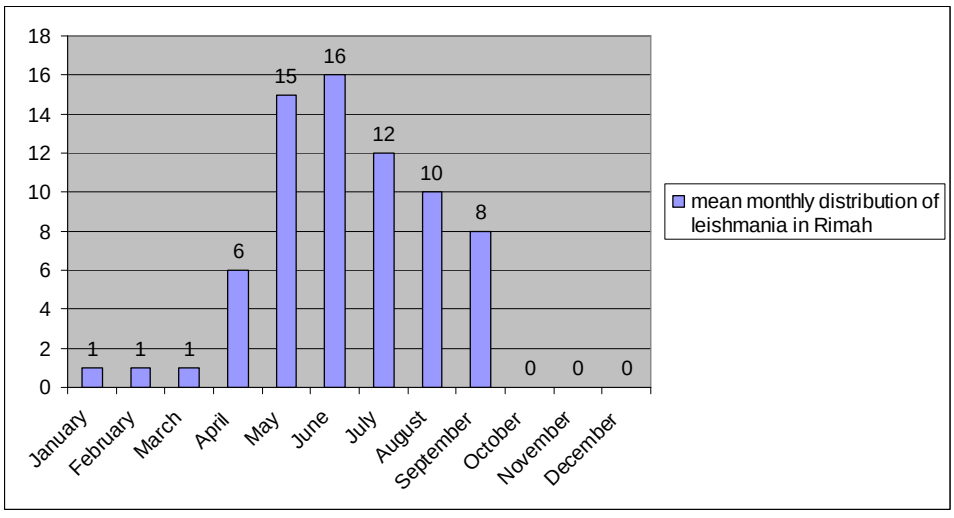

Figure 10: Monthly distribution of leishmaniasis Rimah (Western Yemen)

In the fertile areas in the western part of Yemen, the majority of patients with leishmaniasis are registerd in the middle of summer as exemplified by Figures 10and 11.

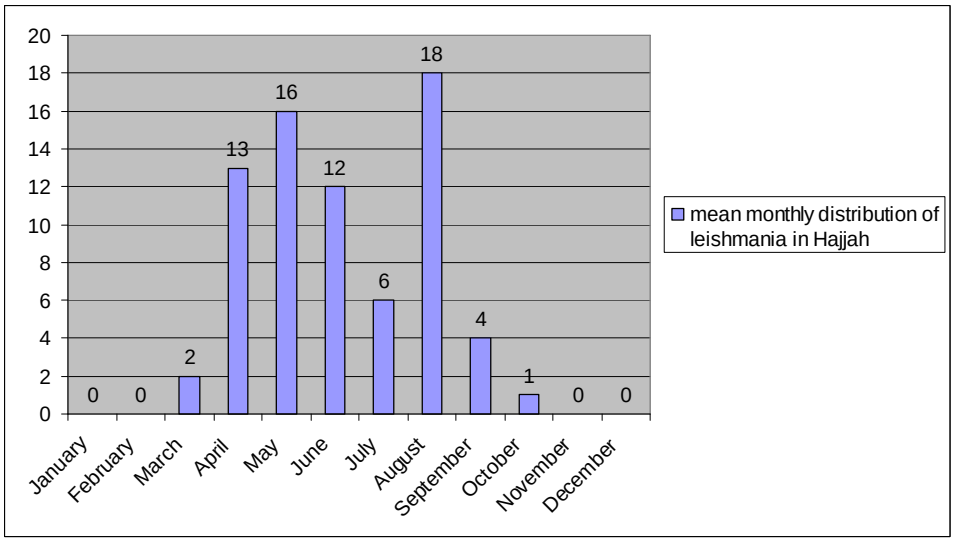

Figure 11: Monthly distribution of leishmaniasis Haija (Western Yemen)

In the arid region in the eastern governates, the majority of cases occurs between January and March (Figure 12). This area is extremely hot im summer time and there is almost no rain. The vectors can only develop during the cooler winter season, and water exists only in so called wadis, deep canyons which carry some water from the mountains only part of the year.

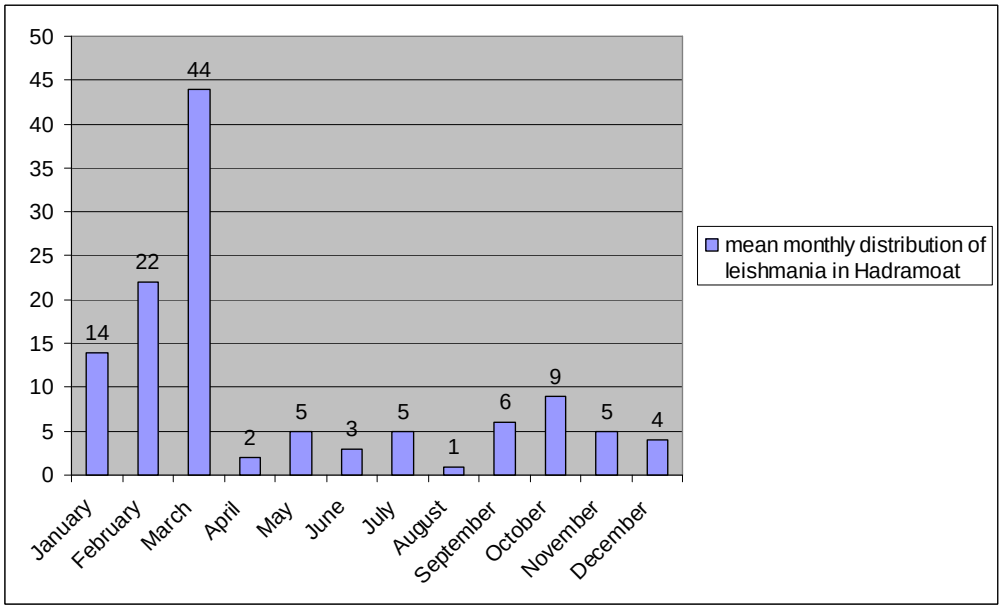

Figure 12: Monthly distribution of leishmaniasis in Hadramont (Eastern Yemen)

3.2 Collecting samples of leishmaniasis patients in Yemen

In order to obtain more data on the epidemiology of leishmaniasis and to collect material for future attempts to develop a new, inexpensive, but highly specific diagnostical procedure, blood samples from patients with suspected leishmaniasis were collected in several locations in the western part of Yemen. The differential diagnosis was generally done based on history of exposure (sandfly bites) and physical examination focussed on ulceric lesions and scar formation. Whenever possible, scrapings from open ulcers were analysed by microscpopy upon Giemsa staining in order to detect amastigote parasites inside of infected cells (see Figure 1). In suspected cases of visceral leishmaniasis, involvement of internal organs was analysed by palpating liver and spleen.

The blood samples were immediately mixed with EDTA (10 mM final concentration) and centrifuged with a manual centrifuge (Roth, Karlsruhe, Germany) in order to sediment the blood cells (Figure 13).

Figure 13: Manual centrifuge used to prepare plasma and buffy coat from blood samples under field condition

The supernatant plasma was withdrawn and mixed with one volume of glycerol. The yellow layer on top of the sedimented erythrocytes (buffy coat, consisting mainly of white blood cells and platelets) was collected with a pipette and mixed with one volume of 5 M guanidinium hydrochloride dissolved in 20 mM Tris buffer pH 7.5 and 50 mM EDTA. By this way, the plasma as well as the DNA were

conserved and could be kept at ambient temperature for several weeks without to be damaged. The samples were transported to Germany for detailed laboratory diagnosis (see below). They served as a basis for developing new diagnostic assays, the plasma for an immunoassay with recombinant antigens (doctoral thesis of Michael Heiman in our group), and the DNA after extraction from the buffy coat for developing new PCR assays as described below.

In addition to patients with open wounds characteristic for cutaneous leishmaniasis, 26 samples were collected from hospitalized persons suspected to have the visceral form of disease. Typical symptoms of visceral leishmaniasis are general weakness, a swollen abdomen (Figure 14) and low counts of white blood cells. Normally, such persons have no cutaneous wounds, but are hospitalized because of their weakness. In the absence of good immunological tests, correct diagnosis is very difficult. In Yemen, a formalin precipitation test is being used (see Materials and Methods), but this test has a low specificity. This test is being used to detect the greatly increased serum proteins in visceral leishmaniasis; one drop of concentrated formalin is added to 1 ml of serum, with rapid and complete coagulation indicating the positive reaction. The parasites could be found microscopically in biopsies from liver or bone marrow, but this test is not performed in Yemen. Blood samples from these persons were collected and processed as described above.

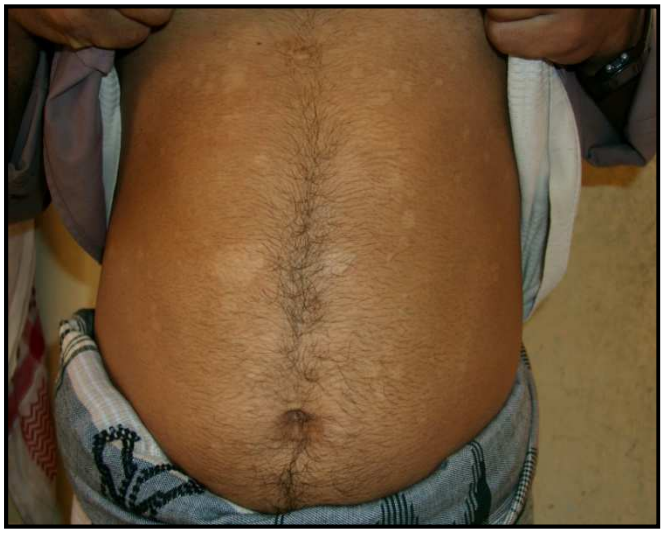

Figure 14: Visceral leishmaniasis

3.3 Laboratory diagnosis of the collected patient samples in Yemen

After collection, samples were taken for laboratory confirmation of leishmaniasis. Basically, there are four major categories of diagnostic methods used routinely: Parasitological methods (for the detection of amastigotes in Giemsa-stained smears of scrapings from the wound, aspirates of bone marrow or lymph nodes), *in vitro* cultivation, immunological tests such as the enzyme-linked immunosorbent assay (ELISA), and molecular methods (polymerase chain reaction, PCR). In Yemen, only microscopical analysis was performed.

Scrapings from the wounds of all 119 patients suspected of having cutaneous leishmaniasis were analysed by microscopical examination in the International Central Blood Bank in Sana'a, Yemen during the years 2005-2006. According to this analysis, 47 of the patients (39 %) were found to be positive. 25 patients were male (53%) and 22 were female (47%). The majority of the positive persons were in the age group 15-34 years (55%). The age group less than 15 years was represented by 14 patients (30%). The highest prevalence rate of disease (74%) was seen in patients comming from rural areas.

It is well known that microscopical analysis has a sensitivity of maximal 70 % only (Al-Jawabreh, 2008), i.e. at least one third of the actual infections could not be confirmed by this way. Furthermore, it is not possible to discriminate different species of *Leishmania* by microscopy. Several of the cases characterized as cutaneous leishmaniasis could have been caused by *L. infantum* and would correspond to visceral leishmaniasis. The samples from the suspected visceral leishmaniasis patients were no futher alanlysed in Yemen. The samples of all patients were transported to the laboratory in Giessen for further analysis.

3.4 Establishing new procedures for the diagnosis of leishmaniasis

Commercially available diagnostic test kits for leishmaniasis are very expensive and not affordable under the social and economical conditions of Yemen. In addition, *Leishmania* strains differ markedly in geographical regions of the world so that tests developed elswhere may not function sufficiently in the country (Burns et al., 1993; Jensen et al., 1999; Kumar et al., 2001; Maalei et al., 2003: Boarino et al., 2005; Chappuis et al., 2005). Appropriate diagnostic procedures should be highly sensitive and specific, but at the same time inexpensive and simply to perform. Two

different approaches to this problem were attemptet in our group in parallel, one included immunoassays with recombinant antigens (Heimann, 2008), and the other, which is topic of this thesis, included molecular methods. PCR is very useful for diagnosis, as it is highly sensitive and specific and can be performed with many different biological samples including skin scrapings, blood or enriched leukocytes and biopsy material such as liver or bone marrow. In addition, PCR diagnosis can be made highly specific and more sensitive than immunological methods. In any case, it is always recommended to use more than one diagnostic test.

To obtain sufficient DNA in pure form to establish PCR assays, two laboratory strains of the parasite, *L. major* and *L. infantum,* were cultivated in tissue culture medium (medium 199 or earle's medium) supplemented with glutamine and fetal calf serum each at 26 ℃ for at least two weeks as described in the Materials and Methods section. Growth of the promastigotes was observed by microscopy. DNA was extracted by the guanidinium silica procedure. Aliquots of the purified DNA were kept at -20℃ and used to set up PCR assays with different sets of primers.

3.5 Establishing new diagnostic PCR procedures

To establish diagnosis of leishmaniasis with PCR, several genes of *Leishmania* have been identified, preferentially targets with multiple copies, for example kinetoplast DNA (kDNA), ribosomal RNA genes (rRNA), mini-exon-derived RNAs (medRNA) and multiple repeats in protein coding genomic regions. *Leishmania* is member of the order kinetoplastida, a group of organisms possessing a unique organelle, the kinetoplast, which is a single mitochondrion containing a characteristic DNA structure known as the kinetoplast DNA network. Kinetoplast DNA comprises two components, maxicircle and minicircle kDNA. Maxicircles, 20.000 - 40.000 basepairs (bp) in length, are present in 30 - 50 copies and carry the genes encoding the mitochondrial enzymes. Minicircles are approximately 1000 bp in length, they encode guide RNA (gRNA) molecules involved in the RNA editing of maxicircle cryptogenes and are present in 10.000 – 20.000 copies per parasite. Because of the high copy number of the minicircles, the kDNA is a prefered target, even though these plasmid-like elements are not fully homogenous, but contain variable sizes and sequences. However, all minicircles contain two highly conserved sequences of 23

and 26 nucleotides which are localized in a distance of approximately 120 bp from each other. Primers directed to these conserved regions can be used to detect all *Leishmania* species. Because the distance between these conserved regions are somewhat variable amongst the different minicircle species, the resulting PCR fragment looks sometimes slightly diffuse. In addition, the small size of the resulting fragment cannot be analysed easily by agarose gel electrophoresis. Nevertheless, there is no comparable highly amplified sequence in the genome of the parasite and kDNA-specific primers have been used by several groups for specific PCR diagnosis of leishmaniasis (Singh et al., 1999; Disch et al., 2005 Reithinger and Dujardin, Bensoussan et al., 2006; 2007; Gomes et al., 2008). A limitation of this highly sensitive test is that it cannot be used to discriminate between different species of Leishmania.

Another widely used target are the approximately 200 copies of ribosomal genes distributed in the different chomosomes of *Leishmania*. Preferntially used by many groups is the coding sequence of the 18S small ribosomal RNA gene (Van Eys et al., 1992; Wortmann et al., 2001; Schönian et al., 2003). This sequence has been determined for more than hundred isolates of different leishmania strains and is highly conserved. At the other hand, this sequence is distant enough from closely related species such as *Trypanosoma cruzi* and *Trypanosoma tarantolae* which both occur in areas of South America in parallel to Leishmania and may even co-infect the patients. As with the kDNA, discrimination between different *Leishmania* species is not feasible, however, specific PCR fragments of 400 to 600 bp can obtained which allow perfect resolution on agarose gels. The disadvantage of a lower copy number can easily be compensated by nested PCR with a second set of primers, although this is not needed in most cases.

To obtain PCR assays which discriminate between different Leishmania strains, repetitive sequences specific for individual *Leishmania* species were selected. As the former sequences, this information was extracted from the complete genomic sequences of *L. major* and *L. infantum* as contained in the Sanger gene data base (GeneDB) (http://www.genedb.org/genedb/leish/index.jsp and http://www.genedb.org/genedb/linfantum) which is freely available in the internet. These sequences consist of approximately 100 million bp each and can be handled only by using appropriate computer software programs. One of these programs is the

“Tandem Repeats Finder” (http://tandem.bu.edu/trf/trf.html) which allows to analyse a whole chromosome (1-2 million bp) of *Leishmania* at once. Large parts of the genomes consits of tandem repeats with lengths between 15 to several hundred bp and it was attempted to find repetitions which were firstly specific for either *L.major*, or for *L. infantum* and secondly of a size easily to be analysed by agarose gel electrophoresis. Two of such sites were selected for designing PCR primers. As the sequences were arranged in mutiple copies as tandem arrays, the resulting PCR product was not a single band, but rather a ladder of fragments corresponding to one, two or more repeats of such sequences. In the following, the design of specific primers for PCR amplification of the above target sites are described. Altogether, four different PCR assays were developed and tested, two of them turned out to be highly useful for diagnosis of Leishmaniasis.

3.5.1 Kinetoplast minicircle (kDNA) specific PCR

In order to define regions in the kDNA appropriate for PCR primer design, a number of representative DNA sequences of relevant Leishmania strains were selected from data bases and aligned by means of the software ClustalW (http://www.ch.embnet.org/software/ClustalW.html). This alignment is shown in Figure 10. Only the most conserved part of the corresponding kDNAs are depicted. Nucleotides identical in all sequences are indicated by an asterix below the lines. As can be seen, only two sequences represent common regions with sufficient length for primer design. The sequence marked in blue was chosen as forward primer (Leishmini-F, ATTTTACACCAACCCCCAGTT) and the sequence marked in blue as reverse primer (Leishmini-R, GGG(G/T)AGGGGCGTTCTGC). The calculated annealing temperature for both primers is the same (approximately 60℃) which is essential for optimal PCR results. The reverse primer contains in position 4 a mixture of the nucleotides G and T in order to match the kDNA sequence of all *Leishmania* species.

Multiple alignment of Leishmania spp minicircle DNA genes

```
L.donovani           CACCCCACACCGCCTATTTTACACCAACCCCCAGTTTCCACCGCCGGAGCCCGGGATT--
L amazon.complex     CCACGCACCGGG--TATTTTACACCAACCCCCAGTTGTGACCGCCGGAG-CCGGAATTC-
L mexicana complex   GAGACCCCCGCCGCTATTTTACACCAACCCCCAGTTTCGAGCCCGGGAG-CCGATTTTCG
L infantum           GGACCACCCGGCCCTATTTTACACCAACCCCCAGTTTCCCGCCCCGGAGGCCAAAAATG-
L.tropica complex    GGACCACCCCCACCTATTTTACACCAACCCCCAGTTTGCAGCCTCCGAGCCCAAAAATG-
L.major complex      GGACCCACCCGGCCTATTTTACACCAACCCCCAGTTTGCACGCCCCGAGGCCAAAAATG-
                        . ..*.     **********************   .      *** **.. ::*

L donovani           ----------TTTGCCCAAAATATGAACGG-GGTTTCTGCACTCATTTTTCCCAAAATCG
L.amazon.complex     ----CCCAAAATTCCCCAAAATATGAACGG-GGTTTCTGCACTCATTTTTCCCAAATTCG
L.mexicana complex   AGGAATTCGAATTCCCCAAAATATGAACGG-GGTTTCTGCACTCATTTTTGCCCTCCCCG
L.infantum           -----ACATTTTCGGGCCAAAAATGAACGGGAATTTCTGCACCCATTTTT-CGGTTTTCG
L tropica complex    ----GCATTTTTTCAGG-ATTTTTGAACGG-GGTTTCTG-TATCAATTTTCGCGATTTTG
L.major complex      ----GCCATTTTCCGTGGATTTTTGAACGG-GGTTTCTG-TATCGATTTTTCGAAATTCG
                              :*      *::::******* ..****** :. *.:****    :    *

L donovani           CAGAACGCCCCTCCCCCACGGGGCAG-AAAAGTTTGGATTTCTGGCCCATTTTTTGCCAC
L.amazon.complex     CAGAACGCCCCTCCCCCACGGGGCAGAAAAGTTTGGG----------------------
L.mexicana complex   CAGAACGCCCCTCCCCCACGG--CAGAAAAGTTTGGAATTCTGGCCATTTTTGACCCCAT
L.infantum           CAGAACGCCCCTCCCCCACGGACCAGAAAAGTTTGGATTTTTAGGCCATTTTTTGGGATT
L.tropica complex    CAGAACGCCCCTACCCGGAG--CTACTCCATGTTTGGAAATTC-CCCCATTTTTGGCCAT
L.major complex      CAGAACGCCCCTACCCGGAGGGCTACTCCATGCTACGATTTCTGCCCCTTTTTTGACGAA
                     ************.*** ..*    *  ..*   *  .
```

Figure 15: Alignment of the conserved kDNA regions of representative Leishmania strains. Complex means a number of geographically distinct strains showing the same sequence. The sequences marked in blue and red were selected to design PCR primers.

Tests with purified DNA from the cultivated strains *L. major* and *L. infantum* led to the expected product of approximately 120 bp by electrophoresis on 6 % polyacrylamide gels (Figure 16). However, using this set of primers in PCR with DNA extracted from buffy coats, no band was detectable in the majority of cases. Only when the PCR was repeated by using 1 microliter of first PCR product as a template and the same set of primers again, the expected band became visible.

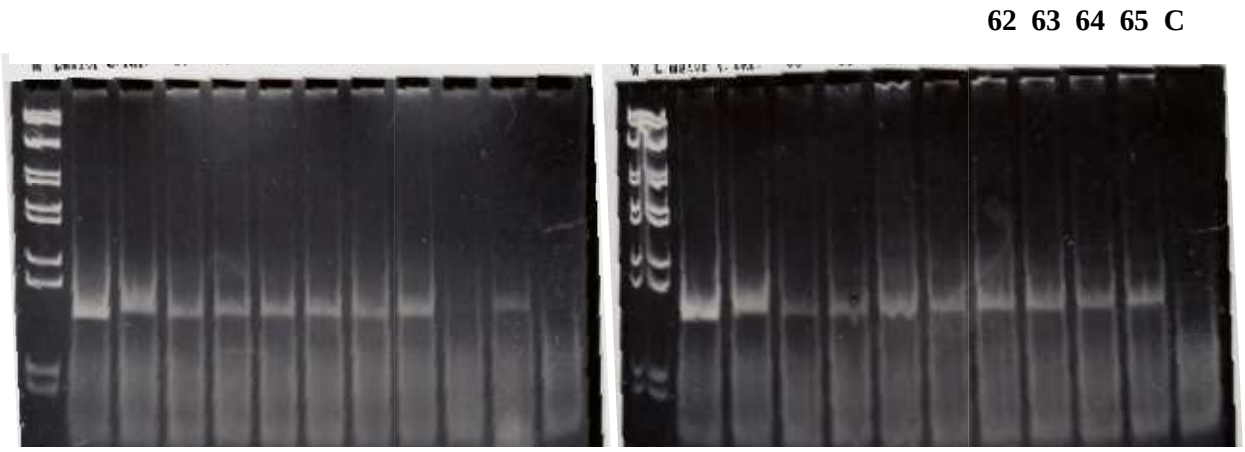

Figure 16: Analysis of Leishmania kDNA PCR products on 6 % polyacrylamide gels. DNA was extracted from buffy coat samples of patients number 50-65 (patients with cutaneous leishmaniasis). m = size marker, Lm = *L. major* positive control, Lin = *L. infantum* positive control, C = water instead of DNA as a negative control.

3.5.2 18S rRNA specific PCR

In principle, the results with kDNA-specific PCR appeared to be sufficiently sensitive and specific, however, the small size of the resulting fragment demands the use of polyacrylamide gels for resolution which makes handling of large numbers of samples demanding. Therefore, the gene for the 18S ribosomal RNA was selected as an alternative target for specific PCR. The gene occurs in approximately 160 identical copies in the genome of *L. brasiliensis* (Villalba and Ramírez, 1982) and in highly related form and probably in similar numbers in other *Leishmania* strains. It has been used as a target for PCR diagnosis by several groups already using different sets of primers. However, these primers had been selected for PCR analysis of Old World *Leishmania* strains and were not sufficiently specific to discriminate *Leishmania* species from *Trypanosoma cruzi, the agent of Chagas disease,* which occurs in Latin America in regions endemic for both, *Leishmania* as well as *T. cruzi*.

```
                                  I--Leish18S-out-F-->
L. tarentolae     AGAAATAGAGTTGTCAGTCCATTTGGATTGTCATTT-CAATGGGGGATATTTAAACCCAT
L. donovani       AGAAATAGAGTTGTCAGTCCATTTGGATTGTCATTT-CAATGGGGGATATTTAAACCCAT
L. amazonensis    AGAAATAGAGTTGTCAGTCCATTTGGATTGTCATTT-CAATGGGGGATATTTAAACCCAT
L. major          AGAAATAGAGTTGTCAGTCCATTTGGATTGTCATTT-CAATGGGGGATATTTAAACCCAT
T. cruzi          AGAAATAGAGCCGACAGTGCTTTTGCATTGTCGTTTTCAATGGGGGATATTTAAACCCAT
                  **********  * **** * **** ****** *** **********************

// 100 nucleotides //

                                        I--Leish18S-inn-F-->
L. tarentolae     TAGTTGAACTGTGGGCTGTGCAGGTTTGTCCCTGGTCGTCCCGTCCATGTCGGATTTGGT
L. donovani       TAGTTGAACTGTGGGCTGTGCAGGTTTGTTCCTGGTCGTCCCGTCCATGTCGGATTTGGT
L. amazonensis    TAGTTGAACTGTGGGCTGTGCAGGTTTGTTCCTGGTCGTCCCGTCCATGTCGGATTTGGT
L. major          TAGTTGAACTGTGGGCTGTGCAGGTTTGTTCCTGGTCGTCCCGTCCATGTCGGATTTGGT
T. cruzi          TAGTTGAATTGAGGGCCTCTAAGGCGCAATGGTT-TAGTCCCATCCACTTCGGATT-GGT
                  ******** ** ****     ***        *  * ***** ****  ******* ***

// 380 nucleotides //

                              <---------Leish18S-inn-R------------I
L. tarentolae     TCGTCCGGCCGTA---------------ACGCCTTT--TCAACTCACGGCCTCTAGGAA
L. donovani       TCGTCCGGCCGTA---------------ACGCCTTT--TCAACTCACGGCCTCTAGGAA
L. amazonensis    TCGTCCGGCCGTA---------------ACGCCTTT--TCAACTCACGGCCTCTAGGAA
L. major          TCGTCCGGCCGTA---------------ACGCCTTT--TCAACTCACGGCCTCTAGGAA
T. cruzi          CTTGCC-TCCGCATATTTTCACATGTGTCATGCCTTCCCTCAACTCACGGCATCCAGGAA
                      **   ** *                * *****   ************ ** *****

                                 <--Leish18S-out-R--I
L. tarentolae     TGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCG
L. donovani       TGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCG
L. amazonensis    TGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCG
L. major          TGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGGGCGTCAGAGGTGAAATTCTTAGACCG
T. cruzi          TGAAGGAGGGTAGTTCGGGGGAGAACGTACTGGTGCGTCAGAGGTGAAATTCTTAGACCG
                  ************************************************************
```

Figure 17: Alignment of part of 18S rRNA genes of different *Leishmania* strains with the corresponding sequence of *T. cruzi*. The complete genes consist of approximately 2200 bp each, however, only the central part (some 600 bp) for most of these sequences is known and was aligned. Regions highly conserved between *Leishmania* and *T. cruzi* have been ommitted (100 nucleotides in the first third, and 380 nucleotides in the middle of the sequence). The locations of the inner (blue) and outer PCR primers (red) are indicated.

PCR primers were selected based on the nucleotide sequence of *L. major* as template. It was attempted to select primers in regions clearly different between

Leishmania and *T. cruzi*. However, only two such regions existed as shown in the above alignment. They were used to design optimal primers with help of the primer seach program “Primer3” (http://frodo.wi.mit.edu/). The resulting PCR fragment was 335 bp. Since it was to be expected that like in the case of kDNA a second PCR would be needed to obtain visible bands upon gel electrophoresis, another set of primers was selected with the same software. As the above PCR product was relatively short, so called “outer” primers were searched in the flanking region of the 18S rRNA sequence. There was no chance to find another set of primers which would discriminate *Leishmania* and *T. cruzi*, sequences with optimal physical characteristics were chosen (Primer3 includes more than 20 different criteria for optimising primers, such as the content of CG versus AT nucleotides, potential hairpin structures, annealing temperature, stability of the hybrid at 3’ and 5’ ends etc.). The outer primers discriminater clearly between *Leishmania* and other organisms including men (not shown), but they cannot well discriminate between *Leishmania* and *T. cruzi*. The reverse primer hybridizes to the identical sequence in both species, for the forward primer there are five base exchanges, at least, They would be sufficient under stringent conditions, however, under such conditions the amplification of DNA is reduced thus reducing the sensitivity of the assay. As a compromise, one would accept limited specificity in the first PCR using the outer primers, and use part of the amplification product as template for the second, clearly discriminatory second PCR. The location of inner and outer primers are indicated in Figure 17.

These primers were used to analyse the DNA extracted from the buffy coat frction of 63 blood samples from Yemenit patients suspected to have cutaneous leishmaniasis. As the expected PCR fragments were 480 bp (outer primers) or 335 bp long (inner primers), 2 % agarose gels could be used simplifying markedly the analysis of results. As can be seen from from the upper halfs of the gels shown in Figure 15, except of the positive control with purified *L. major* DNA, only in rare cases (in numbers 9 and 12) a weak band was visible at position 480 upon staining the gels with ethidiumbromide. However, in the second PCR shown in he lower halfs of the gels, clear-cut positive results were obtained with the majority of samples, whereas other samples were clearly negative.

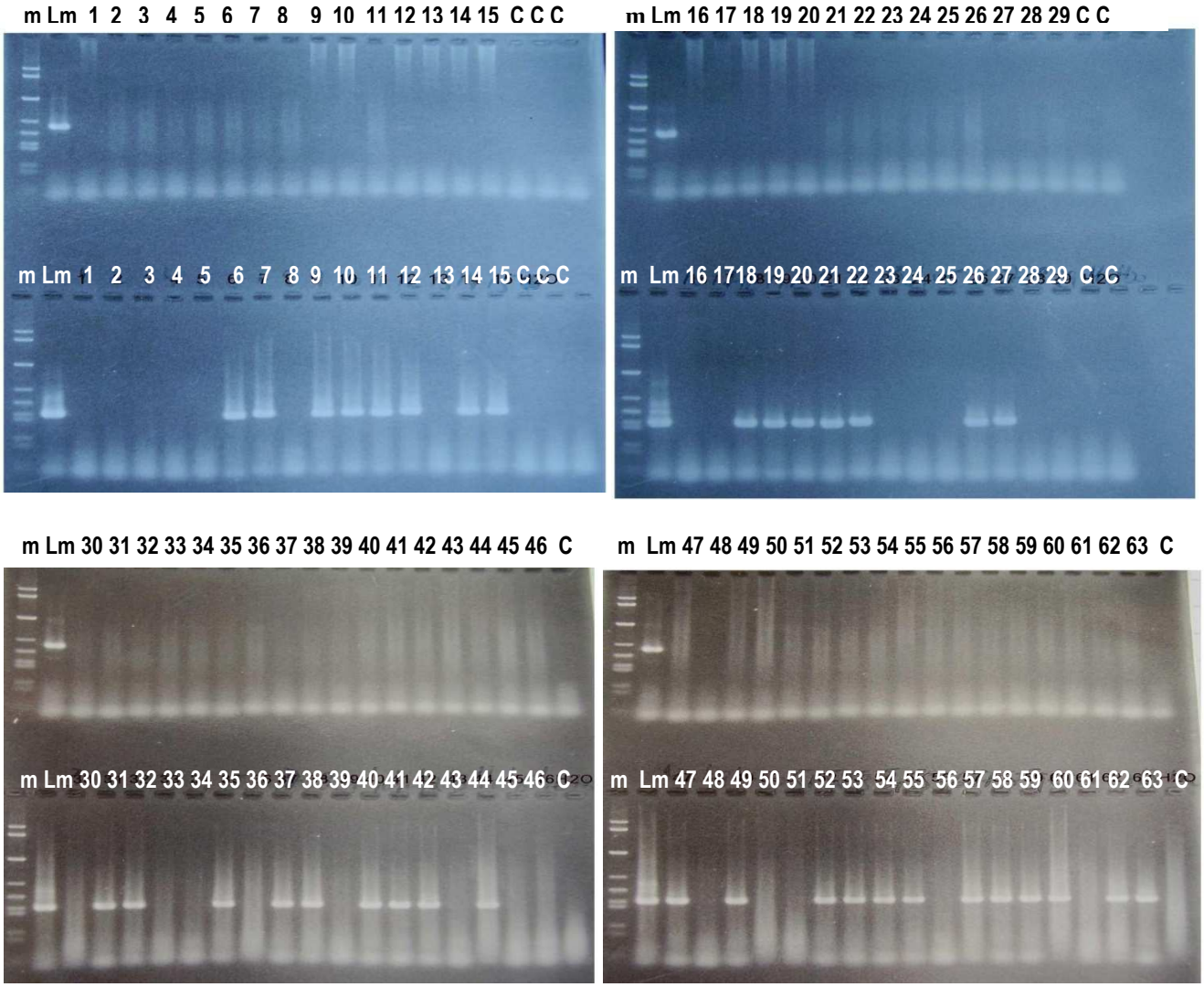

Figure 18. PCR amplification of buffy coat sample obtained from patients with cutaneous leishmaniasis by 18S rRNA-specific primers. m = size marker, *Lm* = *Leishmania major* purified DNA as positive control, and C = water as negative control.

The results with the 18S rRNA specific PCR were consistent with the results obtained with the kDNA specific PCR except of three samples (numbers 50, 51 and 61) which were positive in the latter assay, but negative with the 18S rRNA PCR. This could be explained by the fact, that the kDNA-specific PCR is more sensitive (see Discussion section).

3.5.3 45 basepair repeat-specific PCR

As another approach to a sensitive molecular assay, the amplification of a 45 bp repeat was attempted. Analysis of the genome of *L. major* with Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.html) revealed that this repeat occurred in more than 1000 copies in the genome. The sequence exists in identical form and almost the same frequency also in *L. infantum*, but does not occure in *Trypanosoma cruzi*. It is spread in ten different arrays of 7 to more than 200 repeats over the whole genome. A section of 4 of these repetitive sequence was used to select optimal PCR primers with the computer program Primer3 as shown in Figure 16. The primers were named Leish45-F and Leish45-R.

```
OLIGO            start  len      tm      gc%   any   3' seq
LEFT PRIMER          9   17   61.96   70.59  6.00  1.00 CGGAGCTGCTGCTGGAC
RIGHT PRIMER       181   17   61.65   70.59  3.00  1.00 GGCGTCCTCGTCGTCTG
SEQUENCE SIZE: 225
INCLUDED REGION SIZE: 225

PRODUCT SIZE: 173, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 CGACGGCGCGGAGCTGCTGCTGGACGGCGCAGACGACGAGGACGCCGACGGCGCGGAGCT
              >>>>>>>>>>>>>>>>>

   61 GCTGCTGGACGGCGCAGACGACGAGGACGCCGACGGCGCGGAGCTGCTGCTGGACGGCGC


  121 AGACGACGAGGACGCCGACGGCGCGGAGCTGCTGCTGGACGGCGCAGACGACGAGGACGC
                                                 <<<<<<<<<<<<<<<<<

  181 CGACGGCGCGGAGCTGCTGCTGGACGGCGCAGACGACGAGGACGC
```

Figure 19: Result of the search for an optimal set of PCR primers for the 45 bp repeat of *L. major* using the computer program Primer3. The program was asked for optimal primers producing fragments in a length between 150 and 200 bp on the basis of 4 copies of the repeated sequence. For easier visualisation, the predicted forward and reverse primers have been marked in red and blue. As the sequence is repetitive, each primer occurs for times. The sequence of the recommended right primer (blue) as shown in the upper part of the figure is present in complementary form in the 225 nucleotide sequence which corresponds to the positive DNA strand. Amongst others, the program also indicates the calculated optimal annealing temperature (tm) for each primer.

Because the sequence is repetitive, hybridisation sites for the primers occur every 45 bp in the plus strand as well as in the minus strand of DNA. This leads to the production of not only one PCR product, but to a characteristic ladder of bands differing by the length of 45 bp from each other. Testing these primers in a PCR with

purified DNAs of *L. major* and *L. infantum* resulted in exactly this predicted pattern of DNA bands (Figure 20).

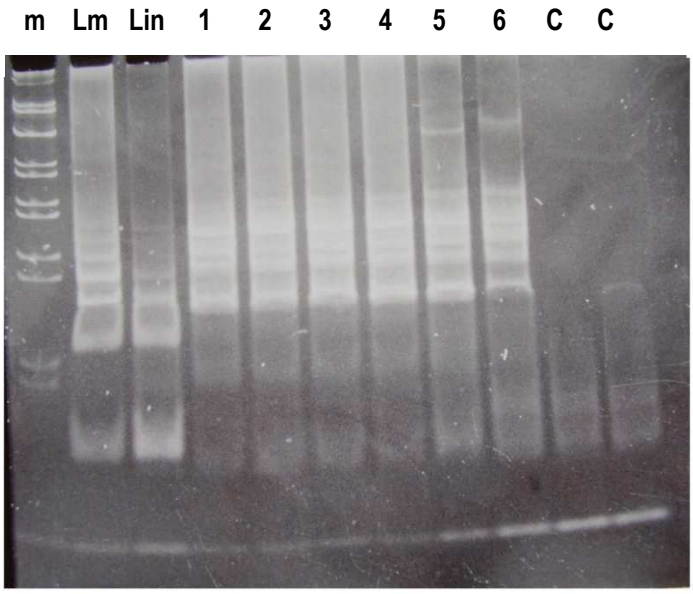

Figure 20: Amplification products obtained with the primers Leish45-F and Leish45-R on a 6 % polyacrylamide gel. In the first two lanes, template DNA is from purified parasites. Lanes 1-6 show results with DNA extracted from buffy coat of cutaneous leishmaniasis patients. Lane H2O is the negative control using water istead of DNA.

As can be seen from Figure 20, PCR with primers Leish45-F and Leish45-R results in the expected ladder of 83, 128, 173, 218, 263, ... bp DNA bands. In combination with the DNA size marker, this pattern would be sufficiently characteristic to identify Leishmania DNA. However, the assays result in clear results with DNA purified from cultivated parasites only. Using crude DNA as isolated from buffy coat, the results are not convincing because of the high background of addidional amplification products which make it difficult to recognize the correct bands. Thus, this approach was no further continued in much detail.

3.5.4 64 basepair repeat-specific PCR

None of the PCR approaches described so far were able to discriminate between *L.* major and *L. infantum*. Thus, it was attempted to search for an appropriate template occuring exclusively in *L. infantum* in order to identify patient at

risk to develop visceral leishmaniasis. Such a sequence was found again using the Tandem Repeats Finder program in the genome of *L. infantum* in the form of a 64 bp tandem repeat. This repeat occurs more than 400 times in different positions of chromosome 27 of the parasite. It is present neither in *L.major* nor in *Trypanosoma cruzi* and appeared to be an ideal target for PCR therefore.

The sequence of this repeat (6 repeats) is depicted in Figure 21. As with the above described Leish45-repeat, Primer3 software was used to select optimal PCR primers.

```
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER        167   20   59.57   45.00  4.00  2.00 AAAACGGGAATGCTCCAGTA
RIGHT PRIMER       361   18   62.64   61.11  7.00  2.00 TTTCGCTGCGAGGGAGAC
SEQUENCE SIZE: 384
INCLUDED REGION SIZE: 384

PRODUCT SIZE: 195, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 CGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAAAACGGGAATGCTCCAGTACT

   61 GGGGCGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAAAACGGGAATGCTCCAG

  121 TACTGGGGCGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAAAACGGGAATGCT
                                                >>>>>>>>>>>>>>

  181 CCAGTACTGGGGCGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAAAACGGGAA
      >>>>>>

  241 TGCTCCAGTACTGGGGCGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAAAACG

  301 GGAATGCTCCAGTACTGGGGCGCGAAGCAATGCTCCCGCGAGGGTCTCCCTCGCAGCGAA
                                              <<<<<<<<<<<<<<<<<

  361 AACGGGAATGCTCCAGTACTGGGG
      <
```

Figure 21: DNA sequence and primer selection of the Leish64 repeat. The sequences of forward primer (red) and reverse primer (blue) are indicated. The corresponding areas on the DNA overlap as indicated (purple and underlined) what holds only for the (hypothetical) shortest PCR fragment.

As can be seen from Figure 19, the primers Leis64-F and Leish64-R would lead to a ladder of DNA fragments with lengths of 67, 131, 195, 259, 323, 387 ... bp. This was tested with DNA extracted from cultivated parasites as well as with buffy coat DNA from patients with visceral and cutaneous leishmaniasis. The results are shown in Figure 22.

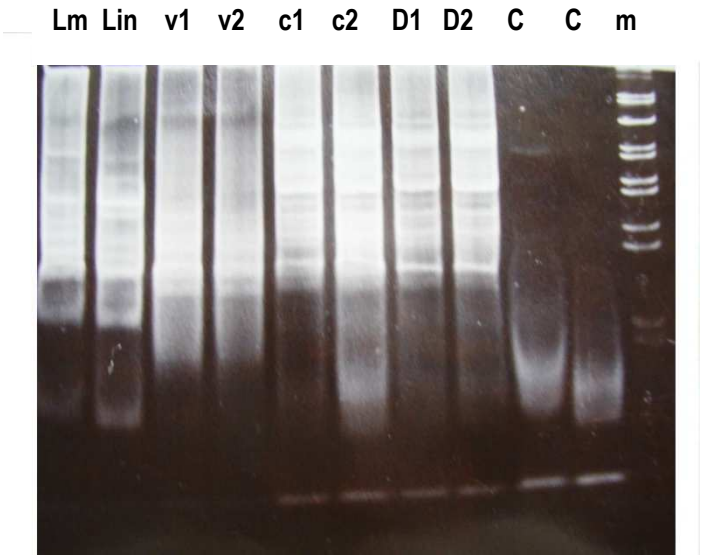

Figure 22: PCR products obtained with primers Leish64-F and Leish64-R with DNA purified from cultivated *L. major* (Lm) and *L. infantum* (Lin) parasites, with DNA from buffy coat of two visceral leishmaniasis patients (v1 and v2), with buffy coat DNA of two cutaneous leishmaniasis patients (c1 and c2) and with buffy coat DNA from two healthy German persons (D1 and D2), C = control PCR in the absence of DNA, m = size marker.

As can be seen from Figure 22, the PCR with the primers Leish64-F and Leish64-R are not appropriate to differentiate between *L. major* and *L. infantum*. The reason for this is the high background arising even with DNA extracted from buffy coat of healthy German blood donors (lanes D 1 and 2). There are obviously sequences in the human genome which ross-react with these primers. The background could not be reduced by using higher annealing temperatures (not shown). Therefore, no further tests were performed with this set of primers.

3.5.5 39 basepair repeat-specific PCR

At last, a set of PCR primers was designed for a 39 bp repetition occuring more than 450 times exclusively in chromosome 22 of *L. infantum* in three different locations. No homologous sequences were detected in the genomes of *L. major* or *Trypanosoma cruzi*. As before for the other tandem repeats, a fragment of five

repeats was used to design optimal PCR primers using Primer3 software. The result of this calculation is shown in Figure 23.

```
OLIGO            start  len      tm     gc%   any   3' seq
LEFT PRIMER         78   25   51.80   32.00  6.00  2.00 CAAAGATGTATATAGACGTTAGTGT
RIGHT PRIMER       163   22   56.11   36.36  2.00  0.00 CATCTTTGTTTGTGTGTTGTGT
SEQUENCE SIZE: 195
INCLUDED REGION SIZE: 195

PRODUCT SIZE: 86, PAIR ANY COMPL: 8.00, PAIR 3' COMPL: 0.00

    1 AAAGATGTATATAGACGTTAGTGTACACAACACACAAACAAAGATGTATATAGACGTTAG

   61 TGTACACAACACACAAACAAAGATGTATATAGACGTTAGTGTACACAACACACAAACAAA
                      >>>>>>>>>>>>>>>>>>>>>>>>

  121 GATGTATATAGACGTTAGTGTACACAACACACAAACAAAGATGTATATAGACGTTAGTGT
                            <<<<<<<<<<<<<<<<<<<<<<<

  181 ACACAACACACAAAC
```

Figure 23: DNA sequence and primer design for the 39 bp repeat of *L. infantum*. The sequences of forward primer (red) and reverse primer (blue) and overlaps (purple and underlined) are indicated as in Figure 19. The calculated size of the amplfication products is a ladder of 86, 125, 164, 203, 242, 281, 320, bp.

Test of these primers with purified Leishmania DNA as template revealed that the primers were highly specific for *L. infantum* exclusively as shown in Figure 24. There was no cross-reaction with DNA from *L. major* (lane Lm) or *Trypanosoma cruzi* (not shown).

With all of the 26 samples from patients suspected to have visceral leishmaniasis there is a amplification of DNA which could correspond to tri- tetra- and pentamers of the 39 bp repeat, even though the band pattern looks diffuse. In some of the samples the bands are better to be recognized than in the others. The *L. major* positive control Is always negative, and so is buffy coat DNA from healthy German persons (data not shown). Thus it looks like PCR using Leish39 primers is specific for the visceral form of leishmaniasis even though PCR products leading to sharper bands would be desirable.

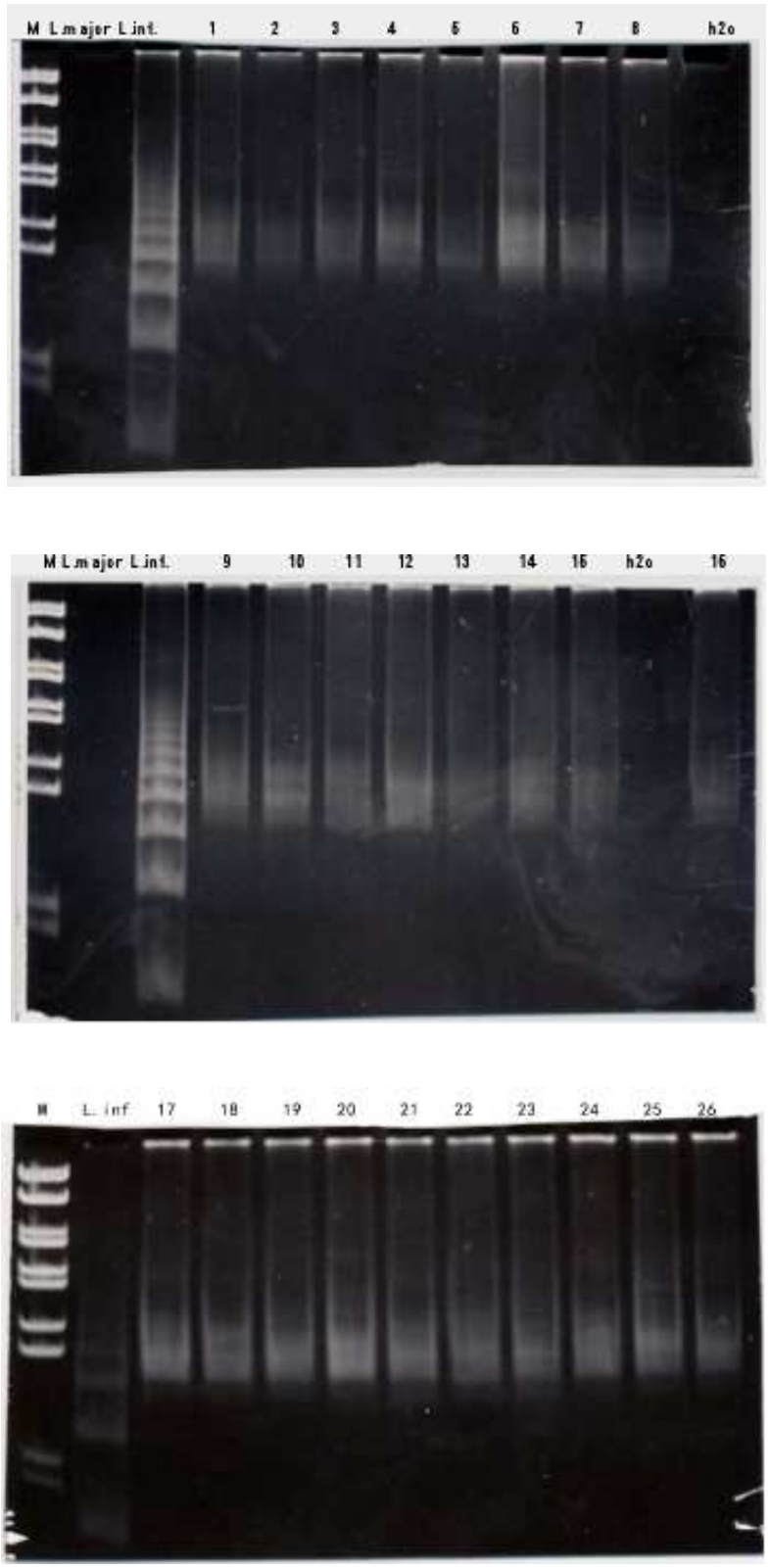

Figure 24: PCR amplification of Leishmania from buffy coat sample (lanes 1-26) obtained from patients with suspected visceral leishmaniasis. M = size marker, L.major = *L. major* positive control, L.inf = *L. infantum* positive control, and h2o = water as a negative control.

3.5.6 Compilation of the PCR results

Even though the PCR procedures developed during this work need to be verified by well characterized patient samples, it is clear from the data that the methods used in Yemen up to now are not sufficiently sensitive.

The primers used for the assays described above are listed in the following Table 5.

Table 5. Primers for diagnsosis of Leishmaniasis

Specificity	Gene	Primer name	Sequence	Size (bp)	Tm (℃)
Leishmania all species	Minicircle (kDNA)	Leishmini-F Leishmini-R	ATTTTACACCAACCCCCAGTT GGG(G/T)AGGGGCGTTCTGC	113	59.5 61.6
Leishmania all species	18S rRNA	Leish18S-out-F Leish18S-out-R Leish18S-inn-F Leish18S-inn-R	TCAGTCCATTTGGATTGTCA AGTACGTTCTCCCCCGAACT CAGGTTTGTTCCTGGTCGTC GAGTTGAAAAGGCGTTACGG	480 335 	64.3 63.2.0 60.5 59.7
Leishmania all species	45 bp repeat	Leish45-F Leish45-R	CGGAGCTGCTGCTGGAC GGCGTCCTCGTCGTCTG	ladder	62.0 61.6
L. infantum	64 bp repeat	Linf64-F Linf64-R	AAAACGGGAATGCTCCAGTA TTTCGCTGCGAGGGAGAC	ladder	59.6 62.6
L. infantum	39 bp repeat	Linf39-F Linf39-R	CAAAGATGTATATAGACGTTAGTGT CATCTTTGTTTGTGTGTTGTGT	ladder	51.8 56.1

During the study, 119 samples from patients with suspected cutaneous leishmaniasis have been collected in different locations in Yemen. These samples have first been analysed by microscopy of smears of wound material. It is agreed in the international literature that microscopical analysis alone has an insufficient sensitivity. Better results could be obtained by addidinal tests such as culivation or immunological techniques. However, these procedures are to expensive for use in Yemen. The results for the 119 patient samples obtained obtained in Yemen by microscopy are shown in the following Table 6.

As can been seen, only 47 cases (39%) of the 119 cases were identified to be positive by microscopic examination. However, by the combined tests with kDNA- and 18S rRNA-specific PCR, 76 (64 %) samples were found to be positive.

Table 6: Clinical and epidemiological features from 119 patients with cutaneous leishmaniasis in Yemen.

Patients features	Positive* (%) n = 47(39)	Negative*(%) n = 72(61)
Sex		
Male	25(53)	49(68)
Female	22(47)	23(32)
Age (years)		
≤15	14(30)	19(26)
15–34	26(55)	39(54)
35–54	5(11)	10(14)
55–64	1 (2)	3(4)
≥65	1(2)	3(4)
Habitation		
Urban	9(19)	13(18)
Periurban	12(26)	18(25)
Rural	26(55)	41(57)
Duration (in months)		
1–6	38(81)	56(78)
7–12	9(19)	12(17)
≥12	1(1)	3(4)
Place of lesions		
Legs and feet	5(11)	13(18)
Arms and hands	8(17)	24(33)
Chest	0	0
Head and face	31(66)	34(47)
Multiple lesions	3(6)	1(1)

* as determined by microscopy in Yemen

Similar results have been obtained with the samples of patients with suspected visceral leishmaniasis. The 26 patients were diagnosis in the central blood bank in Sana'a, Yemen, by both ELISA and formol gel examination. 8 cases have been classified according to these criteria as positive. The data are shown in Table 7.

From the patients identified as positive 4 were male (50%) were and 4 were female (50%). The majority of the positive patients (62%) were in the age group 15-34 years and in the age group less than 15 years (25%). The highest prevalence of disease (62%) was seen in the urban areas.

Table 7: Clinical and epidemiological features from 26 patients with suspected visceral leishmaniasis in Yemen.

Patients features	Positive*(%)	Negative* (%)
	n =8 (31)	n = 18 (69)
Sex		
Male	4(50)	11(61)
Female	4(50)	7(39)
Age (years)		
≤15	2(25)	0
15–34	5(62)	13(72)
35–54	1(12)	4(22)
55–64	0	1(6)
≥65		
Habitation		
Urban	5(62)	13(72)
Periurban	3(37)	4(22)
Rural	0	1(6)
Duration (in months)		
1–6	1(12)	9(50)
7–12	3(37)	7(39)
≥12	4(50)	2(11)
Symptoms		
Hepatomegaly	2(25)	10(56)
Splenomegaly	4(50)	3(17)
Hepatosplenomegaly	2(25)	5(28)

* as determined by the combination of a *Leishmania*-specific ELISA and formol precipitation assay in Yemen

Analysis of buffy coat DNA samples from the same patients in the laboratory in Giessen by the different PCR assays described above yielded a different result. Even if these assays need to be validated by additional tests and there were some inconsitencies between the single PCR runs, it is obvious that the molecular approach is much more sensitive than the traditional procedures used in Yemen. Combination of kDNA-specific PCR and 18S rRNA-specific PCR led to 76 positive results out of the 119 samples indicating that 64 % of the suspected cutaneous leishmaniasis patients were actually infected. By microscopy, only 39 % of the patients had been recognized as positive.

As with the coutaneous leishmaniasis, also the data for the patients with suspected visceral leishmaniasis obtained in Germany were much higher. By combining the PCR data from rDNA-, 18S rRNA- and Leish39-specific PCR, 16

cases (62 %) were found to be positive. Even though the results with the new PCR techniques are preliminary and need to be confirmed, the results show clearly that the occurence of leishmaniasis in Yemen is highly underestimated.

Table 8: Comparison of PCR and traditional methods in patients with leishmaniasis

suspected cutaneous leishmaniasis patients	**PCR**		**Microscopy**	
	positive	negative	positive	negative
119 (100 %)	76 (64 %)	43 (36 %)	47 (39 %)	72 (61 %)

suspected visceral leishmaniasis patients	**PCR**		**ELISA**		**Formol gel**	
	positive	negative	positive	negative	positive	negative
26 (100 %)	16(62%)	10(38%)	2 (8 %)	3 (12%)	6 (23 %)	15(58%)

4. DISCUSSION

The Republic of Yemen is a developing country with very poor health conditions. According to the Ministry of Public Health and the National Center for Surveillance and Disease Control, deaths among children under five years of age are mainly due to infectious diseases such as diarrhea with an estimated 45,000 and malaria reaching about 25.000 cases of death per year. According to the U.S.A. Census Bureau (http://www.census.gov/ipc/prod/wp02/tabA-11.pdf), the total children mortality within the first 5 years of life in Yemen is 92 per 1000, ranging amongst the last 20 % countries worlwide (for comparison: in Europe this number is 6 per 1000). These data are indicatory for the poor quality of life in this country and for the realistic possibilities to improve heath conditions.

The aim of this study was first to shed some light on the real extent of leishmaniasis in Yemen, and second, to contribute to the development of a more trustable diagnosis than the traditional procedures used in the country such as microscopy and serum precipitation with formaldehyde. The limiting sensitivity and specificity of these traditional methods are well known, but the limiting economical conditions in Yemen do not allow the use of more sensitive and specific diagnostic techniques such as modern immunoassays with recombinant antigens. New diagnostic techniques to be established in the country should not only be highly specific and sensitive, but also inexpensive and simple to be applicable under the given limiting conditions. This latter goal was followed in our laboratory at the University of Giessen in two different ways: one approach included the development of recombinant antigens capable to discriminate between cutaneous and visceral leishmaniasis for use in simple and inexpensive immunoassays (ELISA and immunochromatographic test stripes) which was topic of another doctoral thesis (Heimann, 2008). The second approach, which is realised in this work, was the development of molecular diagnostic tests in the form of different PCR assays.

The basis for both approaches were the comlete genomic sequences of three *Leishmania* strains (*L. major, L. infantum and L. brasiliensis*) together with the closely related genome of *Trypanosoma cruzi* wich are available free of charge since a short time in the internet. Many computer programmes needed to handle these huge amounts of genetic information (more than 100 million basepairs) are equally free accessible from the internet.

4.1 Epidemiology of leishmaniasis

There are no solid data on the incidence of *Leishmania* infections in Yemen, but the disease is certainly under-reported, especially in women and children, and may exceed 10.000 cases per year. Most cases correspond to cutaneous leishmaniasis due to infections with *L. major* and *L. tropica* which may not necessarily need treatment, but up to 10 % of the infections may be due to *L. infantum* leading to visceral leishmaniasis which is associated with up to 90% mortality when untreated.

There are only few reports on leishmaniasis in Yemen in the literature (Sarnelli 1933; Rioux 1986; Pratlong et al. 1995: Khatri et al., 2006) which can be used to predict the parasite strains circulating in the country (*L. major* and *L. tropica* for cutaneous leishmaniasis and *L. infantum* for visceral leishmaniasis), but these publications do not indicate the frequency and severity of the disease in the population. In order to get more information on the occurence of leishmaniasis, data collected during the years 2005-2008 by the the National Center of Surveillance of Disease from all governmental hospitals in the 20 governorates were analysed (Table 1). However, these data can only be used in a qualitative way, but they are by no means quantitative. Data obtained during a visit of the (private) Alolfi Hospital in the Alhodeidah governorate indicated 105 cases during the same period of time (2005-2008) in this single place which is twofold the number of cases reported by the government for the whole governate (53 cases).

The closest countries with similar kinds of *Leishmania* strains, phlebotomine vectors and comparable geographic conditions are Oman in the East, Saudi Arabia in the North-West and, in a distance of some 2000 km North-West from Yemen, Jordan and Palestine. Some usful epidemiological data exist from Saudi Arabia (al-Zahrani et al., 1989 and Ibrahim et al., 1992), wheras most informatve data have been published for Jordan and Israel (Nimri et al., 2002; Wasserberg et al., 2002; Al-Jawabreh et al., 2003; Al-Jawabreh, 2005; Singer et al., 2009; Amro et al., 2009;Hamarsheh et al., 2009). These data could form a basis for the understandig of leishmaniasis in Yemen. As in Yemen, two forms of transmission of the disease exist: in urban areas the parasites are transmitted from infected individuals by the bite of phlebotomine sand flies to naïve persons (anthroponotic cutaneous leishmaniasis), whereas In rural areas animals are thought to be the reservoir (zoonotic cutaneous leishmaniasis). The anthroponotic form of disease is frequent in large cities where

many people live close together under poor hygienic conditions. The vectors can multiply in small water resevoirs close to the dwellings as illustrated by the following pictures (Figure 25).

Figure 25: Conditions for anthroponotic cutaneous leishmaniasis. In many cities of Yemen, leishmaniasis is not dependent on animal hosts, but is transmitted by sand flies directly from infected individuals to naïve persons. In such loations the infection rate can be very high. The larvae of the sand flies can perfectly thrive in nearby water pools.

Hosts involved in the transmission of the zoonotic form of the disease include primarily dogs and small rodents living in arid regions. In the neighbouring contries Iran, Jordan and Israel host species such as *Psammomys obesus, Rattus rattus, Gerbillus dasyurus, Nesokia indica, Rhombomys opimus* and *Meriones spp.*

(Wasserberg et al,. 2002; Yaghoobi-Ershadi et al., 2004; Pourmohammadi et al., 2008) and rock hyraxes (Svobodova et al., 2006) have been found to be infected with Leishmania species. Which of these animals occure in Yemen remains to be determined. Among the Saudi populations in the south-west of Saudi Arabia (Asir and Al-Baha provinces), an annual incidence of 12 and 38 per 10,000 respectively was estimated for cutaneous leishmaniasis, mainly caused by *L. tropica* (al-Zahrani et al., 1989), whereas for the Jordan Valley an average of 7.5 cases per 10,000 per year was calculated (Mosleh et al., 2008).

Seasonality of cutaneous leishmaniasis

According to data collected from the Ministry of Health and National Centre for Surveillance and Disease Control the prevalence of leishmaniasis in the coastal governorates of Yemen is saisonal, ranging from October to April. This has been confirmed in this study during collecting samples from October 2004 to March 2005. Another study on patients in mountainous governorates showed a striking difference in seasonality, starting in June and reching till October. The reasons for seasonality of outbreak of the disease are the activity of sand fly vectors and the incubation period of the infection. *L. major* has an incubation period that ranges from a few weeks to a few months, but is longer for *L. tropica* which may take 2 -24 months (Harrison's Principles of Internal Medicine, Chapter 205, Leishmaniasis. 1991). *Ph. papatasi*, vector of *L. major*, has its activity and abundance peaks in the spring (April) and the autumn (October), and was found to be not active in mid-summer (July) (Wasserberg et al., 2002).

Another interesting phenomenon with cutaneous leishmaniasis is the fact, that men and children are by far more affected by the disease than women. This may be explained by behavioural or cultural factors which make males more exposed than females, and lack of immunity to make children more exposed than adults (Al-Jawabreh et al., 2003).

In summary it can be concluded that the epidemiological situation is comparable to the neighbouring countries. As the epidemiology of leishmaniasis of these countries is best described for Palestine, these data may serve as a basis to elucidate the situation in Yemen. However, this was not the purpose of this work, but has to be done by future analyses.

4.2 Diagnosis of leishmaniasis

Health indicators for Yemen are poor, with high infant and maternal mortality rates as stated above. The per capita health expenditures are as low as 33 US Dollar per year, and government spending on health was only 3.3 per cent of total public expenditure, a proportion that is the fourth smallest among World Health Organisation (WHO) member states (CSO [Central Statistical Organization], Republic of Yemen. Demographic and Maternal and Child Health Survey 1998).

This facts have to be kept in mind when planning to improve the health situation in the country by introducing new methods for the diagnosis of disease. Commercial diagnostics for infectious diseases are too expensive to be used under such conditions, however, the progress in molecular biology in the past years allows to produce essential diagnostic tools such as recombinant antigens, monoclonal antibodies or molecular probes at costs which can be afforded also by poor countries. The material costs for highly sensitive and specific tests such as ELISA with recombinant antigens, of molecular diagnosis using PCR are few cents per assay and the tests can be produced independent from external support inside the country when the essential theoretical and practical knowledge has been achieved.

Purpose of the experimental work of this thesis was to develop molecular methods for the diagnosis of *Leishmania* infections in Yemen in a most simple way. This could be realized using DNA sequence data for different *Leishmania* species available through the internet in combination with free available computer programmes which are needed to process the huge amount of genetic information. Five different PCR assays have been designed and tested, and some of them turned out to possess a good potential for specific and sensitive diagnosis of leishmaniasis.

4.3 Collection and processing of patient samples

In planning inexpensive molecular tests for developing countries it is also essential to have appropriate methods for collecting samples from patients, for processing these samples and for conservation of the essential components under field conditions. In general, there is litte chance to keep such samples in a freezer at -20℃, and even normal refrigerators are not always available. Traditionally, srapings from leishmanial ulcers are used for microscopical analysis. However, frequently only few, or no parasites at all, are contained in such samples. Better sources for

parasites are biopsies from the border of the ulcer, from lymph nodes, from bone marrow, or from spleen. However, biopsies can hardly be taken under field conditions, but alternatively, few millilitres of peripheral blood can be taken with a syringe without the risk of accidental infection or cross-contamination. The parasites amplify mainly in macrophages, and even in the chronical stage of disease sufficient infected white blood cells can be detected in the circulation.

A simple method for blood collection and processing under field conditions has been developed in our laboratory in Giessen. The protocol is described in the Materials and Methods section. In short, blood is collected in syringes containing EDTA or citrate as anti-coagulant. Blood cells are sediment by centrifugation (a simple hand-operated centrifuge has been used during this work, see Figure.10), the supernatant plasma was collected and conserved in 50 % glycerol, and finally, the yellow layer of white blood cells and platelets (buffy coat) was harvested with a pipette. The excess of red blood cells in this crude buffy coat preparation were lysed by a low ionic stength buffer, the cells sedimented again by short centrifugation and mixed with a guanidinium hydrochloride solution. The guanidinium hydrochloride has two functions: first, it lyses the cells for later DNA extraction, and second, it protects the DNA for extended periods of time (months) from degradation even at ambient temperatures.

The DNA from the lysed white blood cells was lateron easily purified in the laboratory by adding small amounts of silica (pulverized SiO_2 which binds exclusively DNA under these conditions), several washing steps with diluted ethanol and finally eluted in pure and concentrated form by a low salt buffer. This procedure proved to be very appropriate for field conditions as no toxic chemicals such as phenol and chloroform are needed, and the costs of consumables are minmal. Furthermore, the whole procedure can be carried out in a single tube, which is important in terms of avoiding cross-contamination. DNA obtained by this way was used to test new PCR assays, and the plasma fraction was used to test new immunological assays by another member of the group.

4.4 Kinetoplast DNA (kDNA)-specific PCR

The first test developed was a PCR with primers specific for the kinetoplast DNA of *Leishmania* which consists of approximately 10.000 copies of circular DNA

molecules ("minicircles") containing a conserved sequence of approximately 120 bp. This DNA has been perfered for PCR diagnosis by many groups because due to the high copy number of target sites, less than a single parsite could be detected theoretically. kDNA-PCR has repeatedly been found to be the most sensitive of diagnostic assays for leishmaniasis (Lachaud et al., 2002; Nasereddin et al., 2006; Romero et al., 2009).

The common part of the kDNA minicircles is not perfectly conserved, but consensus sequences can be found at the two ends of a more conserved region of approximately 120 bp. These consensus sequences have been used to develop primers for PCR amplification by others (references see above). According to the Prime3 software used for selection of PCR primers, the published primers appeared to be not optimal, basically because forward and reverse primers revealed differences in the annealing temperatures of more than 20℃. This is not appropriate for specific PCR since the hybridization temperature has to be set according to the primer with the lower binding strength. The other primer can bind under these conditions also to sites which do not match perfectly, thus creating unspecific background amplification. Therfore, it was attempted to predict primers with more equal annealing temperatures. The performance of the resulting (shorter) primers in kDNA-specific PCR assays was compared with the efficiency of the primers used by other groups, but no significant difference in sensitivity and specificity were observed.

The kDNA-specific PCR assay developed by this way appeared to be most sensitive, however, it did not discriminate between cutaneous and visceral leishmaniasis. Furthermore, processing of the results was somewhat demanding because the small resulting amplification product of 113 bp could only be redissolved on 6 % polyacrylamide gels. Therefore, some alternative PCR assays were developed in addition.

4.5 18S ribosomal RNA-specific PCR

Leishmania species contain approximately 160 copies of the genes for the small subunit of ribosomal RNA (18S rRNA or ssu rRNA). These genes have been used by several groups as target for diagnostic PCR, even though most of them were using different sets of primers (Spanakos et al., 2002; Schönian et al., 2003; Chargui et al., 2005). It is agreed within all these groups that 18S rRNA-specific PCR is highly

sensitive, superior to PCR assays with other target genes in the *Leishmania* genome. The genes for the 18S rRNA are highly conserved between the different species of *Leishmania*, thus, discrimination within the genus is not possible. However, clear discrimination is feasible from other species such as the closely related *Trypanosoma cruzi*, or the non-pathogenic *Trypanosoma rangelli* which occurs frequently in Latin America. Only part of the sequence of the gene can be used for establishing discriminatory PCR because of the high degree of homology of 18S rRNA sequences between all organisms, However, some regions differ sufficiently to be appropriate for this purpose.

By means of Primer3 software, two sets of PCR primers were selected to produce a fragment size that could be easily analysed be agarose gel electrophoresis. Due to the lower gene copy number as compared with kDNA, it appeared to be necessary to employ nested PCR using a second set of primers. The disadvantage of a second amplification cycle is compensated not only by higher sensitivity, but also by a higher specificity. The second set of primers leads to enrichment of correct amplification products only, thus avoiding false positive results.

The first PCR with the outer set of primers led only in few cases to a visible band on agarose gels (see Figure 15). This could be due to several reasons. First, one or both primers are not optimal and higher sensitivity could be reached with alternative primers. However, also the set of inner primers, when used alone, did not lead to more sensitivity. Another reason for this fact could have been the relative low amont of DNA used for the assays. The DNA of the samples from Yemen was limited and had to be spared for many other assays. Normally not more than 2 per cent of the DNA isolated from one sample were used per assay. Taking 10 per cent of DNA from a sample would probably improve the sensitivity markedly. Furthermore, in all assays a self-made Taq DNA polymerase was used which may not have the same quality as expensive commercial enzymes. Nevertheless, clear results were obtained with nested PCR using aliquots of the amplification products of the first PCR as template for the second PCR with inner primers. The sensitivity was almost as high as obtained with the kDNA-specific PCR, and the fact that the fragments can be easily separated on agarose gels allows to use this test in large scale. To compare the quality of this test with other 18S rRNA-specific PCR assays, tests with PCR primers used by other groups need to be done.

4.6 PCR assays with tandem repeat sequences

The three completely sequenced Leishmania strains (*L. major, L. infantum* and *L. brasiliensis*) accessible in the Sanger Gene Database contain plenty of tandemly repeated sequences. Using the "Tandem Repeats Finder" computer program (http://tandem.bu.edu/trf/trf.html), the three genomes were analysed for tandem repeats in the DNA. This analysis revealed many different repeats with different lengths of the repetitive unit. The highest number were more than 1000 repeats of a 45 nucleotide sequence, other repeats with lengths between ten and more than 100 nucleotides occured somewhat less frequently, but several hundred repetitions were not uncommon. Approximately 10 per cent of the genomes consist of such repetitive sequences. The repeats were usually fragmented in several subfractions (2-10) distributed in the same, but also in different chromosomes. Some repeats were highly conserved, others differed up to 10 per cent in the nucleotide composition of the single units. Most of the repeats occured in related form in all three Leishmania strains even though in different numbers. However, there were several repeats specific for a single strain thus representing candidates for species-specific PCR. Some repeats were also to be observed in related form in the genome of Trypanosoma cruzi.

The repeats occure in non-coding as well as in protein coding regions of the genomes. In coding regions, they are translated in tandem arrays of amino acid sequences which are in part highly immunogenic leading to a strong antibody reaction. Such tandem arrays of amino acids have been used in recombinant form as antigens for developing specific immunoassays such as the K39 antigen (Burns etal., 1993).

As these tandemly repeated sequences differ in part between the three Leishmania species, it seemed to be interesting to test if they could serve as targets for specific PCR assays. In contrast to normal PCR, the amplification products were not expected as a single band of defined length, but as a ladder of bands with distances between the different steps corresponding to the length of a single repeat unit. By means of the primer3 software appropriate primers were selected for three such repeats: the 45 basepair repeat with approximately 1000 copies occuring in all strains, a 64 basepair repeat with a frequency of approximately 400 copies common

to all strains as well, and a 39 basepair repeat present in L. infantum exclusively. None of these repeats was present in related form in *Trypanosoma cruzi*.

As can be seen from the Results section, the PCR amplification of these repeats worked out in principle as predicted, however, the results were not as convincing as expected. Even though the 45 basepair repeat led to a characteristic ladder with the purified DNAs of *L. major* and *L. infantum*, this ladder diaspeared almost completely when the *Leishmania* DNA was mixed with buffy coat DNA. Obviously, human DNA contains many sequences which serve as template for the primers used in this PCR. The results were not much different under more stringent conditions, i.e when the highest possible annealing temperature for these primers was selected.

The results with the PCR specific for the 64 basepair repeat looked even worse. The primers led to strong background reactions even with the purified parasite DNAs indicating a too low selectivity of this PCR.

However, the 39 basepair specific PCR led to results which look promising for diagnosis. The resulting ladder of bands is specific for *L. infantum*, that means for visceral leishmaniasis (Figure 19). There is no background reaction in controls without DNA and with buffy coat DNA from patients with cutaneous leishmaniasis (not shown in the Figure). The lowest bands of the ladder are not visible in the amplification products with buffy coat DNA from visceral leishmaniasis patients, however, higher multimeric forms are present, at least in some of the lanes. As with the 18S rRNA-specific PCR, the weak reaction may be due to the limiting amounts of DNA added to the assays. In any case it appears to be worthwhile to repeat this approach with more DNA. In addition, the stringency of the assay could probably be improved, and alternative primers specific for the 39 basepair repeat could be tested. The PCR may not be sufficient as only test, but it could be used as a second test after demostration of the infection either by kDNA- or 18S rRNA-specific PCR.

4.7 Concluding remarks

Leishmaniasis is still a widely negected disease which means that even though millions of people in developing countries suffer from the infection, there are neither appropriate diagnostic procedures nor sufficiently effective drugs for treatment available. At present, several groups worldwide study the variability of Leishmania strains with complex

techniques such as analysis of enzyme polymorphismes, restriction length polymorphism, or determination of microsatellite patterns of the organisms. Even though these techniques contribute to evaluate the genetics and epidemiology of the parasite in much detail, they are by far too demanding to be used for answering the simple question: is there an infection with *Leishmania* or not? To reduce suffering from this disease, prove of the infection has to be simple and inexpensive since it affects mainly the poorest of the poor.

The first step of diagnosis has to discriminate between an infection with *Leishmania* and other causes of the dermal ulcer which may have bacterial or fungal origin. Only in a second step is needed to differentiate between cutaneous and potentially visceral leishmaniasis in the Old World, or between cutaneous, and mucocutaneous leishmaniasis in the New World. As up to 20 per cent of all cutaneous cases in Latin America develop to the mucocutaneous form after some time, a proven Leishmania infection is treated anyway and this discrimination may not be needed therefore. A detailed analysis of the specific strain may be helpful for the prognosis of disease and for the prediction of drug-resistancy, but this is not the first priority.

Because of the severe side effects of the drugs, cutaneous leishmaniasis in the Old World is normally only treated if the ulcers are severe and could have disfiguring consequences. However, since also the life-threatening visceral form of the disease begins with a cutaneous ulcer as a first clinical symptom, it will be needed in the future to decide already in this early stage whether the ulcer is caused by a cutaneous form (*L. tropica* and *L. mayor* in the Arabian peninsula), or by the dangerous visceral form (*L*. *donovani* or *L. infantum*). There are good immunodiagnostic assays available, mainly based on the recombinant antigen rK39, but these assays are not sufficient because specific antibodies can persist for many years and it is difficult to decide, wether they derive from an healed infection, or indicate acute disease. Therefore, a simple and inexpensive molecular test would be very helpful.

It was goal of this work to contribute to this aspect. Even though the assays established so far could be improved to a certain degree, it becomes obvious that PCR diagnosis is extremely useful and not as complicated and expensive that it could not be introduced to Yemen. For this purpose it was also essential that procedures basic for PCR diagnosis, the collection of patient samples, the conservation of the essential fractions DNA and plasma, the isolation and purification of DNA and the preparation of Taq DNA polymerase have been simplified in such a way that they can also be performed in a low equiped laboratory and for little money.

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EIDESSTATTLICHE ERKLÄRUNG

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Giessen im Juli 2009

Abdullatif Ali