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Identification of a Novel Recombinant Protein for Improved Diagnosis of Visceral Leishmaniasis in Sudan

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To those who never stop loving me,

my parents, Einas, Gawad, Elwalid and Mustafa.

CONTENTS:

LIST of ABBREVIATIONS:

AA	Amino acid
AIDS	Acquired Immuno-Deficiency Syndrome
AmpR	Ampicillin resistant
APC	Antigen presenting cell
ASC	Asymptomatic case
bp	Base pair
BSA	Bovine serum albumin
CI	Confidence interval
CL	Cutaneous leishmaniasis
cpb	cysteine proteinase B
CR	complement receptor
CVL	Canine visceral leishmaniasis
DAT	Direct Agglutination Test
DDT	Dichloro-Diphenyl-Trichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'- Deoxyribonucleoside-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylendiamintetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal calf serum
FD	Freeze-Dried
FRET	Fluorescence resonance energy transfer
gp63	Glycoprotein 63
H_2O	Water
H_2O_2	Hydrogen peroxide
HASPB	Hydrophilic acylated surface protein B
HIV	Human Immunodeficiency Virus
IFAT	Immunofluorescent antibody test
IFN-γ	Interferon gamma
Ig	Immunoglobulin

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-	Interleukin-
IMTA	Institute of tropical medicine Antwerp
IPTG	Isopropyl-β-D-thiogalactopyranosid
ITN	Insecticide-treated bednet
KA	Kala-azar
Kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani
LD	*Leishmania donovani*
LN	Lymph node
LPG	Lipophosphoglycan
LST	Leishmanin skin test
M	Molar
MC	Mucosal leishmaniasis
MCL	Mucocutaneous leishmaniais
mg	Milligramm
MHC	Major Histocompatibility Complex
MLEE	Multilocus enzyme electrophoresis
MLMT	Multilocus microsatellite typing
MOPS	3-(N-morpholino)propanesulfonic acid
MR	Mannose-fucose receptor
MSF	Medecins Sans Frontieres
NaOH	Sodium hydroxide
Ni-NTA	Nickel-nitriloacetic acid
NK	Natural killer
NNN	Novy-MacNeal-Nicolle
NO	Nitric oxide
NPV	Nagative predictive value
O/N	Over night
OD	Optical density
ORF	Open reading frame
Ori	Origin of replication

PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR-restriction fragment length polymorphism analysis
PKDL	Post-kala-azar dermal leishmaniasis
PMSF	Phenylmethylsulfony fluoride
PPG	Proteophophoglycans
PPV	Positive predictive value
psi	Pound per square inch
q-PCR	Quantitative PCR
rK	Recombinant kinesin
rpm	Round per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SC	Symptomatic case
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sec.	Second
SLA	Soluble *Leishmania* antigen
TAE	Tris (hydroxymethyl) Aminomethan-Acetat-EDTA-buffer
TB	Tuberculosis
TBE	Tris (hydroxymethyl) Aminomethane-Boric acid-Dinatrium EDTA
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGF-β	Transforming growth factor-beta
Th	T helper
TR	Tandem repeat
U	Unit
V	Volts
v/v	Volume per volume
VL	Visceral leishmaniasis
VL/HIV	Visceral leishmaniasis/human immunodeficiency virus
VLS	Visceral leishmaniasis suspect

WHO	World Health Organisation
w/v	weight per volume
WB	Western blot

SUMMARY:

For effective control of visceral leishmaniasis (VL) in East Africa, new rapid diagnostic tests are required to replace current tests with low sensitivity. The aim of this study was to improve diagnosis of VL in East Africa by testing new antigens from an autochthonous *Leishmania donovani* strain.

We cloned and expressed a new antigenic protein (designated rKLO8) of *Leishmania donovani* containing putative conserved domains of significant similarity with immunodominant kinesin proteins of several *Leishmania* strains. rKLO8 exhibited 93% amino acid identity with cloned kinesin proteins of *Leishmania infantum* (rK39) and 88% with *Leishmania donovani* (rKE16). Sequence analysis of rKLO8, rK39 and rKE16 revealed genetic heterogeneity within immunodominant epitopes of these antigens.

Immunoreactivity of the purified recombinant protein rKLO8 was confirmed by Western blot and enzyme-linked immunosorbent assay (ELISA). Importantly, antibody reactivity against rKLO8 was detected in VL patients but not in healthy controls. We successfully developed a diagnostic ELISA based on rKLO8 which was evaluated with sera from VL patients originated from Sudan, India and France. Direct comparison between rKLO8– and rK39 ELISA revealed that our newly developed test system showed similar reactivity with sera of VL patients from France but increased with sera from Sudanese and Indian patients.

Next, we compared the diagnostic performance of rKLO8- and rK39 ELISA with other commercially available tests, including rK39- and rKE16 rapid tests and direct agglutination test (DAT). Results showed that all tests performed very well in India but best sensitivity in all countries was observed with rKLO8- and rK39 ELISA. However in Sudan and France, the two rapid tests showed low sensitivity. DAT showed better sensitivity in Sudan and India than in France. The sensitivity of all tests was markedly reduced in VL patients co-infected with human immunodeficiency virus (HIV). Furthermore, the rKLO8 ELISA was also evaluated with sera of *Leishmania*-infected dogs from Portugal, Croatia and Brazil. The results showed that rKLO8 ELISA was

similar to DAT but more sensitive than the routinely used immunofluorescent antibody test (IFAT).

Thus, increased reactivity of sera from Sudanese VL patients with rKLO8 shows that this antigen is a potential candidate for improving VL diagnosis in Sudan and other regions of East Africa where similar strains of *Leishmania donovani* are endemic.

ZUSAMMENFASSUNG:

Die effiziente Kontrolle der viszeralen Leishmaniose (VL) in Ostafrika hängt ganz besonders von einer schnellen und sensitiven Diagnostik ab. Gegenwärtige Testsysteme sind für die Diagnostik der VL im Sudan leider nicht besonders gut geeignet. Ziel dieses Projektes war die Identifikation und Testung neuer Antigene eines aus dem Sudan stammenden *Leishmania donovani*-Stammes zur Verbesserung der VL-Diagnose in Ostafrika.

Es wurde ein neues Antigen aus *Leishmania donovani* identifiziert und kloniert (rKLO8), das eine hohe Sequenzübereinstimmung mit dem immundominaten Kinesinprotein verschiedener *Leishmania*-Stämme aufweist. Die Immunreaktivität des aufgereinigten rekombinanten Proteins wurde durch Westernblot und ELISA getestet und bestätigt. Es zeigte sich, dass rKLO8 nur mit Seren von VL- Patienten, nicht jedoch gesunden Individuen reagiert.

Zusätzlich wurde ein auf dem rKLO8-Protein basierter Test (ELISA) etabliert und mit Patientenseren aus dem Sudan, Indien und Frankreich evaluiert. Eine vergleichende Studie zeigte, dass das diagnostische Potential des neu entwickelten rKLO8 Tests im Sudan und Indien gegenüber dem derzeit verwendeten Testantigen, rK39, deutlich besser ist.

Weiterhin wurde das diagnostische Potential der rKLO8 - und rK39 ELISA mit verschiedenen kommerziellen Tests, den rK39- und rKE16-Schnelltests und dem direkten Agglutinationstest (DAT) verglichen. Alle Tests zeigten bei Patienten aus Indien ähnlich gute Ergebnisse, bei VL-Patienten aus anderen Ländern jedoch zeigten der rKLO8- und rK39-ELISA die höchste Sensitivität.
Ein weiterer Befund war, dass die Koinfektion mit dem HI-Virus die Sensitivität aller Testsysteme beträchtlich reduzierte.

Zuletzt wurde der neu entwickelte rKLO8-Test auch mit Seren VL-infizierter Hunde aus Portugal, Kroatien und Brasilien getestet. Der ELISA war in seiner diagnostischen Potenz ähnlich dem DAT, im Vergleich zum routinemäßig eingesetzten immunofluoreszenz-basierten Antikörpertest (IFAT) jedoch deutlich sensitiver.

Zusammengefasst stellt rKLO8 aufgrund seiner erhöhten Reaktivität mit Patientenseren aus dem Sudan ein potentielles Antigen dar, mit dem die VL-Diagnostik im Sudan und anderen *Leishmania donovani* endemischen Regionen Ostafrikas verbessert werden kann.

1. INTRODUCTION:

1.1 General introduction:

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus *Leishmania* and is transmitted by the bite of phlebotomine sandfly vectors. It has world-wide distribution, affecting millions of people in 98 countries on 5 continents with approximately 350 million people being under the risk (Desjeux, 2004; Alvar et al., 2012). It is classified as one of the most neglected diseases because of the limited resources invested for the control of the disease (Yamey and Torreele, 2002).

Leishmaniasis has three major clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL), the latter is the most severe form and is caused by various species of *Leishmania (L.) donovani* complex (Lainson and Shaw, 1987; Jamjoom et al., 2004). Of particular concern is the increase and expansion of VL in areas in East Africa that were previously non-endemic (Marlet et al., 2003; Alvar et al., 2007b). The mass population movement caused by the armed conflicts (e.g. Somalia) or peace (e.g. Sudan) let to increase exposure to sandfly vectors and contributed to increase geographical areas of the disease (Reithinger, et al., 2007).

Sudan is one of the six VL-endemic countries that constitute 90% of global VL cases and has the highest incidence of post-kala-azar dermal leishmaniasis (PKDL) in the world (Ghalib and Modabber, 2007; Alvar et al., 2012). The country has experienced several outbreaks in the last two decades, the recent ones occurred between 2009 - 2011 in South Sudan (the new Republic of South Sudan) where more than 10,000 patients were reported (Doctors Without Borders, kala-azar home page, http://www.doctorswithoutborders.org/). The disease is further complicated by the spreading of HIV in VL-endemic areas. In Africa, the number of VL/HIV co-infections is increasing; approximately 30% of all VL patients in Ethiopia are also infected with HIV (Alvar et al., 2008).

1.2 History of leishmaniasis:

Leishmaniasis was first reported in Jessore (now in Bangladesh) in 1824 during outbreak of fever that was thought to be due to malaria but failed to respond to quinine. In 1862, the disease spread to Burdwan in India, where it became epidemic. It is accepted that the Scottish pathologist, William Leishman and Professor Charles Donovan discovered the parasite in the spleen of an Indian patient in 1903 (see Cox, 2002). In early 1900s, leishmaniasis was reported in a Sudanese child who had come from Bahr el Gazal in South Sudan, which was the first reported case in East Africa (Neave, 1904).

A full description of *Leishmania* in skin lesions was given by James Homer Wright, an American pathologist (Wright, 1903). However, David Cunningham might be the first who described the parasite in 1885 in specimen of skin lesion (see Write, 1903). The role of *Phlebotomus* sandflies in transmission of the infection to humans was experimental confirmed in 1921 by the Sergent brothers, Edouard and Etienne. In 1911, a Brazilian pathologist Gaspar Vianna showed that *Leishmania* in South America are different from those in Africa and India. His observation let to discovery of a new species, *Leishmania braziliensis (*see Cox, 2002*).*

The origin of *Leishmania* parasites is not well understood. Recent studies have shown that Sudan might be the original focus of leishmaniasis; *Leishmania donovani* DNA was detected in bone marrow samples from ancient Nubian mummies from North Sudan dated back to around 4000 BC (Zink et al., 2006; Pratlong et al., 2001). Subsequently, the disease could have been introduced to the Indian subcontinent and the New World in early migrations (Ibrahim, 2002). Recent spreading of *L. infantum* from Southwest Europe to South America has occurred during European colonization of the New World, where the parasite was designated a distinct species name, *Leishmania chagasi* (Kuhls et al., 2011).

1.3 Taxonomy of *Leishmania*:

Historically, classification of *Leishmania* was based on eco-biological criteria such as sandfly species, geographical distribution, and clinical presentation of the disease. But these methods were insufficient and therefore other methods have

been used such as the patterns of polymorphism in kinetoplast DNA (kDNA), proteins or antigens. *Leishmania* and *Viannia* subgenera are separated based on their location in intestine of the sandfly; *Leishmania* develop within the midgut and foregut of the host while *Viannia* undergo an additional developmental phase within the hindgut. Other authors used isoenzyme analysis to define species complexes within the subgenera. Since 1970s, immunological, biochemical and genetic criteria are used for the current taxonomic classification (**Fig. 1.1**), which was developed by the World Health Organization (see Cupolillo et al., 2000; Banuls et al., 2007).

Recently, a new taxonomic classification was introduced for the *L. donovani* complex including *L. donovani*, *L. infantum*, *L. chagasi*, and *L. archibaldi*, the causative agents of VL. Here all strains of *L. donovani* complex were phylogenetically grouped in two main clusters: *L. donovani*– strains from East Africa, India and Middle East; *L. infantum*– parasites from Europe, North Africa and Latin America. Therefore, *L. donovani* and *L. infantum* are considered the only recognized species within *L. donovani* complex. The degree of diversity among strains of *L. chagasi* and *L. infantum* is lower than that found within *L. donovani* strains (Mauricio et al., 2000; Lukes et al., 2007).

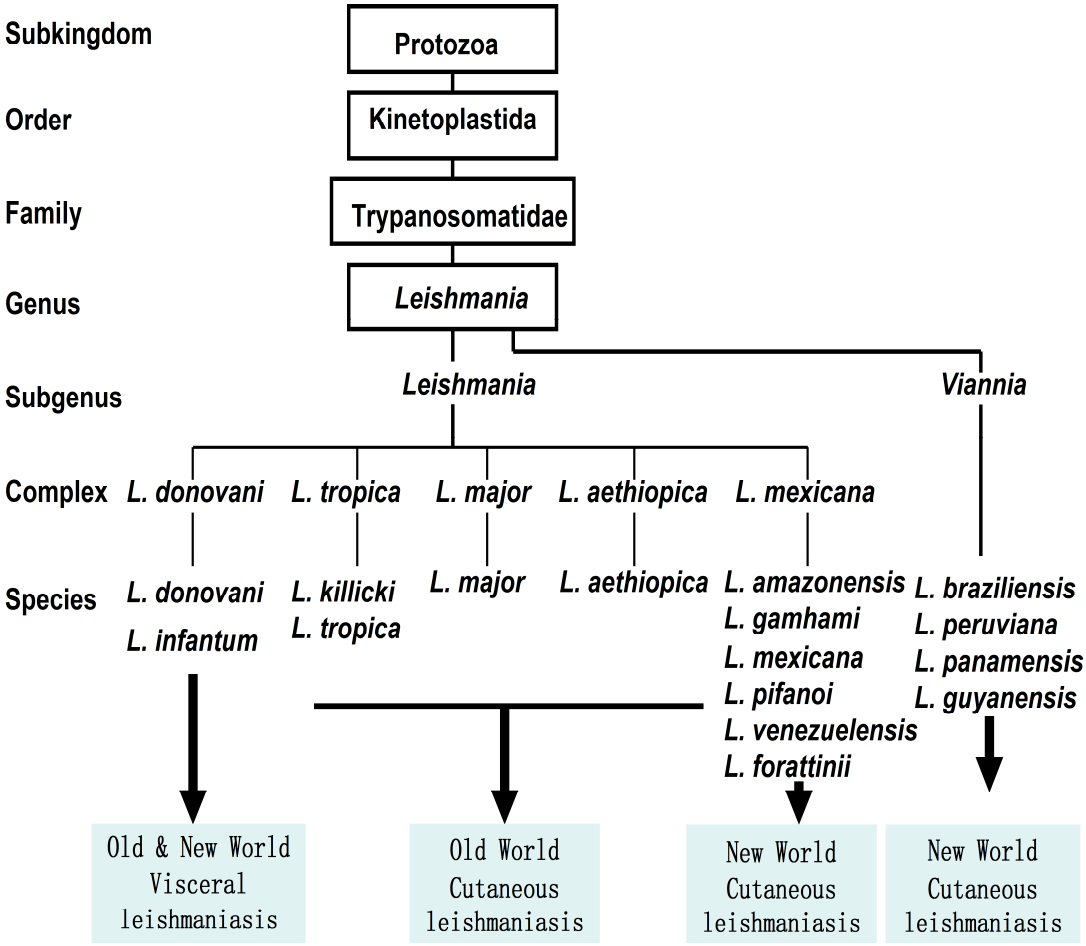


Fig. 1.1: C**lassification of the genus *Leishmania***. The diagram shows the two subgenera and species of medical importance with their related diseases. Taxonomy is based on a scheme published by the World Health Organisation (WHO, 1990) with modification from the literature (Mauricio et al., 2000; Lukes et al., 2007; Bates, 2007).

1.4 Clinical pictures of leishmaniasis in humans:

Clinical symptoms of *Leishmania* infection in humans depend on parasite species and infected tissues, and represent a wide range of clinical manifestations (**Fig. 1.2**). After infection, many people remain asymptomatic for a certain time period, reflecting differences in parasite virulence and host population characteristics within the host (Chappuis et al., 2007). The most common forms of leishmaniasis in humans with their respective parasites are as follows:

1.4.1 Cutaneous leishmaniasis (**CL**):

CL is the most common clinical form, which causes skin sores (ulcers) at the site of sandfly bite. It is mainly caused by *L. amazonensis, L. braziliensis, L. guyanensis, L. mexicana, L. panamensis, L. naiffi, L. venezuelensis, L. lainsoni and L. shawi* in the New World and *L. major, L. aethiopica, L. tropica, L. arabica and L. gerbilli* in the Old World. In Sudan, it is caused by *L. major* and *L. tropica.* However, other species such as *L. donovani* and *L. infantum* can also cause the disease (see Banuls et al., 2007). Few weeks after infection, patients develop skin ulcers in the exposed areas such as face, arms and legs, which usually heal within few months leaving scars. The disease may show different clinical manifestations in regard to the number of lesions and whether lesions are self-healing or require treatment (see Modabber et al., 2007).

1.4.2 Mucocutaneous leishmaniasis (**MCL**):

MCL is most common in Latin America. In contrast to CL, lesions lead to extensive destruction of the mucous membranes of the nose, mouth and throat cavity and surrounding tissues. MCL is mostly caused by *L. braziliensis* in Central and South America, but other species may also cause the disease, such as *L. panamensis and L. guyanensis;* in Ethiopia and Kenya it caused by *L. aethiopica* (see Banuls et al., 2007).

1.4.3 Diffuse cutaneous leishmaniasis (**DCL**):

DCL is a chronic form of the disease which occurs in individuals with impaired cell-mediated immune responses (Gaafar et al., 1995). It is characterized by non-ulcerating, non-necrotizing nodular lesions spread over the body that never heal spontaneously and tend to relapse after treatment, resembling lepromatous leprosy. It caused by *L. aethiopica* in Ethiopia and Kenya, *L. major* in Sudan and by *L. amazonensis* and *L. braziliensis* in Central and South America (see Zerpa et al., 2007).

1.4.4 Visceral leishmaniasis (VL):

VL (also known as kala-azar) is the most severe form of the disease with 100% mortality rate if not treated. It affects vital organs of the reticuloendothelial system such as spleen, liver, bone marrow, lymph nodes. VL is caused by species which belong to *L. donovani* complex that includes *L. donovani* in East Africa and the Indian subcontinent, *L. infantum* in Europe, North Africa and Latin America (Lainson and Shaw, 1987; Mauricio et al., 2000; Jamjoom et al., 2004; Lukes et al., 2007). In most cases, patients develop the disease weeks to months after infection. Symptoms start with irregular fever, headache, sometimes with cough, abdominal pain, diarrhoea, vomiting, epistaxis and anaemia. Weeks later, patients lose weight and may show severe malnutrition. Signs include splenomegaly, hepatomegaly and/or lymphadenopathy, which is more frequent in Sudan than in other countries. Without treatment, patients may suffer from bacterial infection and severe anaemia (Jeronimo et al., 2007). However, co-infections with other pathogens such as *Plasmodium* species, *Mycobacterium tuberculosis* and HIV may give rise to atypical clinical pictures (de Beer et al., 1991; van den Bogaart et al., 2012). Other factors that may additionally complicate the infection is the occurrence of different *Leishmania* species or strains in the same host (Ibrahim et al., 1994; Martinez et al., 2002).

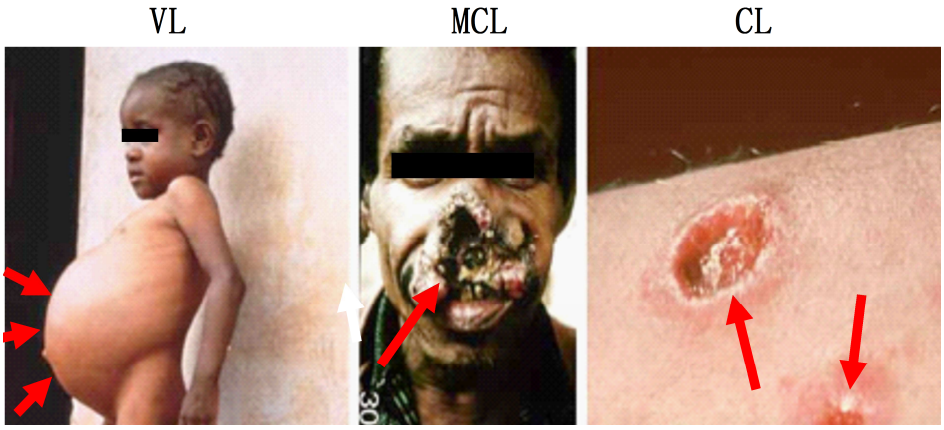


Fig. 1.2: Clinical symptoms of leishmaniasis in humans: visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL) and cutaneous leishmaniasis (CL). Adopted from: http://www.stanford.edu/group/parasites/ParaSites2007/ImmuneEvasion/.

1.4.5 Post-kala-azar dermal leishmaniasis (PKDL):

PKDL is a skin condition that develops several months or years after successful VL treatment in individuals who are otherwise healthy (see Zijlstra et al., 2003). It occurs in 50% and 5-10% in treated VL patients in Sudan and India, respectively. However, it may also appear in individuals with no history of VL (Uranw et al., 2011). Typically, it starts on the face as papules which can spread to other parts of the body. It may increase in size to become macular, papular or nodular lesions in which the parasites can be detected (**Fig. 1.3**) (see Zijlstra et al., 2003).

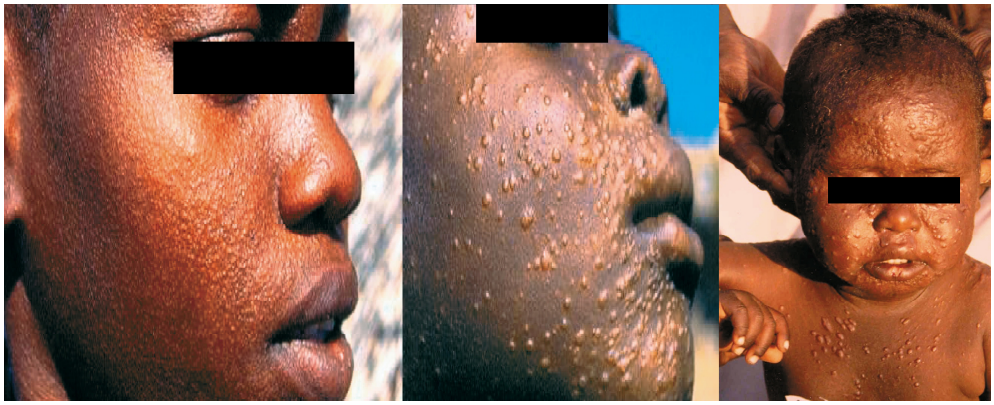


Fig. 1.3: Sudanese post-kala-azar dermal leishmaniasis (PKDL): From left to right: micropapular, papular and nodular lesions. Adopted from Zijlstra et al., (2003).

1.5 Epidemiology and impact of leishmaniasis:

Leishmaniasis has a world-wide distribution in areas ranging from rain forests to deserts, which cover some parts of the Old World (Africa, Asia, Europe) and the New World (the Americas). Approximately, 0.2 - 0.4 million visceral leishmaniasis (VL) cases and 0.7 - 1.2 million cutaneous leishmaniasis (CL) cases occur annually in 98 countries (**Fig. 1.4** & **1.5**). More than 90% of global VL cases occur only in six countries: India, Bangladesh, Sudan, South Sudan, Brazil and Ethiopia with approximately 20,000 – 40,000 deaths annually (Alvar et al., 2012). The disease affects both humans and animals with higher prevalence in poor rural communities than urban areas. Climate and economic changes are considered as

factors that affect the migration of people and expand the geographic range of the sandfly vectors (Desjeux, 2001; Reithinger, et al., 2007).

VL in Asia is endemic in 6 countries: India, Bangladesh, Nepal, Bhutan, Sri Lanka and Thailand. This region is considered the biggest focus of VL in the world, with an estimated annual incidence of 162,100 – 313,600 (Alvar et al., 2012). The disease affects mainly poor people in rural areas; approximately 80% of all cases in the region come from the state of Bihar in India. There are approximately 200 million people are at risk for VL in Indian subcontinent (Joshi et al., 2008).

East Africa is the second major endemic region of VL in the world. The disease occurs either as sporadic severe outbreaks or as endemic disease (Ibrahim, 2002). The seven countries where VL is endemic with high annual incidence are Sudan, Ethiopia, Kenya, Somalia, Uganda and Eritrea. Approximately, 29,400 – 56,700 new cases occur each year (Alvar et al., 2012; Mueller et al., 2012).

In Europe, VL is endemic in Mediterranean countries including Italy, France, Spain, Portugal, Greece, Croatia, Albania, Malta, Cyprus (see Schallig et al., 2013), with an annual incidence of 1,200 – 2,000 (Alvar et al., 2012). The disease has spread also to neighbouring countries such as Bosnia and Herzegovina, Bulgaria, Hungary, Macedonia, Monaco, Romania, Azerbaijan, Georgia, Kazakhstan, Tajikistan, Turkey, Turkmenistan and Uzbekistan (WHO, 2004). Due to human-made environmental and global climate changes, the disease is thought to be increasing (Dujardin et al., 2008). In non-endemic countries of Europe, the majority of infections occur through travelling to endemic countries. However, reports indicate that VL is spreading in central Europe such as Germany where the disease was diagnosed in humans and dogs who had never been in known-endemic areas (Gothe, 1991; Bogdan et al., 2001; Mencke, 2011).

In the New World, VL has been reported in several countries, covering parts of Southern USA, Mexico, North of Argentina, Brazil, Paraguay, Bolivia, Venezuela, Suriname, Guyana, Colombia, Honduras, Panama, Costa Rica, El Salvador, Guadeloupe, Guatemala and Nicaragua (see Kuhls et al., 2011). The annual incidence of VL throughout America is 4,500 – 6,800 (Alvar et al., 2012). Brazil is the country with the highest prevalence in this region, representing approximately

90% of the total cases (see Romero and Boelaert, 2010). In this country, the epidemiology of the disease is previously associated with migration to urban areas, causing several outbreaks in large cites (Albuquerque et al., 2009; Harhay et al., 2011). Until 1993, more than 90% of VL cases were reported in the north and north east of Brazil. However, in 2003 the disease spread to the south east and central west of the country. This increase was due to construction of a major road that allowed movement of people with their infected dogs to new areas (see Palatnik-de-Sousa and Day, 2011).

CL is more widely distributed than VL, with about one-third of cases occurring in each of the three regions, America, Mediterranean area, and Asia. The ten countries with the highest numbers of CL cases are Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Sudan, Costa Rica and Peru, together they account for 70% to 75% of the global estimated CL incidence (Alvar et al., 2012). In Sudan, most of the reported cases are from west and central regions where several outbreaks were reported: in Shendi- Atbara region (1976 - 1977), in Tuti Island where approximately 10,000 cases were recorded (1985 – 1987) and along the Nile, north of Khartoum to the border with Egypt (Malaria Consortium, 2010).

In some regions, outbreaks of CL are associated with armed conflicts and travel. In 2005, cases of CL were reported among International Security Assistance Force and the local population of Mazar-e Sharif, North Afghanistan (Faulde et al., 2008). In Syria, two years after the civil war started, an alarming increase in CL cases has been reported, with expansion of the disease to South Turkey (Alasaad, 2013). In the United States, infection is associated with travel and immigration; most of the cases are travellers that acquired the infection in Latin America, such as Costa Rica (Centres for Disease Control and Prevention, Epidemiology and Risk factors home page http://www.cdc.gov/parasites/ leishmaniasis/epi.html).

Fig. 1.4: Global distribution of visceral leishmaniasis. Sources: http://www.sanofi-paediatrics.com/web/endemic/leishmaniasis/pathology.

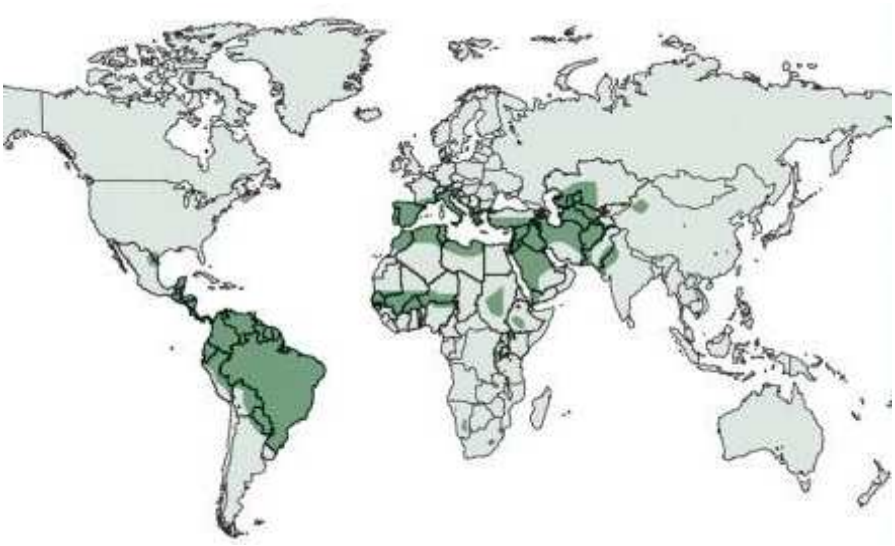


Fig. 1.5: Global distribution of cutaneous leishmaniasis. Adopted from Reithinger et al., (2007).

1.6 Leishmaniasis in dogs:

Leishmaniasis in dogs or canine VL (CVL) is endemic in more than 70 countries in the world including South Europe, Africa, Asia, South and Central America (Baneth et al., 2008). The disease has been also reported in some non-endemic countries such as North America, United Kingdom, Netherland and Germany (Gothe et al., 1991; Petersen and Barr, 2009; Shaw et al., 2009). In these countries, infection is restricted to dogs that travelled to or have been imported from endemic countries in South Europe (Spain, France, Italy, and Turkey) (Gothe et al., 1991; Petersen and Barr, 2009). The risk of introducing leishmaniasis to Central Europe from Mediterranean countries depends on the climate and environmental conditions (Ready, 2010). The maximum northern latitude for the survival of sandflies may exceed the northern boundaries of Germany, creating better opportunities for survival of the sandfly vectors (Desjeux, 2001).

Leishmaniasis in dogs causes a wide range of clinical signs including lymphadenopathy, weight loss, emaciation (extremely thin), hepatosplenomegaly, conjunctivitis, keratitis, onychogryphosis (thickening and increase in curvature of the nail) and lesions on skin, limbs and ears (**Fig. 1.6**). Infected dogs can also remain asymptomatic (Marzochi et al., 1985; Slappendell, 1988; Berrahal et al., 1996).

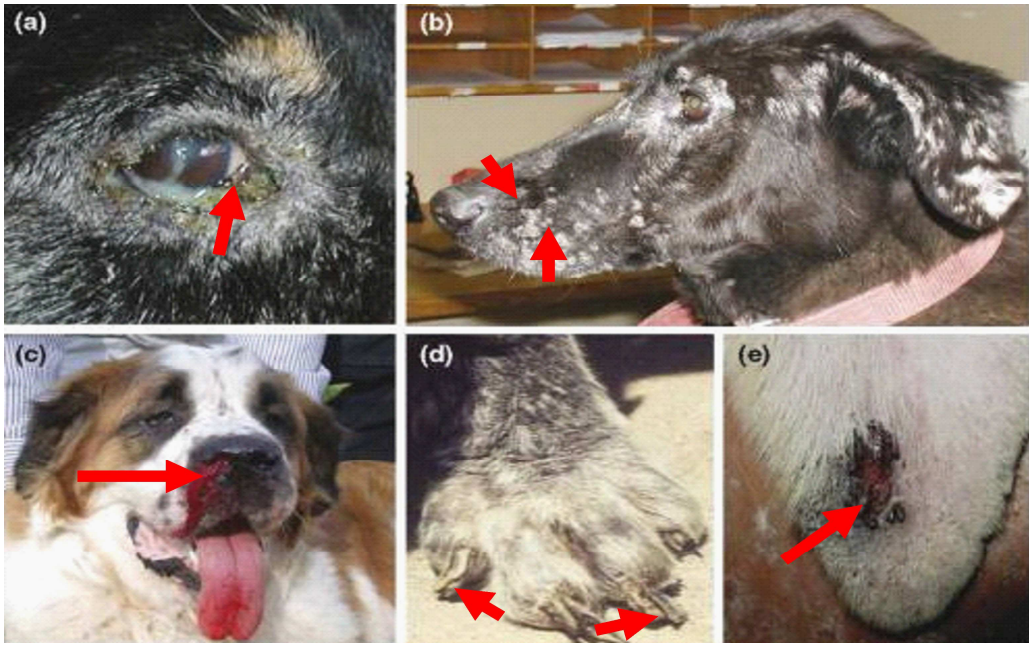


Fig. 1.6: Signs of leishmaniasis in dogs: (a) purulent keratoconjunctivitis, (b) facial skin lesions, (c) epistaxis, (d) onychogryposis and (e) skin ulceration on the ear. Adopted from Baneth et al., (2008).

1.7 Transmission and life cycle:

Classically, leishmaniasis is a zoonotic disease, but human-to-human transmission has been reported in some regions (Molina et al., 2003). Different developmental cycles occur in humans, reservoirs and the sandfly vectors. An important feature is that, *Leishmania* transmission occurs in certain locations such as forests. However, epidemiology of the disease has changed and became more domestic and in settled areas due to deforestation and agricultural development (Walsh et al., 1993).

The parasite has a complex life cycle, involving two developmental forms (digenetic parasite) in two different host species: a flagellated-extracellular promastigote stage within the sandfly and a non-flagellated-intracellular amastigote stage within the mammalian (human or animal) hosts. The different stages represent an adaptation to the internal environment within the hosts (Besteiro et al., 2007).

Sandfly:

The sandfly (**Fig. 1.7**) is smaller than mosquitoes, with a body length of 2-3 mm. It belongs to the subfamily Phlebotominae, which includes two genera of medical importance: *Phlebotomus* in the 'Old World', and *Lutzomyia* in the 'New World'. Out of the 500 known phlebotomine species, 31 are known as vectors for *Leishmania* and 43 as possible vectors. As in the case of mosquitoes, female sandflies require blood for egg production (haematophagous). Some phlebotomine species such as *Phlebotomus papatasi* and *Phlebotomus sergenti* are restricted vectors supporting the growth of certain species of *Leishmania*. Other species such as *Lutzomyia longipalpis* and *Phlebotomus argentipes* can support growth of several *Leishmania* species (see Banuls et al., 2007). In Sudan, *Phlebotomus orientalis* transmits the disease in the North and the former southern part of the country (Ashford et al., 1992). This species lives in areas with *Acacia seyal/Balanites aegyptiaca* vegetation and black cracking cotton soil (**Fig. 1.8**) (Elnaiem et al. 1998). In sandfly, *Leishmania* transforms to promastigotes, a motile extracellular flagellated form (**Fig. 1.9**) (see Banuls et al., 2007).

Fig. 1.7: Female *Phlebotomus* sandfly. Source: National History Museum, UK. (http://www.nhm.ac.uk/).

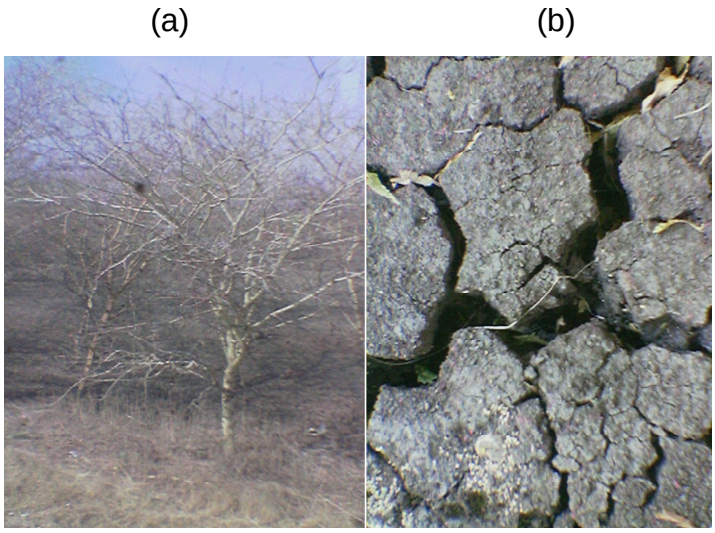


Fig. 1.8: Sandfly breading sites: (a) *Acacia Seyal* trees and (b) black cotton soil. Photos were taken by the author (E. Abass) around Doka village, Gadaref State, a known VL-endemic area in Eastern Sudan.

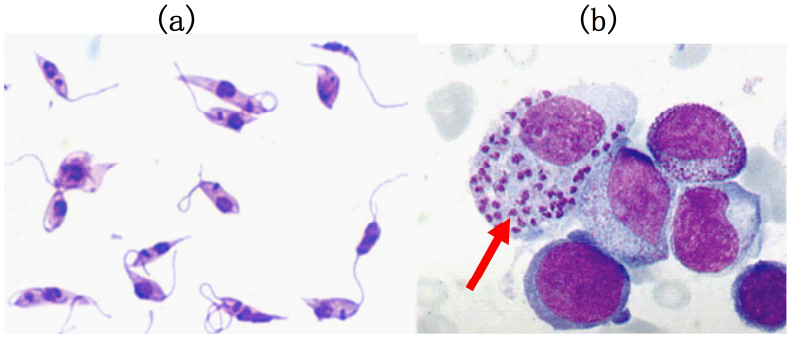


Fig. 1.9: Morphological forms of *Leishmania* parasites: (a) the flagellated-extracellular promastigote stage and (b) the non-flagellated-intracellular amastigote stage in a macrophage cell. Source: http://www.leishinfonet.com/Morphology.php.

Mammalian Host:

An important feature of *Leishmania* is their infectious potential of host cells such as macrophages. Beside humans and dogs, *Leishmania* can infect also other species such as rodents and canids. These species are considered potential reservoirs (Lainson and Shaw, 1987). In the mammalian host, *Leishmania* transforms into a non-flagellated intracellular ovoid amastigote form (2.5–5 µm diameter) with a well defined large nucleus and a smaller kinetoplast. (**Fig. 1.9**).

Life Cycle (**Fig. 1.10**):

Infected female sandflies inject a small number of infectious metacyclic promastigotes into the skin. These forms are efficiently opsonized by serum components and taken up by macrophages, where they live in phagolysosomes and transform into non-flagellated amastigotes forms. Infected macrophages are taken up by sandflies during blood meals and lysed in the midgut of the sandflies. Here the parasites are released that transform into non-infectious promastigotes (procyclic promastigotes). These forms attach to the midgut wall and differentiate into non-dividing metacyclic promastigotes, which can be transmitted when the sandfly takes another blood meal (Sacks and Noben, 2002; Besteiro et al., 2007).

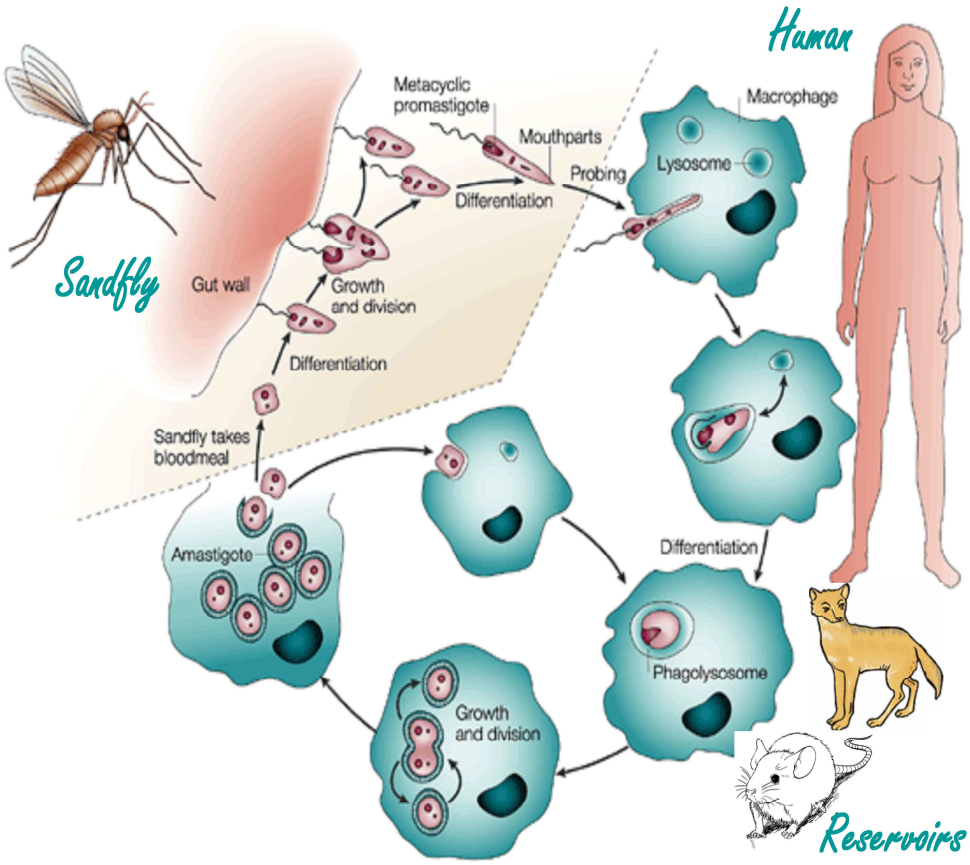


Fig. 1.10: Life cycle of *Leishmania* parasites. Modified from Sacks and Noben (2002).

1.8 Visceral leishmaniasis in Sudan and South Sudan:

Today, Sudan and South Sudan belong to the six VL-endemic countries in East Africa, which constitute 90% of global VL cases (Alvar et al., 2012). In these countries, the disease affects poor communities in remote rural areas where many patients have no access to diagnosis and standard treatment services and often succumb undetected by the local health information system (Collin et al., 2006). Since its discovery in 1904 in Sudan, VL is one of the biggest health problems in the country (see Zijlistra and Elhassan 2001).

In North Sudan, Gedaref State is the main endemic focus with an incidence of 6.6 - 8.4 cases per 1000 persons during 1996 to 1999. The main affected region is along two rivers "Atbarah and Rahad", in area with high rainfall (Ritmeijer and

Davidson, 2003; Elnaiem et al., 2003). Cases were also reported in small foci in West Sudan including Nuba Mountains and Darfur (Zijlstra and El-Hassan, 2001). Another endemic focus is the White Nile State in Central Sudan, where the disease reappeared after it had been under control for 25 years (Khalil et al., 2008). In this area, cases were reported from 5 villages on the west bank of the White Nile with children (2-14 years) being most affected (Walyeldin et al., 2010). Due to the lack of health care in this area, patients had to travel for more than 100 km to be treated in Omdurman Hospital in the capital.

The armed conflicts over the last decades have caused serious environmental changes such as widespread destruction of houses and health infrastructures that resulted in increased exposure to sandfly and less accessibility to health care facilities. Non-immune or infected populations moved to VL-endemic or non-endemic areas, causing major epidemics (Reithinger et al., 2007). Among several epidemics that occurred in the 20th century, the most severe was in the Upper Nile province (now in the new Republic of South Sudan) during 1984-1994 where one third of the population (100,000) were killed and 40,000 patients were treated (de Beer et al., 1991; Gorski et al., 2010). The problem has further been complicated by the massive exodus to South Sudan after independence. More than 18,000 cases were recorded since the outbreak started in September 2009, with children mostly affected. At least 720 people died, however the number is likely to be much higher (United Nation, News Centre, http://www.un.org/apps/news/story.asp?NewsID=40232#.UZiUE6DCd48).

1.9 Visceral leishmaniasis and HIV co-infection:

Visceral leishmaniasis and human immunodeficiency virus (VL/HIV) co-infection is a global health problem in several countries (Desjeux, 1995). HIV-infected individuals are highly susceptible to VL, which then enhances HIV progression to AIDS (Alvar et al., 2008). These cases frequently develop atypical VL symptoms and thus complicate clinical diagnosis (Desjeux, 1995). Co-infections have been reported in 35 countries, most of the cases from countries of southern Europe. In these countries, 25-70% of adult VL patients also have HIV infection and 1.5-9% of AIDS cases have newly acquired or reactivated VL. Those patients have a

much shorter survival period than other AIDS patients (Desjeux, 1995; Alvar et al., 2008).

VL/HIV co-infection has been also reported in East African countries such as Ethiopia, Kenya, Malawi, and Sudan. In Ethiopia, HIV co-infection was reported in 92 (38%) patients with VL. These cases showed much more treatment failure and death (Hurissa et al., 2010). In Sudan, the reported prevalence was 5% (3/60) in Khartoum between 1998 and 1999, 9.4% (5/53) in 2002, 8.1% (3/37) and 3.6% (3/84) in 2002 and 2003, respectively, in Gedaref State (Alvar et al., 2008). In a cross-sectional study included 204 VL patients from Sudan during 2003 – 2007, HIV co-infections were confirmed in 4.9% of the cases, all of them were male aged between 25 and 46 years (Abass and Elhussein, 2009).

1.10 Laboratory diagnosis:

1.10.1 Cutaneous and mucosal leishmaniasis:

In areas with high endemicity, appearance of characteristic lesions is sufficient to establish clinical diagnosis. However, laboratory procedures are required to differentiate leishmaniasis from other dermal conditions such as tropical ulcers, impetigo, infected insect bites, leprosy, lupus vulgaris, tertiary syphilis, yaws, blastomycosis, skin cancer (see Singh, 2006). Diagnostic procedures are based on:

(i) Parasitological methods by direct detection of *Leishmania* in lesional specimens of Giemsa-stained smears or culture. The latter is useful for the identification of the species and selection of therapy. These methods are time consuming, laborious and have low sensitivities (Weigle et al., 1987).

(ii) Molecular biology using PCR techniques are very sensitive and useful to determine the species of the parasite but require invasive procedures for specimen collections (de Oliveira et al., 2003; Bensoussan et al., 2006).

(iii) Serologically methods based on antibody detection are easy and fast but lack enough sensitivity due to the low number of circulating

antibodies and probably antigenic diversity of the parasites causing the disease (Reithinger and Dujardin, 2007; Szargiki et al., 2009).

(iv) Leishmanin skin test (LST) is a useful marker for cutaneous and mucocutaneous leishmaniasis (Weigle et al., 1991). It is used to measure cell-mediated immune responses, which is an important feature of cutaneous leishmaniasis. The antigen is a suspension of killed promastigotes of *L. major* (Reithinger and Dujardin, 2007).

(v) Isoenzyme characterization and monoclonal antibodies are used for species typing.

1.10.2 Visceral leishmaniasis:

Diagnosis of VL is complex because the common clinical symptoms are shared with other diseases that are endemic in the same regions such as malaria, typhoid and tuberculosis. These diseases may occur together with VL in the same patients (de Beer et al., 1991; van den Bogaart et al., 2012). VL should be suspected if a patient has persistent fever (>2 weeks) and splenomegaly and is living in or visited a VL-endemic area (WHO, 1996). Definitive diagnosis of VL is typically done by laboratory tests. These tests are based on:

(i) Detection of *Leishmania donovani* (L.D) in aspirates of lymph node, bone marrow or spleen by light microscope. These techniques have varying diagnostic sensitivities, depending on the type of specimen. Spleen aspiration has the highest sensitivity (93.1% - 98.7%) (Siddig et al., 1988; Zijlstra et al., 1992), but can be associated with serious complications such as bleeding or even rupture of the spleen (Chulay and Bryceson, 1983). Bone marrow aspiration has lower sensitivity (52% - 85%) but less invasive than spleen aspiration (Zijlstra et al., 1992; Bryceson, 1996). Lymph node aspiration is safe but has the lowest sensitivity (52% - 58%) (Siddig et al., 1988; Zijlstra et al., 1992). In a hospital based study in Sudan, detection of *Leishmania* in lymph nodes showed a sensitivity of only 20%, lower than been described before (Walyeldin et al., 2010). In cases of co-infection with HIV, the parasites can be also detected in peripheral blood smears (Delgado et al., 1998).

(ii) Detection of the parasites in culture. Samples can be cultured in NNN-medium or golden hamsters. However, the latter is not applied in clinical practice. For cases of VL/HIV co-infection, buffy coat or blood cultures can be used with varying sensitivity, 67% - 92% (Lopez-Velez et al., 1995; Salam et al., 2012).

(iii) Detection of *Leishmania* DNA in clinical samples using polymerase chain reaction (PCR). These assays include several formats. In conventional assays, specific PCR amplicons are resolved by electrophoresis after cleavage with restriction enzymes such as PCR-restriction fragment length polymorphism analysis (PCR-RFLP). In other assays, PCR products are analyzed during amplification cycles (real-time PCR) after staining with SYBR-green I dye or hybridization with fluorogenic probes (TaqMan or fluorescence resonance energy transfer (FRET) (see Reithinger, et al., 2007). These techniques are very sensitive for diagnosis of VL, even in patients with negative parasitological- and Leishmanin skin tests (Marques et al., 2006; Srivastava et al., 2011a). However, PCR is not a good marker for clinical VL as it may remain positive after treatment (Deborggraeve et al. 2008). In addition, the need for standard laboratory equipments and the risk of contamination limit their routine use.

(iv) Immunodiagnostic methods, which include serological tests for antibody detection and assays to measure *Leishmania*-specific cell-mediated immunity. Specific antibodies can be detected through different methods such as Enzyme-linked Immunosorbent Assay (ELISA), Immuno-fluorescent Antibody Test (IFAT), Direct Agglutination Test (DAT) and Immunochromatographic rapid tests.

Direct Agglutination Test (DAT):

DAT is an agglutination assay that uses intact stained promastigotes either in suspension or in a freeze-dried form (Harith et al., 1986; Meredith et al., 1995). The test is simple to perform and requires no sophisticated machines, thus being an ideal test for field use. It has proven to be a useful tool for diagnosis of VL in several countries including Sudan (Mengistu et al., 1990; Shiddo et al., 1995;

Boelaert et al., 1999a; Abass et al., 2007; Hamzavi et al., 2012). The stability of DAT for field application was improved by using freeze-dried and glycerol preserved antigens which do not require storage at 4℃ (Meredith et al., 1995, Harith et al., 2003). However, the need for overnight incubation gives limitation for the field use.

Enzyme Linked Immunosorbent Assay (ELISA):

ELISA is easy to standardize and practical for routine application. The performance of the test depends on type of antigen and can be improved by using recombinant proteins. Conventional water soluble antigens are highly sensitive but less specific (Bray, 1985). Several recombinant antigens have been characterized and used in ELISA for serodiagnosis of VL (Burns et al., 1993; Sivakumar et al., 2006; Carvalho et al., 2002; Goto et al., 2006; Pattabhi et al., 2010). Among these antigens, the rK39, which is a kinesin protein of *L. infantum* (synonymus *L. chagasi*), has shown the best performance in several endemic regions (see Chappuis et al., 2007; Maia et al., 2012; Machado de Assis et al., 2012).

Immunochromatographic rapid tests (RTs):

RTs are strip-based assays that can be carried out directly on-site without large efforts: only a blood sample of the patient is required. This format has the advantages of being ready for use and rapid. Commercially available RTs for VL are either based on rK39 of *L. infantum* (Burns et al., 1993) or rKE16 of *L. donovani* (Sivakumar et al., 2006). These tests are quite effective in diagnosing VL in several countries (Singh et al., 1995; Badaró et al., 1996; Ozensoy et al., 1998; Medrano et al., 1998; Houghton et al., 1998; Maalej et al., 2003). But, their use for East Africa is not satisfactory (Zijlstra et al., 2001; Veeken et al., 2003; Ritmeijer et al., 2006). Recently, it has been shown that RTs based on either rK39 or rKE16 have considerable variation in the major endemic regions for VL (Indian subcontinent, East Africa, Brazil). These tests have been performed with high sensitivity in the Indian continent (92.8%-100%) but not in Brazil (61.5%-91%) and East Africa (36.8%-87.2%) (Cunningham et al., 2012).

Indirect Fluorescent Antibody Test (IFAT):

IFAT is routinely used for serodiagnosis of leishmaniasis in dogs. It has similar sensitivity as DAT (88.3% and 88.5%) but lower specificity, 83% for IFAT and 95.4% for DAT (Machado de Assis et al., 2012). IFAT is difficult to standardize and to interpret and is not suitable for screening of large numbers of samples.

Western Blot (WB) analysis:

Application of WB for detection of *Leishmania*-specific antibodies is limited only for research purposes and is not adopted for routine diagnosis.

Leishmanin Skin Test (LST):

LST is typically used as a marker for successful VL treatment (Weigle et al., 1991). During active VL, patients show no cell mediated immune response and thus test negative, but convert to positive after successful treatment (Zijlstra and El-Hassan, 2001).

1.11 Immunity against *Leishmania*:

Immunity against *Leishmania* parasites is mediated by both innate (neutrophils, macrophages, and dendritic cells) and adaptive immune mechanisms (Kedzierski, 2010). The parasite is able to infect a variety of host cells and manipulate signalling pathways, which are involved in killing of pathogens or in stimulating immune responses (Shio et al., 2012). Neutrophils are the earliest cells recruited to the site of infection, also representing the first infected cells. They serve as intermediate host cells, helping the parasite to enter macrophages silently (van Zandbergen et al., 2004). *Leishmania* enters macrophages through a classical receptor-mediated process. This includes several parasite and macrophage surface molecules such as the complement receptors (CR)1, CR3 (Mac-1), fibronectin receptor and the mannose-fucose receptor (MR) on the surface of macrophages. These receptors recognise special structures on the surface of promastigotes include surface lipophosphoglycan (LPG), major surface protease (GP63), and proteophophoglycans (PPG) of *L. major* promastigotes (Liu and Uzonna, 2012).

Macrophages and dendritic cells, the two major antigen presenting cells (APCs), play an essential role in susceptibility to or resistance against *Leishmania* infection (Liu and Uzonna, 2012; McCall et al., 2013). They initiate adequate defence mechanisms by presenting antigens of the parasite via the major histocompatibility complex class II (MHC-II) molecules to T cells (Lang et al., 1994). Subsequently, T cells produce cytokines, influencing various immune responses by which *Leishmania* can evade or interfere with immune mechanisms (see Banuls et al., 2007).

In humans, different patterns of immune responses exist which are associated with the various clinical forms of the disease and parasite species. Active disease is characterized by strong Th2 responses and absent or low Th1 responses (Reed and Scott, 1993; Herwaldt, 1999). In VL, several reports have shown impaired lymphocyte function with suppression of type 1 cytokines such as IL-2, IFN-γ and IL-12, which is associated with increased type 2 cytokines including IL-4 and IL-10 (see Dey et al., 2008). Other authors have shown mixed Th1/Th2 immune responses with production of IFN-γ and IL-10, which significantly decrease after cure. This suggests that both cytokines are involved in the regulation of the immune responses against VL (Ghalib et al., 1993; Kenney et al., 1998; Ansari et al., 2006). Individuals with subclinical or asymptomatic infections demonstrate peripheral blood mononuclear cell (PBMC) proliferation with production of IL-2, IFN-γ and IL-12 (Kemp et al., 1993).

IFN-γ exerts different immune-protective mechanisms in *Leishmania* infection. It mediates macrophage activation and production of nitric oxide (NO), which plays an important role in intracellular killing of *Leishmania* (Liu and Uzonna, 2012). To prevent killing by macrophages, *Leishmania* manipulates macrophage activity by impairing the ability to produce IL-12 (an important cytokine for $CD4^+$ Th1 development and IFN-γ production) via synthesis of immuno-regulatory cytokines such as IL-10 and TGF-β (Liu and Uzonna, 2012). Increased levels of IL-10 and TGF-β are associated with disease progression through counteracting IFN-γ effects and deactivation of macrophage function (Barral-Netto et al., 1992; Cillari et al., 1995). Both IL-4 and IL-10 have shown

to inhibit intracellular killing of *L. infantum* and *L. major* by human macrophages through inhibiting NO production (Vouldoukis et al., 1997).

VL patients demonstrate increased humoral antibody responses, including IgG, IgM, IgE and IgG isotypes (Chatterjee et al., 1998; Anam et al., 1999; Ravindran et al., 2004). These antibodies have little effect in clearance of the parasite, but their specific function is still unclear. Miles and colleagues (2005) have shown that IgG not only fails to provide protection against *Leishmania* but also causes disease progression via inducing IL-10 production in macrophages. IgG-coated *Leishmania* can bind to macrophage Fc receptors (FcγR) and facilitate phagocytosis. It also activates downstream pathways and thus prevent killing and promote intracellular survival and growth of the parasites (Miles et al., 2005).

1.12 Variation and genetic polymorphism in *Leishmania* parasites:

Leishmania is a group of parasites with a wide ecological, epidemiological and clinical diversity (Shaw, 1997). These parasites have adapted to survive in diverse environments and are able to infect different mammalian hosts. Climatic and environmental factors impose strong selective pressure and may affect population genetics. Data indicate that the geographical distribution, the range of vectors and/or mammalian hosts have an influence on the genetic heterogeneity of *Leishmania* (see Banuls et al., 2007).

Genetic variations and heterogeneity of *Leishmania* parasites have been shown in several studies where *L. braziliensis* complex and *L. mexicana* complex revealed more diversity than *L. guyanensis* and *L. donovani* complexes (see Banuls et al., 2007). Species of *L. donovani* complex from different countries exhibit extensive genetic variation, which has been confirmed using different parasite-specific sequences. These parasites are grouped in six main genetically distinct populations based on multilocus enzyme electrophoresis (MLEE), where different allelic diversities were detected with a high degree in Mediterranean region, intermediate in Africa and lowest in India (Kuhls et al., 2007).

Analysis of *L. donovani* strains from East Africa and India show considerable heterogeneity with the existence of more than one genetic variant or mixed population in the same region (Kuhls et al., 2007; Srivastava et al., 2011b). East African strains are grouped into two genetically and geographically populations, strains from South Ethiopia and Kenya in one population and strains from North Ethiopia and Sudan in a second population. These parasites are derived from two different sandfly vectors, *Phlebotomus orientalis* in North Ethiopia and Sudan, *Phlebotomus martini* in South Ethiopia and Kenya. The existence of distinct clonal populations with putative hybrid genotypes at the same region indicates a mixed-mating system (Gelanew et al., 2010). In particular, strains of *L. donovani* from East Sudan are markedly diverse (heterogeneic) (Hamad et al., 2010).

Antigens of *Leishmania* reveal also marked heterogeneity among different species and within strains from the same regions. Kinesin proteins of *L. donovani* exhibit polymorphisms in the immunodominant repeats with multiple amino acid substitutions among East African and Asian strains. Coding sequences of rK39 homologues of East African and Asian *L. donovani* strains significantly differ from the rK39 of *L. chagasi*, with much more heterologeneity among strains from East Africa (Bhattacharyya et al., 2013). Other *Leishmania* antigens demonstrate also genetic polymorphism. The cysteine proteinase B (cpb) and gp63 exhibit intragenic and intergenic regions specific polymorphism among subspecies of *L. donovani* complex (Tintaya et al., 2004). HASPB (Hydrophilic acylated surface protein B) contains polymorphisms in the immunodominant repeat regions of *L. donovani* and *L. infantum* (Zackay et al., 2013; Bhattacharyya et al., 2013).

1.13 Control strategies of visceral leishmaniasis:

Efficient control strategies against human VL are primarily based on the control of reservoir hosts and the vector as well as diagnosis and treatment of patients. Detection and treatment of infected dogs is limited by the fact that dogs may relapse or re-infected after successful treatment (see Chappuis et al., 2007). Studies have shown that culling of infected dogs reduces incidence of the disease in both dogs and humans, but this strategy is not accepted ethically (see Romero

and Boelaert, 2010). Vector control through insecticide spraying is a useful strategy in India because the sandfly lives in and around homes. In other endemic areas such as Sudan, infections occur outside villages and therefore spraying of houses and insecticide-treated bednets (ITNs) are not useful. Early detection is essential for treatment of patients and infection control. Without treatment, VL patients are sources for spread of the parasite. Thus, early case detection and treatment remains an efficient strategy for VL control (see Chappuis et al., 2007).

1.14 Visceral leishmaniasis treatment:

Treatment of VL is based on anti-leishmanial drugs and management of other complications such as secondary bacterial or parasitic infections and anaemia. Pentavalent antimonials, sodium stibogluconate (Pentostam®) and meglumine (*Glucantim*®), are used as the first-line treatment in most VL endemic areas. These drugs are very toxic and associated with severe side effects such as cardiac arrhythmia and acute pancreatitis. Antimonials should be given with caution for children and old patients (<2 and >45 years) and to patients with advanced disease or malnutrition. Resistance to antimonials are common in several countries, which may reach >60% (see Chappuis et al., 2007).

Amphotericin B is an alternative drug and currently used in some countries such as India. Its side effects include infusion-related fever, chills, rigor, hypokalemia (low potassium levels in the blood), nephrotoxicity (toxic to kidney cells) and first-dose anaphylaxis. Liposomal amphotericin B is the best available drug and is currently used in Europe and the United States. Its use in developing countries is limited by the high cost (see Chappuis et al., 2007). Miltefosine is the first and still the only oral drug available for VL and CL. It has a high cure rate and less toxicity but has mild gastrointestinal side effects (Dorlo et al., 2012). Paromomycin, which is an aminoglycoside antibiotic, has high anti-leishmanial efficacy and acts also against bacterial infections. Combination therapy was suggested to increase efficacy, lower drug resistance and reduce duration of treatment. Combination of sodium stibogluconate and paromomycin has shown to be safe and effective. Other trial includes the use of liposomal amphotericin B and miltefosine (see Chappuis et al., 2007).

1.15 Rational and objectives:

1.15.1 Rational:

Diagnosis of VL is a major health problem for poor communities with limited resources. To date, there are no reliable diagnostic tests for VL in East Africa. Available tests have many limitations, none of them is sufficient to identify all positive and negative cases. A negative result doesn't rule out infection and false positives are common. Patients co-infected with other diseases such as HIV and malaria may show atypical clinical pictures with marginal antibody titres and are inaccurately diagnosed. In addition, VL shares clinical features with other diseases such as typhoid, malaria and tuberculosis. These diseases often overlap the same VL-endemic areas and thus making clinical diagnosis difficult.

In Sudan, detection of *Leishmania* parasites in lymph nodes and direct agglutination test (DAT) are currently used for the diagnosis. These tests, however, are either not sensitive and invasive or need overnight incubation, giving limitations for the field use. The available rapid tests which are usually based on a single antigen of parasite from Brazil (*L. chagasi*) are inaccurate in Sudan. In addition, these tests can neither differentiate between active and past infections nor between symptomatic and asymptomatic infections.

Due to their role in transmission cycle of VL, infected dogs should be controlled. If not detected and treated, infected dogs are continuous sources of the parasites for the sandfly vectors. As for humans, currently used tests for canine VL have low sensitivity. Cross-reactivity with other diseases and low sensitivity in detecting asymptomatic dogs are further limitations.

For all these reasons, research on *Leishmania* is necessary to develop better diagnostic procedures for VL especially for those in rural areas of East Africa. In particular, there is a need for simple-rapid, cheap and accurate tests with high sensitivity and specificity. These tests should be able to discriminate between VL and potential co-infections and to detect asymptomatic infections in humans and dogs.

1.15.2 Aim and Objectives:

The aim of this study was to integrate current knowledge about genetic variability of *Leishmania* and heterogeneity of antigens in order to develop a better diagnostic test for VL. Specific objective are:

i- To improve diagnosis of VL in Sudan by using antigens from autochthonous *L. donovani* strain.

ii- To evaluate serodiagnostic tests in major endemic regions for human and canine VL in order to identify appropriate diagnostic tests for different regions.

iii- To explore whether heterogeneity of *Leishmania* antigens has influence on reactivity and thus performance of the serological tests.

2. MATERIALS AND METHODS:

2.1 MATERIALS:

2.1.1 Chemicals and reagents:

Name	Company
Acrylamide mix	Carl Roth
Agar	Merck
Agarose	Biozym
Ammonium persulfate	Sigma
Ampicillin	Sigma
BM blue POD substrate	Roche
BSA	Sigma
Coomassie brilliant blue	Merck
DMSO	Fluka
DNA ladder standard	Fermentas
DNA loading dye (6X)	Fermentas
Donkey anti-Human IgG	JaksonImmunoResearch Labs
EDTA	Sigma
Ethanol	Sigma
Ethidium bromide	Fluka
FCS	Sigma
Gelatine	Fluke
Glycerine	Carl Roth
Glycin	Carl Roth
H_2O_2	R&D system
Imidazol	Carl Roth
IPTG	Carl Roth
Kanamycin	GERBU Biotechnik
LB broth	Invitrogen
NaCl	Sigma
$NaHCO_3$	Sigma
$NaPO_4$	JT Baker
PBS Dulbecco	Biochrom
PMSF	Sigma
Potassium acetate	Carl Roth
Rabbit anti-Dog IgG	JaksonImmunoResearch Labs
Sodium dodecyl sulfate (SDS)	Sigma
ß-mercaptoethanol	Sigma
Sucrose	Sigma
TEMED	Sigma

Name	Company
Tertamethylbenzidine	R&D system
Tris-Base	Acros
Tris-HCl	Carl Roth
Tween 20	OmniPur

2.1.2 Enzymes:

Enzyme	Company
Antarctic phosphatase	New England Biolabs
*Bam*HI	Fermentas
Benzonase nuclease	Novagen
DNA ligase	New England Biolabs
DNase1	Invitrogen
*Eco*RV	Fermentas
Lysozyme	Carl Roth
Phusion DNA polymerase	Finnzymes
Phusion Hot start II DNA Polymerase	Thermofisher
RNase	Biozyme
*Sal*I	Fermentas

2.1.3 Commercial kits:

Kit	Company
Crystal KA rKE16	Span Diagnostic, India
Gentra Puregene cell kit	Qiagen
IT LEISH rK39	Bio Rad
ITMA-DAT/VL	Institute of Tropical Medicine, Antwerp
Ni-NTA column	Qiagen
Nucleospin Gel and PCR clean-up	Macherey- Nagel
NucleoSpin Plasmid Midi kit	Macherey- Nagel
NucleoSpin Plasmid Mini kit	Macherey- Nagel

2.1.4 Culture media:

RPMI-1640 (Sigma): Supplemented with L-glutamine (0.3 g/L), $NaHCO_3$ (2.0 g/L) and 10% (v/v) fetal calf serum.

Luria Bertani (LB) broth and agar media:

Standard ingredients for 1 Liter:

- Tryptone 10 g
- Yeast Extract 5 g
- NaCl 10 g

20 g LB broth base (Invitrogen) was dissolved in 1 litre of distilled water. For LB agar, 15 g agar (Merck) was added. The mixture was heated to boil and dissolve the agar which was then sterilized by autoclaving for 15 minutes at 121℃ (15 psi).

Additives:

Antibiotics and additives were prepared as stock solutions, sterilized using 0.22 µm membrane filters (Millipore) and then added to the media after autoclaving.

Table 2.1: Culture medium additives:

Substance	Solvent	Stock concentration	Final concentration
Ampicillin (Sigma)	H_2O	100 mg/ml	100 µg/ml
Kanamycin (GERBU Biotechnik)	H_2O	10 mg/ml	25 µg/ml
IPTG (Carl Roth)	H_2O	1 M	1 mM

2.1.5 Solutions and buffers:

β-ME stock solution (10 mM):

2-ME	700 µl
PBS	1000 ml

Sterilize by filtration.

Freezing media:

For *Leishmania*:

RPMI 1640 contains 10% FCS	8 ml
FCS	1.2 ml
DMSO	1 ml

For bacteria:

Bacterial culture	900 µl
DMSO	100 µl

DNA isolation buffers:

Cell Lysis Solution: 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% (v/v) SDS.

1M Tris-HCl	1 ml
0.5M EDTA	5.0 ml
20% SDS (w/v)	2.5 ml
H_2O	91.5 ml

Protein Precipitation Solution: 5 M Ammonium Acetate, NH_4OAc, Mr = 77.09.

NH_4OAc	19.27 g
H_2O	to 50 ml

DNA electrophoresis buffers:

5X TBE Buffer:

Tris-Base	540 g
Boric acid	2.75 g
0.5 M EDTA (pH 8.0)	200 ml
Water	up to 10 L.

50X TAE Buffer:

Tris-Base	2.42 kg
Acetic Acid	571 ml
0.5 M EDTA (pH 8.0)	1 L
Water	up to 10 L.

6X DNA loading Buffer:

Sterile H_2O	1 ml
Glycerol	1 ml
Bromophenol blue	~ 0.05 mg

Buffers for chemically competent *E. coli*:

RF1 buffer: 1 M potassium acetate, pH 7.5

1 M potassium acetate	15 ml
Rubidium chloride (RbCl)	6 g
Manganese chloride ($MnCl_2$ 4 H_2O)	4.95 g
Glycin	75 g
Water	Complete to 500 ml

Adjust pH to 5.8, sterilize by autoclaving.

RF2 buffer:

MOPS (3-(N-morpholino)propanesulfonic acid) (0.5 M, pH 6.8)	10 ml
RbCl	0.6 g
$CaCl_2$ X2 H_2O	5.5 g
Glycin	75 g
Water	Complete to 500 ml

Adjust pH to 5.8, sterilize by autoclaving.

Plasmid cracking solution:

2 N NaOH	100 µl
10% SDS (w/v)	50 µl
Sucrose	0.2 g
H_2O	complete to 1 ml (~ 850 µl)

Protein purification buffer:

Table 2.2: Imidazol buffers:

Stock	Final concentration			
	10 mM	20 mM	60 mM	400 mM
10X PBS pH 7.4	10 ml	10 ml	10 ml	10 ml
β-ME	4 µl	4 µl	4 µl	4 µl
1 M imidazol	1 ml	2 ml	6 ml	40 ml
0.1 M PMSF	1 ml	1 ml	1 ml	1 ml
H_2O	84 ml	83 ml	79 ml	49 ml

SDS-polyacrymide gel:

Resolving gel 12%: for 5 ml

H_2O	1.6 ml
30% acrymide mix	2.0 ml
1.5 M Tris-HCl (pH 8.8)	1.3 ml
10% SDS	0.05 ml
10% ammonium persulfate	0.05 ml
TEMED	0.002 ml

Stacking gel 5%: for 1 ml

H_2O	0.68 ml
30% acrymide mix	0.17 ml
1.5 M Tris-HCl (pH 8.8)	0.13 ml
10% SDS (w/v)	0.01 ml
10% ammonium persulfate	0.01 ml
TEMED	0.001 ml

Protein electrophoresis and WB buffers:

10X electrophoresis running buffer:

2 M glycin

0.25 M Tris-Base

1X electrophoresis buffer:

10X running buffer	100 ml
10% SDS (w/v)	10 ml
H_2O	To 1000 ml

4X sample buffer:

150 mM Tris-HCl (pH 6.8)
1.2% SDS
3.3% glycerine (v/v)
1.8% bromphenol blue (w/v)

10X WB transfer buffer:

Glycin 2 M
Tris-HCl 250 mM pH 8.8

1X WB transfer buffer:

10X transfer buffer	30 ml
Methanol	60 ml
H_2O	210 ml

WB wash buffer

100 mM NaCl
10 mM Tris-HCl pH 7.5
0.05 Tween 20 (v/v)

WB blocking buffer

100 mM NaCl
10 mM Tris-HCl pH 7.5
0.05 Tween 20 (v/v)
5% BSA (w/v)

ELISA buffers:

Coating buffer:

0.1 M $NaHCO_3$ (MW = 84.01g/mol) pH 9.6

Blocking and sample buffer:

BSA	3 g
PBS	100 ml
Tween 20	0.05 ml

Wash buffer:

0.05% Tween 20 in PBS (v/v)

2.1.6 *Leishmania* strains:

L. donovani LO8 was used for amplification and expression of the immunodominant kinesin protein gene. For sequence comparison, similar published DNA and amino acid sequences of other *L. donovani* strains (**Table 2.3**) were obtained from public domain of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Table 2.3: *Leishmania donovani* strains and isolates:

Strain	Geographical origin	Reference code/isolate
L. donovani LO8	Sudan	Sudanese isolate
L. donovani 1S	Sudan	MHOM/SD/62/1S-CL2D
L. donovani KE16	India	MHOM/IN/1998/KE16
L. donovani DD8	India	MHOM/IN/DD8/1968
L. donovani Morena	India	Indian isolate
L. donovani DD8	Bangladesh	Bangladeshi isolate
L. chagasi BA-2	Brazil	MHOM/BR/82/BA-2
L. infantum LON49	Iran	MCAN/IR/96/LON-49

2.1.7 Bacterial strains:

Different bacterial strains (**Tables 2.4**) were used for expression of the recombinant protein. Expression in M15 *E. coli* was found to be better and thus this strain was used for up-scaling protein production.

Table 2.4: Bacterial strains:

Name	Characteristics	Source
E. coli Top10	Host for cloning and plasmid propagation.	Invitrogen
E. coli M15[1]	Contains the repressor pREP4 which permits high- level protein expression with pQE-vectors.	Qiagen
E. coli XL1 blue[2]	Harbors the *lacI*^{q} mutation (lacIqZΔM15 gene) to increase transcription.	
E. coli TG1[2]		

E. coli strains were kindly offered by Prof. Mohamed Marahiel, Biochemistry- Marburg University[1] or Dr. Maria Llamazares-Prada, Department of Dermatology and Allergy, Marburg University[2].

2.1.8 PCR primers:

Oligonucleotide primers for amplification of the immunodominant kinesin protein gene of *L. donovani* (*KLO8)* were designed according to published *L. chagasi* gene for kinesin-related protein (GenBank: L07879.1). To express the recombinant protein, *KLO8* was subcloned into the His-tag vector pQE41 (Qiagen GmbH, Germany) using primers designed to contain an extra bases sequence at the 5´ end of the primers overhang specific sequence of the vector. Primers included also the restriction sites *Bam*HI and *Sa*lI (underlined).

Primers for cloning *KLO8*:

Forward: 5'- GAGCTCGCAACCGAGTGGGAGG–3´

Reverse: 5'- GCTCCGCAGCGCGCTCC–3'

Primers for subcloning:

Forward: 5'-GTGGAATTCTGCAGATGGATCCATGGAGCTCGCAACC–3´

Reverse: 5'-GCCGCCACTGTGCTGGATGTCGACGCTCC–3´

Sequencing primers for pcDNA 3.1(+) vector:

Forward (T7 promoter): 5'TAATACGACTCACTATAGGG-3'

Reverse (BGH): 5'-TAGAAGGCACAGTCGAGG-3'

Sequencing primers for pQE41 vector:

EApQE41 forward: 5'- TTCACACAGAATTCATTAAAG-'3

EApQE41 reverse: 5'- TCTATCAACAGGAGTCCAAGC-'3

2.1.9 Plasmids:

Table 2.5: Plasmid vectors:

Plasmid	Description	Source
pcDNA3.1(+)	Used for cloning and plasmid propagation, carries ampicillin resistant gene (Amp^R).	Invitrogen
pQE41[1]	A His-tag protein expression vector, carries Amp^R gene.	Qiagen
pcDNA/KLO8	A new generated plasmid carrying *KLO8* which was cloned into pcDNA3.1(+) *Eco*RV site.	Generated in this study
pQE41/KLO8	A new generated plasmid carrying *KLO8*, was inserted into pQE41 *Bam*H1-*Sal*1 site and down stream of a 6x His-tag. Used for expression of the recombinant protein KLO8	

[1] Kindly offered by Dr. Cassian Sitaru, Department of Dermatology, University medical centre- Freiburg.

2.1.10 Analysis and bioinformatics online tools:

Software	Website
ApE Plasmid Editor	http://biology.utah.edu/jorgensen/wayned/ape/
BLAST analysis tool	http://blast.ncbi.nlm.gov/blast
ClustalW2-Sequence Alignment	http://www.ebi.ac.uk/Tools/msa/clustalw2/
ExPASy Proteomics server	Htt://web.expasy.org/translate/
GraphPadPrism4	http://www.graphpad.com
Sequin	http://www.ncbi.nlm.nih.gov/Sequin/
Tandem Repeat Finder	http://tandem.bu.edu/trf/trf.html

2.1.11 Serum samples:

Human sera:

A total of 324 human serum samples of patients and controls were obtained from established serum collection banks (**Table 2.6**). Sera were collected in 3 endemic regions of VL in Doka-East Sudan, Bihar-North India and South France. Samples included 158 sera of VL, 11 sera of proven VL/HIV co-infection cases. 24 sera were from symptomatic patients with unconfirmed VL diagnosis (VL suspects, VLS) from India and France. Twenty-five sera were from asymptomatic cases (ASC) from the same VL-endemic area in France. Control samples included sixty sera from healthy individuals with no previous history of leishmaniasis collected in a non-endemic areas (non-endemic control, NEC) or from the same VL-endemic area (endemic controls, EC). The control samples also included sera from patients with malaria, TB, leukaemia and toxoplasmosis.

Selection criteria and characteristics of sera:

Diagnosis of VL was established by detection of *Leishmania* amastigotes in Giemsa-stained smears of lymph nodes (Sudan), spleens (India) or bone marrows (France) aspirations and/or by culture in Novy, Nicolle and McNeal (NNN) medium (reference method). Patients with positive smear or culture results were categorised confirmed VL. Diagnosis was performed in the same endemic area by experienced personnels. Smears were repeated for

symptomatic patients with negative results. Patients with repeated negative results were defined as unconfirmed VL suspects (VLS). All sera of VLS from France have shown positive reactivity in ELISA and Western blot (WB) analysis using *L. infantum* soluble antigen (SLA). This strain was characterised by zymodeme analysis and found to be representative for the local strains in France. Confirmed VL sera from France were classified based on their HIV status into VL with HIV-negative or HIV-positive (VL/HIV). Sera of asymptomatic cases were selected based on their positive reactivity to 14 and 16 kDa antigens in WB using the SLA, which are common in all *Leishmania* species (Mary et al., 1992).

Table 2.6: Origin, source and number of human serum samples:

Origin (no. of sera)	Supplier	Clinical condition (no. of sera)	
[1] Doka, Eastern Sudan (n=183)	Dr. Durria Mansour, Biomedical Research Laboratory of Ahfad, Omdurman, Sudan	VL Healthy Malaria TB Leukemia	(n=106) (n=50) (n=11) (n=10) (n=6)
Bihar, North India (n=66)	Prof. Peter Walden, Charité-Universitätsmedizin-Berlin	VL VLS Healthy Toxoplasmosis Malaria	(n=26) (n=11) (n=10) (n=9) (n=10)
South France (n=75)	Prof. Renaud Piarroux, University of the Mediterranean, Marseille-France	VL VL/HIV VLS ASC	(n=26) (n=11) (n=13) (n=25)
Total			**324**

Abbreviations: VL, visceral leishmaniasis; TB, pulmonary tuberculosis; VLS, VL suspect; VL/HIV, VL/HIV co-infection; ASC, asymptomatic cases. Healthy control sera from Sudan included 20 of individuals from a non-endemic area (non-endemic control, NEC) and 30 from the same endemic area (endemic control, EC) as VL sera. Healthy sera from India were from the same endemic area (endemic control, EC). [1] Some sera were collected from non-endemic area, as described in section 2.1.11.

Dog sera:

A total of 200 serum samples of symptomatic, asymptomatic and healthy dogs were included. Sera were collected in 3 endemic regions of canine visceral leishmaniais (CVL) in Portugal, Brazil and Croatia (**Table 2.7**). Sera included 9 samples of confirmed CVL and 21 symptomatic cases (SC) of unconfirmed diagnosis. Dogs were considered symptomatic if they showed at least one of the common symptoms. Confirmation of diagnosis was based on detection of *Leishmania* amastigotes in stained lymph node (popliteal or prescapular) smears. 168 sera were from asymptomatic cases (ASC) from the same regions. Control samples included 20 sera of healthy dogs from Croatia. These sera were used to calculate ELISA cut off value. Dogs were clinically and parasitologically evaluated at the time of diagnosis and serum samples were collected. In addition, sera were also tested for the presence of anti-*Leishmania*-specific antibodies using the direct agglutination test (DAT) or indirect immunofluorescence antibody test (IFAT) as previously described (Martinkovic and Marinculic, 2006).

Table 2.7: Origin and number of dog sera:

Origin (no. of sera)	Supplier	Clinical condition (no. of sera)
Évora, South Portugal (n=27)	Dr. Saul Santos, Department of veterinary medicine, University of Évora, Portugal	CVL (n=9)
		SC (n=18)
Caicó Paraíba, Brazil (n=16)	Prof. Paulo Andrade, Department of Genetics, Federal University of Pernambuco, Recife, Brazil. & Dr. Maria Melo, Laboratory of Molecular Genetics and Immunology, Federal University of Campina Grande, Patos, Paraíba, Brazil.	SC (n=3)
		ASC (n=13)
Croatia (n=157)	Dr. Franjo Martinkovic, Department for Parasitology and Parasitic Diseases, faculty of Veterinary Medicine University of Zagreb	ASC (n=137)
		Healthy (n=20)
Total		**200**

CVL, canine visceral leishmaniais; SC, symptomatic cases; ASC, asymptomatic cases.

2.1.12 Commercial recombinant antigen and serological tests:

Recombinant K39 antigen:

Recombinant lipoprotein antigen rK39 of *L. infantum* (synonym. *L. chagasi)* was purchased from Rekom Biotech, S.L., Granada Spain. It contains repetitive immunodominant epitopes of kinesin-related protein. It was expressed as 6 x His-tagged His-rK39 fusion protein at the C-terminus of the kinesin-related protein with 100% identity with the accession number AAA29254.1. Upon receipt, the protein concentration was verified as described for rKLO8 protein (see section 2.2.9). Aliquots were kept at -80℃.

rK39 strip test:

Individual IT LEISH dipstick kits using recombinant K39 antigen (Burns et al., 1993) for detection of human VL antibodies were purchased from Bio Rad, France. The test was performed and interpreted as recommended by the manufacturer. Sera were considered positive when a dark purple control band appeared. Samples with invalid results were repeatedly tested.

rKE16 rapid test (Signal-KA):

Signal KA flow through test kit, purchase from Span Diagnostics Ltd (Surat, India), was used for qualitative determination of *Leishmania*-specific antibodies. The test is based on the recombinant KE16 antigen of *L. donovani* KE16 strain isolated in India (Sivakumar et al., 2006). Test were performed and interpreted as described by the manufacturer. Briefly, 50 µL of patients' serum diluted 1:5 was added onto a nitrocellulose membrane which has been immobilised with the recombinant antigen. Sera were allowed to soak completely before carrying out the next step. The test was read within 10 minutes. Sera were considered positive if two magenta red dots, one for control and other for test, appeared. A single dot in the control area indicated a negative result. In the absence of a control dot, tests were considered invalid.

Direct agglutination test, DAT:

The DAT (ITMA-DAT/VL) kits (Lot 11D1B1) were purchased from the Institute of Tropical Medicine, Antwerp-Belgium (ITMA). The antigen is a freeze-dried suspension of trypsin-treated, fixed and stained promastigotes of *L. donovani*

strain 1-S (Harith et al., 1988; Meredith et al., 1995). The test was performed in 96 V-shape microplates (Greiner BioOne, Germany) according to the manufacturer's instructions. Besides internal controls, positive and negative pooled sera were included in each plate and results were read after overnight incubation at R/T. Samples with titres of 1:≥3200 were considered positive, whereas samples with titres of 1:800 and 1:1600 were considered as borderline and were repeated.

Table 2.8: Overview of *Leishmania* antigens and parasites used in the serological tests:

Test	Antigen	Parasite	Source
rKLO8 ELISA	rKLO8	*L. donovani* LO8	This study
rK39 ELISA	rK39	*L. infantum* LEM 589	Rekom Biotech, Spain
IT LEISH	rK39	*L. chagasi* BA-2	Bio Rad, France
Signal-KA	rKE16	*L. donovani* KE16	Span Diagnostics, India
ITMA-DAT/VL	DAT	*L. donovani* 1S	ITMA, Belgium

DAT, direct agglutination test; ITMA, Institute of Tropical Medicine, Antwerp; VL, visceral leishmaniasis.

2.2 METHODS:

2.2.1 *Leishmania* culture:

L. donovani reference strain LO8 was kindly provided by Prof. Bernhard Fleischer, Bernhard Nocht Institute for Tropical Medicine (BNITM), Hamburg. The strain was originally isolated in Sudan from a confirmed case of visceral leishmaniasis. The parasite was cultured at 28℃ in RPMI-1640 supplemented with L-glutamine, $NaHCO_3$ (Sigma-Aldrich) and 10% (v/v) fetal calf serum (Sigma-Aldrich).

2.2.2 Isolation and purification of *Leishmania* genomic DNA:

Genomic DNA was isolated from *L. donovani* LO8 culture using Gentra Puregene Cell Kit.

Protocol:

- Add 20 million cells in culture medium to a 1.5 ml microfuge tube.
- Centrifuge at 13,000 x g for 5 min. to pellet cells. Remove supernatant leaving behind 10-20 µl residual liquid.
- Pour off supernatant and resuspend cells in remaining liquid by vortexing.
- Add 300 µl Cell Lysis Solution to the resuspended cells and pipet up and down.
- Add 1.5 µl RNAse A (10 mg/ml) and mix the sample by inverting the tube 25 times. Incubate at 37℃ for 30 min.
- Add 100 µl Protein Precipitation Solution to the cell lysate.
- Vortex vigorously at high speed for 30 seconds.
- Centrifuge at 13,000 x g for 1 min. to pellet proteins.
- Pour supernatant into a fresh 1.5 ml microfuge tube containing 300 µl isopropanol and invert 50 times to precipitate genomic DNA.
- Centrifuge at 13,000 x g for 1 min. to pellet the DNA.
- Discard the supernatant and add 300 µl 70% ethanol and invert the tube several times to wash the DNA. Invert the tube until the pellet is no longer stuck to the tube.
- Centrifue at 13,000 x g for 1 min. to pellet DNA. Discard supernatant and allow to air dry.

- Rehydrate DNA in 50 µl water and incubate at 65℃ for 1 hr then store at -20℃.

2.2.3 Polymerase chain reaction (PCR):

Partial gene fragment encoding the immunodominant repeats of *L. donovani*, designated *KLO8*, was amplified from promastigote genomic DNA using the forward (5'-GAGCTCGCAACCGAGTGGGAGG–3´) and reverse (5'-GCTCCGCAGCGCGCTCC–3') primers, designed according to the published *L. chagasi* gene for kinesin-related protein (GenBank: L07879.1). PCR reaction was performed using Phusion® High-Fidelity DNA Polymerase (FINNZYMES OY, Finland) in a total volume of 50 µl, containing 3 % (v/v) DMSO, 10µl HF buffer, 10mM dNTPs mix-OLS (OMNI life science) and 100 ng genomic DNA. Concentration of DNA was measured by NanoDrop 1000 spectrophotometer (peQLab).

Table 2.9: PCR protocol (Phusion® high-Fidelity DNA polymerase):

Component	Volume /50 µl	Final concentration
H2O	28 µl	-
5X Phusion HF buffer	10 µl	1 x
10 mM dNTPs	1 µl	200 µM
Primer 1	2 µl	0.5 µM
Primer 2	2 µl	0.5 µM
Template DNA	5 µl	100 ng
DMSO	1.5 µl	3%
Phusion DNA polymerase	0.5 µl	0.02 U/µl

Cycling condition:

Cycle step	Temp.	Time	Cycles
Initial denaturation	98^{o}C	30 sec.	1
Denaturation Annealing Extension	98^{o}C 61.0 – 71.0^{o}C 72.0^{o}C	10 sec. 20 sec. 20 sec.	25
Final extension	72.0^{o}C 4^{o}C	5 min. Hold	1

2.2.4 Agarose gel electrophoresis:

The DNA was separated by electrophoresis and visualized by ethidium bromide.

Preparing 1% (w/v) agarose gel:

- Take 1 g agarose powder and add it to a 250 ml flask
- Add 100 ml TBE or TAE Buffer to the flask.
- Melt the agarose in a microwave until the solution becomes clear.
- Let the solution cool to about 55-60℃.
- Pour the melted agarose solution into the casting tray and let cool until it is solid.
- Pull out the combs and remove the tape.
- Place the gel in the electrophoresis chamber.
- Add enough TBE or TAE Buffer over the gel (about 2-3 mm).

Protocol:

- Add 6X Sample Loading dye to an appropriate volume of PCR reaction
- Carefully pipette 10 µl of each sample/Sample Loading dye mixture into separate wells in the gel.
- Pipette 5 µl of the DNA ladder standard into one well of each row on the gel.
- Run at 100 - 120 volts.
- Stain with 0.5 mg/ml ethidium bromide for about 20 minutes.

2.2.5 DNA ligation and restriction digestion:

KLO8 of *L. donovani* (883bp) was gel purified, digested with *Eco*RV and cloned into the plasmid vector pcDNA3.1(+) (Invitrogen life technologies, USA)

generating the non-tagged KLO8 construct, pcDNA/KLO8. Protocols were according to the manufacturer' instructions and as follow:

Restriction digestion protocol (Invitrogen):

- *Eco*RV 1 µl
- 10X Buffer H 2 µl
- Substrate DNA 10 µl (~ 200 ng)
- Sterile water 17 µl
- Incubate at 37℃ for 30 mins.

Buffer Compositions:

10X Buffer H

- 500 mM Tris-HCl, pH 7.5
- 100 mM $MgCl_2$
- 10 mM Dithiothreitol (DTT)
- 1000 mM NaCl

Gel clean-up (Macherey- Nagel):

- Excise gel slice containing the DNA fragment and determine its weight.
- For each 100 mg of agarose gel add 200 µl buffer NT.
- Incubate at 50℃ for 5-10 min.
- Load into the extraction column and centrifuge for 1 min at 11,000 x g. Discard flow-through.
- Add 600 µl buffer NT3 and centrifuge for 1 min at 11,000 x g. Discard flow-through.
- Centrifuge 2 min at 11,000 x g and incubate 2-5 min at 70℃.
- Add 15-50 µl water and incubate at R/T for 1 min. Centrifuge for 1 min at 11,000 x g.

Vector Dephosphorylation Protocol (New England Biolabs):

- 10X Antarctic Phosphatase Reaction Buffer 3 µl
- Vector DNA cut with restriction enzymes 25.5 µl (1 µg)
- Antarctic Phosphatase 1.5 µl (5 units).

Incubate for 30 min. at 37℃. Heat inactivate for 5 minutes at 65℃.

Ligation protocol (New England Biolabs):

- 10X T4 DNA ligase buffer 2 µl
- Dephosphorylated vector DNA 10 µl (50 ng)
- Gel purified and cleaned PCR product 6 µl
- T4 DNA ligase 1 µl
- Mix reaction tubes by pipetting up and down. Incubate at 16℃ for overnight.

Preparation of competent *E. coli* cells:

To allow uptake of DNA, E. coli cells were manipulated with rubidium chloride method as following:

- Prepare 2 ml O/N LB culture at 37℃.
- Inoculate 5 ml LB broth with 500 µl O/N culture and shake at 37℃ until OD600 reach 0.6-0.7 (3.3 McFarland 'mcf').
- Centrifuge 8 min at 1880 x g at 4℃.
- Resuspend the pellet in 33 ml RF1 buffer and incubate for 15 mins on ice.
- Centrifuge 8 min at 1880 x g at 4℃.
- Resuspend the pellet in 5 ml RF2 buffer and incubate 15 mins on ice.
- Put 100 µl aliquots in a liquid nitrogen container then store at -80℃.

2.2.6 Transformation of *E. coli*:

The recombinant DNA was transformed into Top10 *E. coli* cells and cultured on LB agar containing 100 µg/mL ampicillin as a selective agent.

Protocol:

- Thaw a vial (100 µl) of competent *E. coli* on ice.
- To the aliquots add content of a ligation tube or 100 – 200 ng plasmids.
- Incubate 30 min on ice.
- During this time pre-heat water bath to 42℃ and warm 2 ml LB broth medium at 37℃ in a 15 ml Falcon tube.
- Incubate samples at 42℃ in water bath for exactly 90 sec.
- Immediately transfer samples on ice, leave for 1-2 minutes.
- Add 900µl of LB medium to each sample.
- Incubate at 37℃ for 60 minutes with shaking at 200 rpm.

- Centrifuge samples for 5 min at 2000 rpm and discard supernatant.
- Plate 100 µl of bacterial pellet on LB agar plate with ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Incubate plates at 37℃ f or overnight.

2.2.7 Isolation of plasmid DNA and sequence analysis:

Recombinant plasmids were isolated by plasmid DNA purification method (Macherey-Nagel). DNA inserts were confirmed by restriction digestion (Fermentas GmbH, Germany) and by sequence analysis using extended HotShot sequencing reactions at Seqlab-Sequence Laboratories Göttingen GmbH. Each insert was sequenced at least twice. Digested plasmids were resolved by agarose gel electrophoresis and visualized with ethidium bromide.

Restriction digestion protocol (Fermentas):

- PCR product 2 µl
- *Bam*H1 1 µl (5 minutes)
- *Sa*l1 1 µl (60 minutes)
- 10X Buffer 2 µl
- Sterile water 14 µl

  Incubate at 37℃.

2.2.8 Protein expression:

For expression of the recombinant protein, *KLO8* was subcloned into the His-tag vector pQE41 (Qiagen GmbH, Germany). The DNA construct pcDNA/KLO8 was used as template with the forward (5'-GTGGAATTCTGCAGATGGATCCATGGAGCTCGCAACC–3´) and reverse (5'-GCCGCCACTGTGCTGGATGTCGACGCTCC–3´) primers. For amplification of the entire tandem repeats of *KLO8*, primers were designed to contain an extra bases sequence at the 5´ end of the primers overhang specific sequence of the vector. Primers included also restriction sites *Bam*HI and *Sa*lI (underlined) (**Fig. 2.2**).

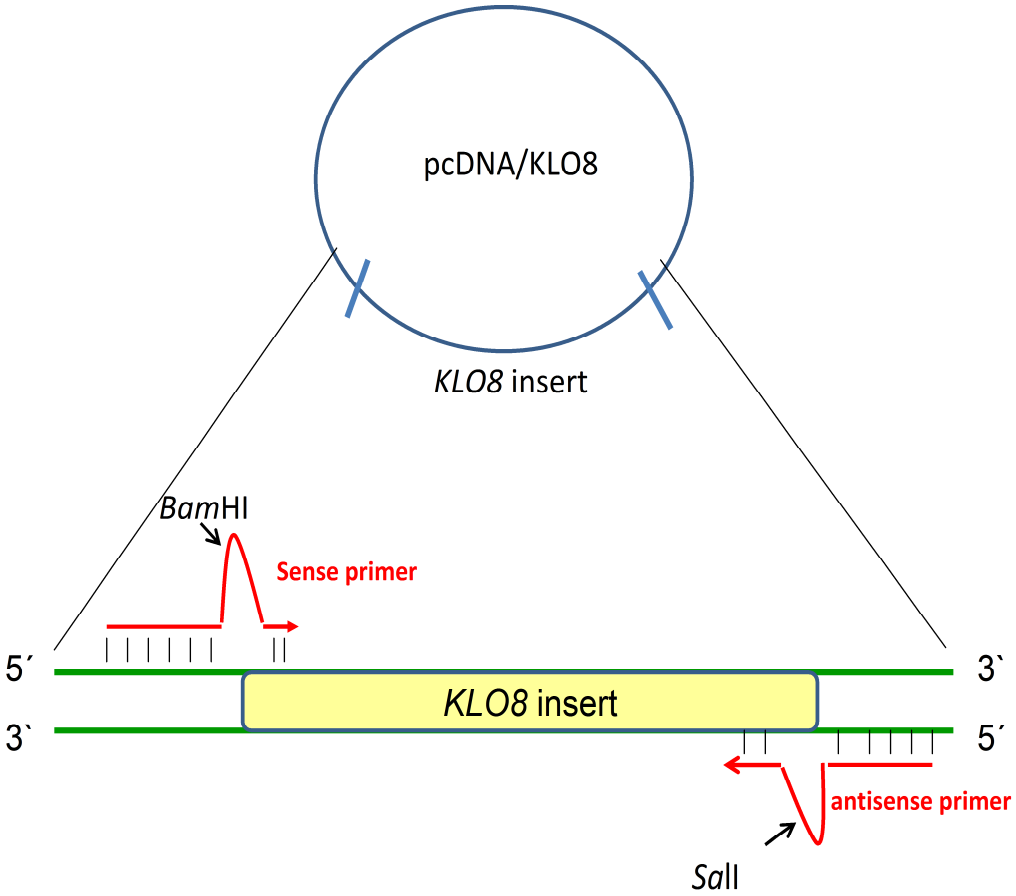


Fig. **2.2** PCR strategy for amplification of *KLO8* tandem repeats:

Amplification was performed using Phusion® Hot Start II DNA Polymerase (Thermofisher Scientific, USA) as recommended by the manufacturer. Amplified DNA fragments were digested with the same restriction enzymes and cloned in-frame and down stream of 6x His-tag into the corresponding sites of the vector pQE41 to generate the plasmid construct carrying the target gene, named as pQE41/KLO8. The recombinant plasmid was verified by DNA sequencing and restriction analysis and then transformed into competent *M15 E. coli* cells (Qiagen GmbH, Germany). *E. coli* were grown at 37℃ in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin (Sigma-Aldrich, Germany) and 25 µg/ml kanamycin (Sigma-Aldrich, Germany) to a optical density (OD_{600}) of 0.8 (BioPhotometer, Eppendorf). Recombinant protein expression was induced by adding 1mM isopropyl β-D-thiogalactoside (IPTG; Roth, Germany) for 4 hours. *E. coli* cells were harvested by centrifugation at 3340 g for 10 min at 4℃. Bacterial pellets were then lysed in PBS (p H 7.4) containing 0.25 mg/ml

lysozyme (Roth, Germany), 25 U/ml benzonase nuclease (Novagen, Germany), 10 mM imidazol (Roth, Germany), 1 mM PMSF (Sigma, USA) and 2µM β-mercaptoethanol (Sigma, USA). Subsequently, bacterial lysates were sonicated 6 times (Bandelin Sonorex, Germany) on ice for 10 seconds each with >10 seconds rest and stored at -20℃. The rKLO 8 was expressed as 6 x His-tagged His-rKLO8 fusion protein and was recovered in the soluble fraction of the bacterial lysate by SDS-PAGE.

Table 2.10: PCR protocol (Phusion® Hot start II high-Fidelity DNA polymerase):

Component	Volume /50 µl	Final conc.
H2O	28.5 µl	-
5X Phusion GC buffer	10 µl	1 x
10 mM dNTPs	1 µl	200 µM
Primer 1	2.5 µl	10 pmol/µl
Primer 2	2.5 µl	10 pmol/µl
Template DNA	5 µl	100 ng
Phusion DNA polymerase	0.5 µl	0.02 U/µl

Cycling condition:

Cycle step	Temp.	Time	Cycles
Initial denaturation	98^{o}C	30 sec.	1
Denaturation Annealing Extension	98^{o}C 61.0 – 71.0^{o}C 72.0^{o}C	10 sec. 20 sec. 30 sec.	35
Final extension	72.0^{o}C 4^{o}C	10 min. Hold	1

2.2.9 Protein purification:

Purification was carried out using nickel nitrilotriacetic (Ni-NTA) columns (Qiagen GmbH, Germany). The supernatant was loaded into a Ni-NTA column, which was pre-equilibrated with PBS, pH 7.4, containing 10 mM imidazole, 1 mM PMSF and 2 µM β-mercaptoethanol. The recombinant protein was eluted with the same buffer containing 400 mM imidazole. Salts and imidazole were removed by dialysis in PBS buffer. Protein concentration was determined

using the Bradford assay compared to bovine serum albumin (BSA) as standard. Protein aliquots were kept at -80℃.

Large-scale Culture Growth for Protein Expression:

1. Inoculate 2 ml of LB broth containing 100 µg/ml ampicillin and 25 µg/ ml kanamycin from a fresh plate with expression-vector strain. Grow O/N at 37℃ with shaking.
2. Inoculate 400 ml (X2) of antibiotic-containing media with 400 µl from the 2 ml culture (1:1000 dilution of starter culture). Grow at 37℃ with shaking at 150 rpm until OD_{600} reach 0.8 (~ 3 hrs).
3. Collect 50 ml to be used as non-induced fraction.
4. Induce protein synthesis by adding 400 µl IPTG 1M (final conc. 1 mM). Incubate for 4 hrs at 37℃ with shaking.
5. Collect pellet by centrifugation for 10 min. at 3300 x g in 50 ml falcon tubes.
6. Wash with cold PBS and repeat centrifugation X1.
7. Freeze pellet at -20℃ and thaw (pellets can be stored frozen for the following day).
8. For each pellet from 400 ml culture, add:
    - 10 ml 10 mM imidazol buffer (lysis), vortex 1 min
    - 20 µl 0.1 M PMSF
    - 2 µl benzonase nuclease (Novagen)
    - 20 µl lysozyme (stock 25 mg/ml)
9. Mix well and incubate 1 hr at 4℃ with shaking a t 350 rpm.
10. Freeze and thaw X3 at -20℃.
11. Sonicate (in glass bottles) 6X for 10 s with > 10 s rest at 4℃.
12. Freeze and thaw X1.
13. Centrifuge at 16400 x g for 30 mins at 4℃ in e ppendorf tubes.
14. Collect supernatants and pellets.
15. Add supernatant to ~ 2 ml Ni- agarose beats column previously 2-3 X washed with water to remove ethanol and equilibrated 2-3 X with 10 ml 10 mM imidazol buffer.
16. Collect flow-through (unbound fraction).
17. Wash with 15 ml 20 mM imidazol buffer, collect wash (wash fraction 1).
18. Wash with 15 ml 60 mM imidazol buffer (wash fraction 2).
19. Elute with 10 ml 400 mM imidazol buffer and collect 1-1.5 ml fractions.

2.2.10 SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot (WB) analysis:

Fractions of bacterial cell lysates or the purified protein were loaded on a 12% SDS-PAGE under denaturing conditions (Laemmli, 1970) and then stained with Coomassie Brilliant Blue G250 (Merck KGaA, Germany) or transferred to a nitrocellulose transfer membrane (Whatman GmbH, Germany) using the Bio-Rad Semi-dry Trans-Blot at 200 mA for 1hr. The membrane was blocked with 5% BSA (w/v) in 100 mM NaCl, 0.05% Tween 20 (v/v) and 10 mM Tris-HCl, pH 7.4 (blocking buffer) and subsequently incubated for 18 hrs at 4℃ with sera from patients– or healthy controls, diluted 1:1000 in blocking buffer. After washing, blots were incubated for 1 hr at room temperature (R/T) with Peroxidase-conjugated Donkey Anti-Human IgG (H+L) (Jackson Immunoresearch Laboratories, USA) diluted 1:10000. The protein bands were revealed with Maximum Sensitivity Substrate system (Thermo Scientific, USA).

2.2.11 ELISA:

The optimal protein concentration and serum dilutions were determined using 10 pooled VL sera and 10 control sera from non-endemic areas in Sudan. To select optimal conditions for the discrimination between positive and negative sera, protein concentrations were titrated against serial dilutions of positive or negative sera. MaxiSorp™ high protein-binding capacity polystyrene 96 ELISA plates (NUNC TM Serving Life Science, Denmark) were used. Protein concentrations of 5 - 50 ng/well were tested for coating ELISA plates overnight at 4℃ in 0.1 M $NaHCO_3$ buffer, pH 9.6. Plates were washed with PBS containing 0.05% (v/v) Tween-20 and then blocked with 3% (w/v) BSA, in the same buffer at R/T for 1-2 hours. After additional washes, 50 µl diluted positive or negative serum samples were added to each well, and plates were incubated at R/T for 45 minutes. After washing, 50 µl/well peroxidase-conjugated AffiniPure Donkey anti-Human IgG (H+L) or rabbit anti-Dog IgG (H+L), diluted 1:10000, were added to each well and plates were incubated at R/T for further 1 hr. Colour was developed with hydrogen peroxide and tetramethylbenzidine (R&D Systems, USA). Reaction was stopped with 2N sulfuric acid after 10 minutes incubation in the dark. The optical density (OD)

was measured at 450 nm using an ELISA microreader (FLUOstar Omega, BMG LABTECH). Each sample was run in duplicates and the mean of absorbance values were calculated. Samples showing invalid or inconsistent results were repeated.
As control, pooled positive and negative sera were included in each plate, when testing individual sera.

2.2.12 Bioinformatics analysis:

Amino acid sequence of *KLO8* was determined with the ExPASy Proteomics Server of the Swiss Institute of Bioinformatics (http://web.expasy.org/translate/) and was compared with published sequences obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Amino acid sequence of KLO8 was aligned with published kinesin-related proteins of seven strains belonging to *L. donovani* subspecies using ClustalW2-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Homology search performed with BLASTP 2.2.1 showed that KLO8 has the highest identity with an amino acid fragment encoded by the 756 bp repeat of K39 kinesin protein of *L. donovani* 1S (2564-3319). Therefore, this region was used in the alignment. Immunodominant repeats of KLO8 (294 AA), K39 (252 AA) and KE16 (155 AA) were also aligned. DNA sequence of *KLO8* was analyzed to locate and display tandem repeats using Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) (Benson, 1999). Nucleotide and amino acid sequences have been deposited in GenBank (accession numbers KC788285 and AGL98402) using Sequin software tool (http://www.ncbi.nlm.nih.gov/ Sequin/).

2.2.13 Statistical analysis:

Data were analyzed using GraphPad Prism software (GraphPad Prism Inc., San Diego, Ca). Significance of antibody responses was assessed using unpaired *Student t* test or one way *ANOVA* test and p values < 0.05 were considered significant. Cut off values for each recombinant protein were defined as mean absorbance values of 30 sera of healthy controls from Sudan or 20 dogs' sera from Croatia plus 3 standard deviations (SD). Sensitivity,

specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated to assess usefulness of the diagnostic assays at 95% confidence intervals. Sensitivity was defined as the percentage of serum samples of confirmed human or canine VL with positive test results. Specificity was assessed as percentage of total serum samples from non-VL cases with negative test results. This group included healthy controls, malaria-, TB-, toxoplasmosis-, and leukemic patients. PPV was calculated as number of true positives divided by the number of true positives and false positives. NPV was calculated as number of true negatives divided by the number of true negatives plus number of false negatives.

Formulae:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + False negatives}} \times 100\%$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + False positives}} \times 100\%$$

$$\text{PPV} = \frac{\text{True positives}}{\text{True positives + False positives}} \times 100\%$$

$$\text{NPV} = \frac{\text{True negatives}}{\text{True negatives + False negatives}} \times 100\%$$

3. RESULTS:

3.1 Identification, characterization and serological evaluation of a novel *L. donovani* antigen rKLO8 for visceral leishmaniasis in Sudan:

3.1.1 Identification and characterization of *KLO8:*

A Partial gene fragment encoding immunodominant kinesin protein of *L. donovani*, designated *KLO8*, was amplified from promastigote genomic DNA from promastigotes using primers designed according to the published *L. chagasi* gene for kinesin-related protein (GenBank: L07879.1). The amplified products revealed multiple bands with sizes equivalent to 117bp repeats (**Fig. 3.1A**). The largest amplification product was digested with *Eco*RV and cloned into the corresponding site of the plasmid vector pcDNA3.1(+) to make the nontagged KLO8 construct, pcDNA/KLO8. The insert was confirmed by restriction digestion with *Bam*HI and *Xba*I (**Fig. 3.1B**) and by sequencing. Sequence analysis revealed a partial open reading frame of 883 bp, lacking the start codon ATG at the 5` end of the sense sequence.

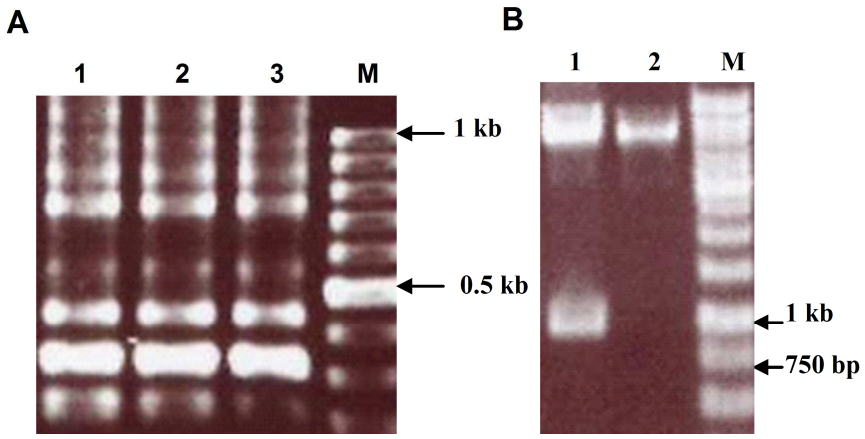


Fig. 3.1: Amplification and cloning of *KLO8*. Gene fragment encoding KLO8 of *L. donovani* was amplified and cloned into pcDNA3.1(+). (A) PCR products were resolved by agarose electrophoresis showed multiple repeat bands equivalent to 117 bp repeats. Lanes 1-3, DNA products amplified at 61.0 - 71.0℃; Lane M, 100 bp DNA Ladder. (B) Recombinant plasmid was isolated and screened by restriction digestion with *Bam*HI and *Xba*I and resolved by agarose gel electrophoresis. Lane 1, digested plasmid carrying the *KLO8* gene; Lane 2, empty vector digested with the same enzyme as control; M, 1 kb DNA Ladder.

Predicted amino acid (AA) sequence, generated with ExPASy (http://web.expasy.org/translate/), showed that *KLO8* encodes a protein of 294 AAs with a predicted molecular mass of 32.4 kDa and isoelectric point (IP) of 4.39. Nucleotide sequence of *KLO8* was submitted to GenBank under the accession number KC788285 (see Appendix). Homology search in protein database (http://www.ncbi.nlm.nih.gov/) showed that KLO8 protein contains putative conserved domains of significant similarity with kinesin proteins of strains belong to *L. donovani* subspecies (**Table 3.1**). KLO8 exhibited 93% and 88% AA identities with kinesin protein K39 of *L. infantum* (synonymous *L. chagasi*) strain BA-2 from Brazil (GenBank: AAA29254.1) and KE16 of *L. donovani* strain KE16 from India (GenBank: AAT40474.1), respectively. These two proteins are currently used in the known commercially available diagnostic rapid tests for VL. Of interest, KLO8 exhibited 97% identity with the kinesin protein Ldk39 of *L. donovani* 1S-CL2D from Sudan (GenBank: ABI14928.1), which however was never processed for development of a diagnostic test.

Identities were high to moderate with other kinesin proteins of *L. donovani*, ranging from 90 – 69%. Moreover, an identity of 79% was found with K28 fusion protein (GenBank: ADR74368.1), a synthetic protein construct derived from *L. donovani*, which consists of multiple tandem repeats of *L. donova*ni haspb1 (hydrophilic acylated surface protein B1) and K39 of *L. chagasi* fused to the complete haspb2 protein (Pattabhi et al., 2010). The fact that there is still 3% sequence variations between KLO8 and Ldk39 proteins, although derived from strains from the same region (Sudan), demonstrates that AA sequences of kinesin proteins vary even between different strains from the same region.

Table 3.1: Amino acid (AA) sequence identities of *KLO8* (GenBank: KC788285) with similar published kinesin proteins of *L. donovani:*

Strain	Geographic origin of the strains	AA identity	GenBank no.
L. donovani 1S	Sudan	97%	ABI14928.1
L. chagasi BA-2	Brazil	93%	AAA29254.1
L. donovani KE16	India	88%	AAT40474.1
L. donovani DD8[1]	India	69%	AAT40475.1
L. donovani Morena	India	83%	ABG43049.1
L. donovani DD8[2]	Bangladesh	90%	BAF34578.1
L. infantum LON49	Iran	90%	ACF77142.1

[1] A WHO reference strain from India (MHOM/IN/DD8/1968). [2] *L. donovani* isolate from Bangladesh (Shamsuzzaman et al., 2000).

3.1.2 Tandem repeats analysis of kinesin protein genes in *Leishmania*:

Proteins containing tandem repeats are potential candidates for detection of immune responses against various parasites including *Leishmania* (Goto et al, 2010). Thus, *KLO8* was checked to determine whether it meets the tandem repeats (TRs) definition using Tandem Repeat Finder (http://tandem.bu.edu/ trf/trf.html). The program calculates an alignment score based on specific features such as period cycle of the repeat, copy numbers and percent of similarities between the repeats. TRs in other similar published protein genes of *Leishmania* were compared with those of *KLO8* (**Table 3.2**). The K39 kinesin protein gene (9831bp) of *L. donovani* 1S from Sudan showed 22 tandem repeats of different period cycle. DNA fragment compose of 742 bp between nucleotide positions 2113 and 2854 showed higher identity with *KLO8*. This region was, therefore, used in the analysis.

Results showed conservation of a repeat of 117 bp in all *L. donovani* strains. Said 117 bp sequence encodes protein with 39 AA each. Copy number of repeats showed considerable variation among the different strains, which was higher in the strains from Sudan, Brazil and Bangladesh than Indian and Iranian strains. Importantly, tandem repeat score of *L. donovani* LO8 was higher (1299) than those of *L. chagasi* BA-2 and *L. donovani* KE16; 1276 and 709, respectively. Since the level of protein immunogenicity depends to a greater extent on the period cycle and copy number of repeats and thus tandem repeat score, rKLO8 may therefore provide a significant contribution in evaluating immune responses to *Leishmania*.

Table 3.2: Characteristics of tandem repeats in kinesin protein genes of *Leishmania:*

Strains	Tandem repeats charactaristics				
	GC-(%)	Period Cycle (bp)	Copy no.	Similarity between repeats (%)	Score
L. donovani LO8	66	117	6.3	94	1299
L. donovani 1S	67	117	6.3	91	1213
L. chagasi BA-2	68	117	6.4	96	1276
L. donovani KE16	68	117	4.0	91	709
L. donovani DD8[1]	66	117	3.2	78	392
		117	3.1	77	356
		234	1.9	80	523
L. donovani Morena	67	117	3.9	92	798
L. donovani DD8[2]	69	117	7.4	97	1618
L. infantum LON 49	68	117	3.0	92	575

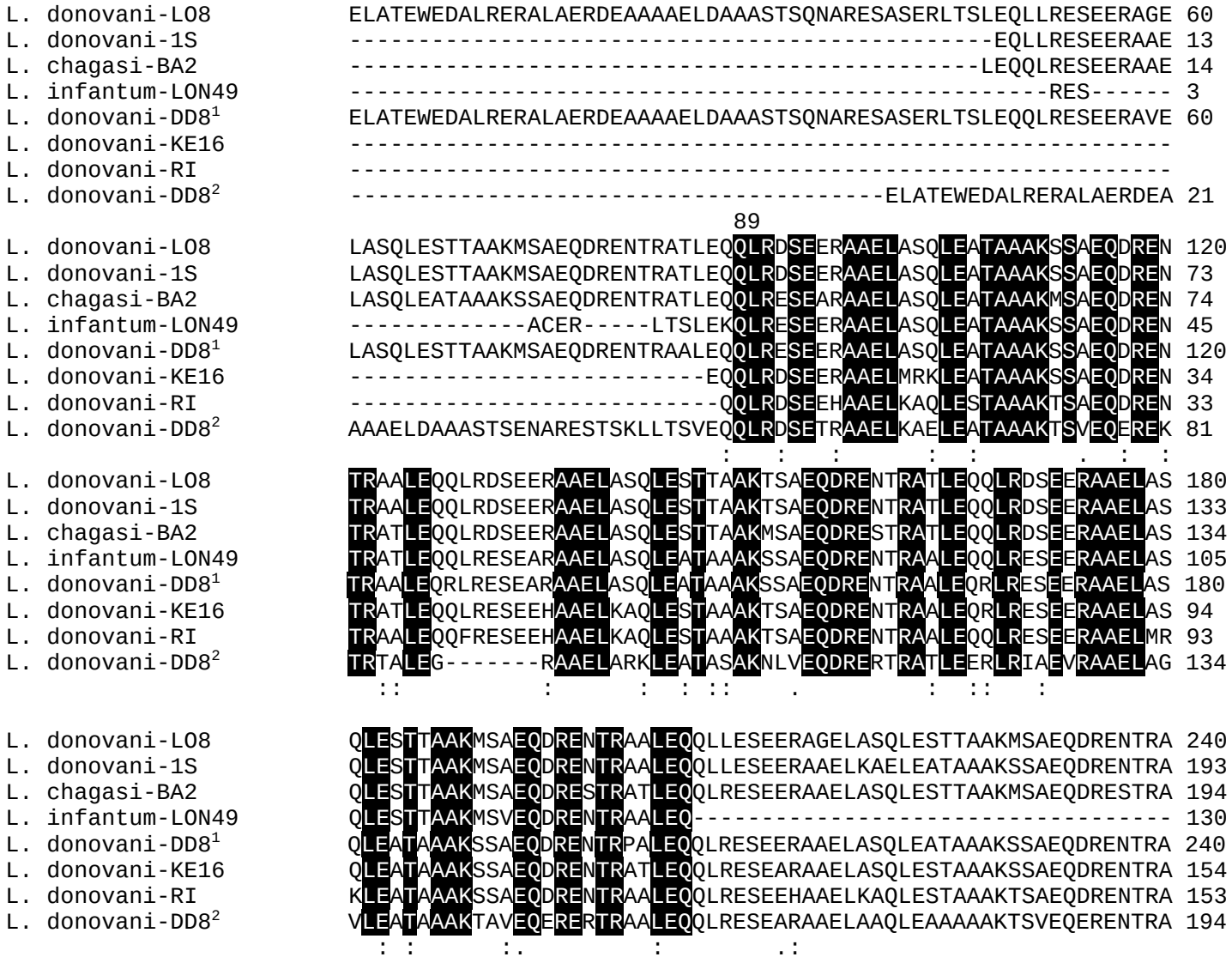
[1] *L. donovani* strain (MHOM/IN/DD8/1968) from India (Sivakumar et al., 2008).

[2] *L. donovani* strain DD8 from Bangladesh (Shamsuzzaman et al., 2000).

3.1.3 Variation of AA composition in kinesin proteins of *L. donovani*:

To identify differences in composition of amino acids in kinesin proteins of *L. donovani*, multiple sequence alignment was performed using ClustalW2- Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). In this analysis aligned regions are jointed creating gaps to show the shared and variable regions. KLO8 was aligned with closely related kinesin proteins of 7 *Leishmania* strains obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). These strains represent *L. donovani* subspecies from the various endemic regions in Sudan, India, Bangladesh, Brazil and Iran. KLO8 showed the highest identity with a 252 AA sequence fragment of K39 kinesin protein of *L. donovani* 1S (GenBank: DQ831678.1). Therefore, this region was used in the analysis. As shown in **Fig. 3.2**, kinesin proteins of *Leishmania* are highly conserved across the examined strains. However, variation in amino acid composition was observed. Interestingly, none of these sequences showed complete homology, even between strains from the same areas (Sudanese and Indian strains).

To identify differences within immunodominant epitopes of the three *Leishmania* antigens, KLO8 was aligned with AA repeats of K39 (GenBank: L07879.1) and KE16 (GenBank: AY615886.1). As shown in **Fig. 3.3**, immunodominant epitopes of the three antigens show presence of non-conservative amino acids. Differences in AA composition were highlighted in grey and identical regions were left unmarked. Altogether, these results confirm the variability in immunodominant repeats of kinesin proteins among different species and within the same species of *L. donovani*.


L. donovani-LO8 ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESASERLTSLEQLLRESEERAGE 60
L. donovani-1S ----------EQLLRESEERAAE 13
L. chagasi-BA2 ----------LEQQLRESEERAAE 14
L. infantum-LON49 ----------RES------ 3
L. donovani-DD8[1] ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESASERLTSLEQQLRESEERAVE 60
L. donovani-KE16 ----------
L. donovani-RI ----------
L. donovani-DD8[2] ----------ELATEWEDALRERALAERDEA 21
89
L. donovani-LO8 LASQLESTTAAKMSAEQDRENTRATLEQQLRDSEERAAELASQLEATAAAKSSAEQDREN 120
L. donovani-1S LASQLESTTAAKMSAEQDRENTRATLEQQLRDSEERAAELASQLEATAAAKSSAEQDREN 73
L. chagasi-BA2 LASQLEATAAAKSSAEQDRENTRATLEQQLRESEARAAELASQLEATAAAKMSAEQDREN 74
L. infantum-LON49 ----------ACER-----LTSLEKQLRESEERAAELASQLEATAAAKSSAEQDREN 45
L. donovani-DD8[1] LASQLESTTAAKMSAEQDRENTRAALEQQLRESEERAAELASQLEATAAAKSSAEQDREN 120
L. donovani-KE16 ----------EQQLRDSEERAAELMRKLEATAAAKSSAEQDREN 34
L. donovani-RI ----------QQLRDSEEHAAELKAQLESTAAAKTSAEQDREN 33
L. donovani-DD8[2] AAAELDAAASTSENARESTSKLLTSVEQQLRDSETRAAELKAELEATAAAKTSVEQEREK 81
TRAALEQQLRDSEERAAELASQLESTTAAKTSAEQDRENTRATLEQQLRDSEERAAELAS 180
L. donovani-LO8 TRAALEQQLRDSEERAAELASQLESTTAAKTSAEQDRENTRATLEQQLRDSEERAAELAS 180
L. donovani-1S TRAALEQQLRDSEERAAELASQLESTTAAKTSAEQDRENTRATLEQQLRDSEERAAELAS 133
L. chagasi-BA2 TRATLEQQLRDSEERAAELASQLESTTAAKMSAEQDRESTRATLEQQLRDSEERAAELAS 134
L. infantum-LON49 TRATLEQQLRESEARAAELASQLEATAAAKSSAEQDRENTRAALEQQLRESEERAAELAS 105
L. donovani-DD8[1] TRAALEQRLRESEARAAELASQLEATAAAKSSAEQDRENTRAALEQRLRESEERAAELAS 180
L. donovani-KE16 TRATLEQQLRESEEHAAELKAQLESTAAAKTSAEQDRENTRAALEQRLRESEERAAELAS 94
L. donovani-RI TRAALEQQFRESEEHAAELKAQLESTAAAKTSAEQDRENTRAALEQQLRESEERAAELMR 93
L. donovani-DD8[2] TRTALEG-------RAAELARKLEATASAKNLVEQDRERTRATLEERLRIAEVRAAELAG 134
QLESTTAAKMSAEQDRENTRAALEQQLLESEERAGELASQLESTTAAKMSAEQDRENTRA 240
L. donovani-LO8 QLESTTAAKMSAEQDRENTRAALEQQLLESEERAGELASQLESTTAAKMSAEQDRENTRA 240
L. donovani-1S QLESTTAAKMSAEQDRENTRAALEQQLLESEERAAELKAELEATAAAKSSAEQDRENTRA 193
L. chagasi-BA2 QLESTTAAKMSAEQDRESTRATLEQQLRESEERAAELASQLESTTAAKMSAEQDRESTRA 194
L. infantum-LON49 QLESTTAAKMSVEQDRENTRAALEQ---------- 130
L. donovani-DD8[1] QLEATAAAKSSAEQDRENTRPALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRA 240
L. donovani-KE16 QLEATAAAKSSAEQDRENTRATLEQQLRESEARAAELASQLESTAAAKSSAEQDRENTRA 154
L. donovani-RI KLEATAAAKSSAEQDRENTRAALEQQLRESEEHAAELKAQLESTAAAKTSAEQDRENTRA 153
L. donovani-DD8[2] VLEATAAAKTAVEQERERTRAALEQQLRESEARAAELAAQLEAAAAAKTSVEQERENTRA 194

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L. donovani-LO8          ALEQQLRDSEERAAELASQLESTTAAKMSAEQDRENTRATLEQQLRDSEERAAE------ 294
L. donovani-1S           ALEQQLRDSEERAAELASQLESTTAAKTSAEQDRENTRATLEQQLRDSEERAAE------ 247
L. chagasi-BA2           TLEQQLRDSEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRDSEERAAELASQ-- 252
L. infantum-LON49        ------------------------------------------------------------
L. donovani-DD8[1]       ALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRESEERAAELASQLE 300
L. donovani-KE16         T----------------------------------------------------------- 155
L. donovani-RI           T----------------------------------------------------------- 154
L. donovani-DD8[2]       TLEERLRLAEVRAAELAARLKSTAAVKSAMEQDRENTRAT-------------------- 234

L. donovani-LO8          ------------------------------------
L. donovani-1S           ------------------------------------
L. chagasi-BA2           ------------------------------------
L. infantum-LON49        ------------------------------------
L. donovani-DD8[1]       ATAAAKSSAEQDRENTRAALEQRLRESEARGAELASQ 337
L. donovani-KE16         ------------------------------------
L. donovani-RI           ------------------------------------
L. donovani-DD8[2]       ------------------------------------
```

Fig. 3.2 Sequence alignment of kinesin proteins of *L. donovani*. KLO8 was aligned with similar published proteins of 7 strains of *L. donovani* using ClustalW2-Multiple Sequence Alignment. These parasites included *L. donovani* 1S, *L. chagasi* BA-2, *L. infantum* LON 49, *L. donovani* DD8[1] isolated in Bangladesh, *L. donovani* DD8[2] from India, *L. donovani* KE16, and a recent isolate (RI) of *L. donovani* from India (GenBank numbers are shown in **Table 3.1**). K39 sequence fragment of *L. donovani* 1S that showed highest homology with KLO8 was used in this analysis. Dashed lines indicate gaps. Conserved amino acids were highlighted in black; (**:**), very similar; (**·**), similar. Length of each sequence is indicated at the 3' end of the sequence.

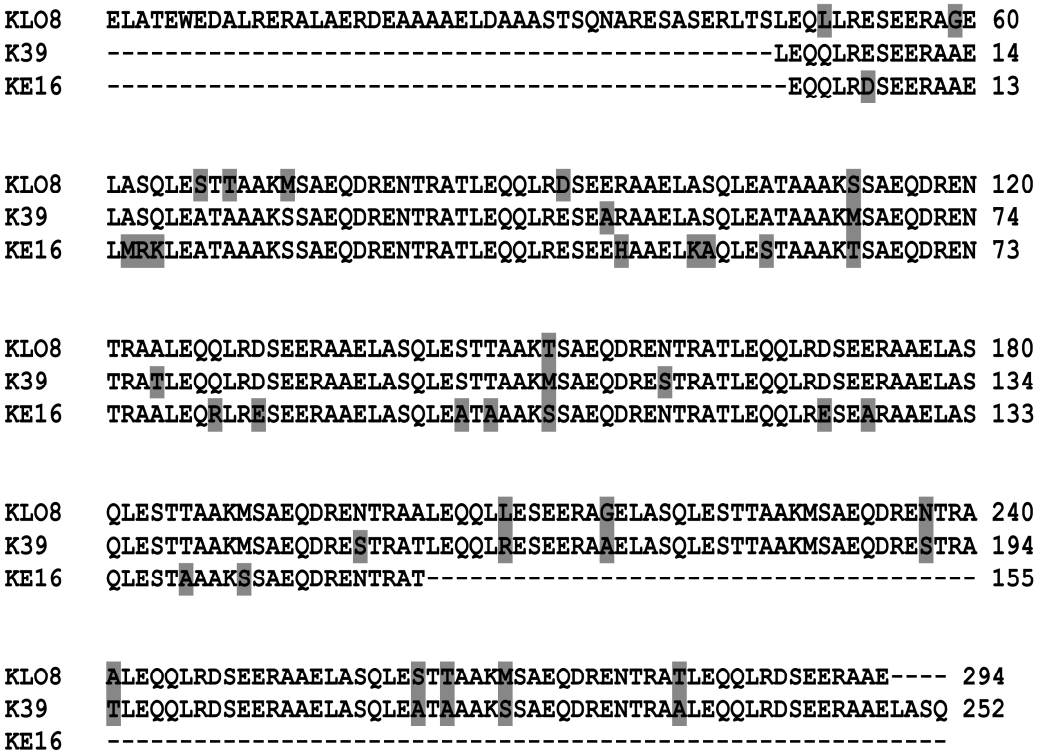

Fig. 3.3 Sequence alignment of KLO8, K39 and KE16 antigens: Immunodominant repeats of KLO8 (294 AA), K39 (252 AA) and KE16 (155 AA) were aligned using ClustalW2-Multiple Sequence Alignment program. Different residues were highlighted in grey and identical were left unmarked. Dashed lines indicate gaps.

3.1.3 Recombinant protein KLO8 as a suitable candidate for detection of *Leishmania*-specific antibodies:

Since KLO8 differs from other *Leishmania* antigens, it was necessary to investigate its reactivity with sera of VL from Sudan. KLO8 was expressed as a His-tagged recombinant protein in M15 *E. coli.* Expression was confirmed by SDS-PAGE. As shown in **Fig. 3.4A** (lane 3), the apparent molecular weight of the His tagged fusion protein was 35kDa. The reactivity of purified recombinant protein rKLO8 was assessed in Western blot analysis using pooled sera from 10 VL patients or 10 healthy controls. As shown in **Fig. 3.4B**, the positive sera recognized the recombinant protein (lane 2 & 3), while the negative sera did not (lane 1). These results demonstrate that the recombinant protein rKLO8 is suitable for detection of *Leishmania*-specific antibodies in sera of Sudanese VL patients.

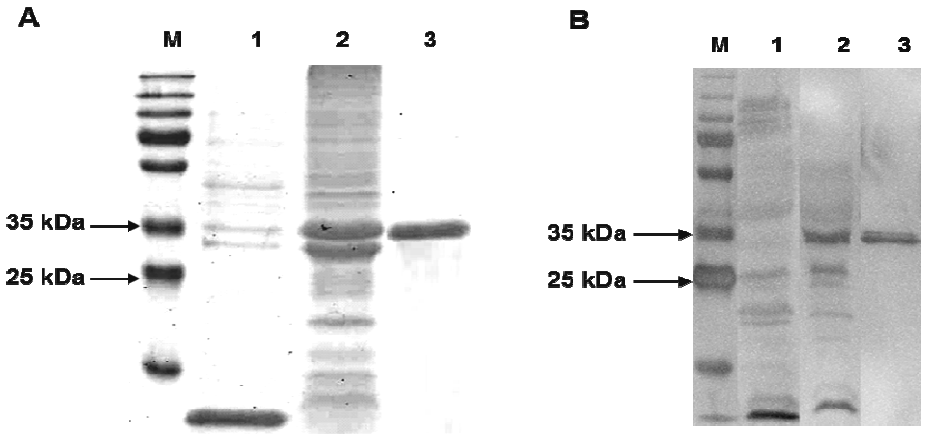

Fig. 3.4 SDS-PAGE and western blot analysis. KLO8 was expressed as 6 x His-tagged protein in M15 *E. coli* and purified on a Ni-NTA column. (A) Protein expression was checked on a 12% acrylamide gel stained with Comassie blue; lane 1 and 2, bacterial lysates from uninduced or 1mM IPTG-induced cultures, respectively; lane 3, purified rKLO8; M, Protein ladder. (B) Reactivity of KLO8 was tested in WB analysis using 10 pooled VL sera or 10 pooled healthy control sera from Sudan; lanes 1 and 2, lysates of IPTG induced cultures blotted with negative or positive sera, respectively; lane 3, purified rKLO8 blotted with positive sera; M, Protein ladder.

3.1.4 rKLO8 ELISA for diagnosis of visceral leishmaniais:

Next, an indirect IgG ELISA system using the recombinant protein rKLO8 was established for detection of *Leishmania*-specific antibodies in sera of VL patients from Sudan. As shown in **Fig. 3.5**, all tested protein concentrations (50-5 ng/well) were detected by pooled VL sera and did not cross-react with pooled sera from healthy individuals. ODs of positive sera were at least 4-fold higher compared to negative sera; however this ratio changed to much higher values when sera were more diluted. Coating ELISA plates with a concentration of 5 ng rKLO8 protein was sufficient for positive detection of sera from VL patients diluted up to 1:25600. As a result of these titrations, a protein concentration of 5 ng/well and serum dilutions of 1:800 were selected and used as standard conditions in subsequent experiments. In some experiments, VL sera with negative results at 1:800, were re-tested at a serum dilution of 1:100.

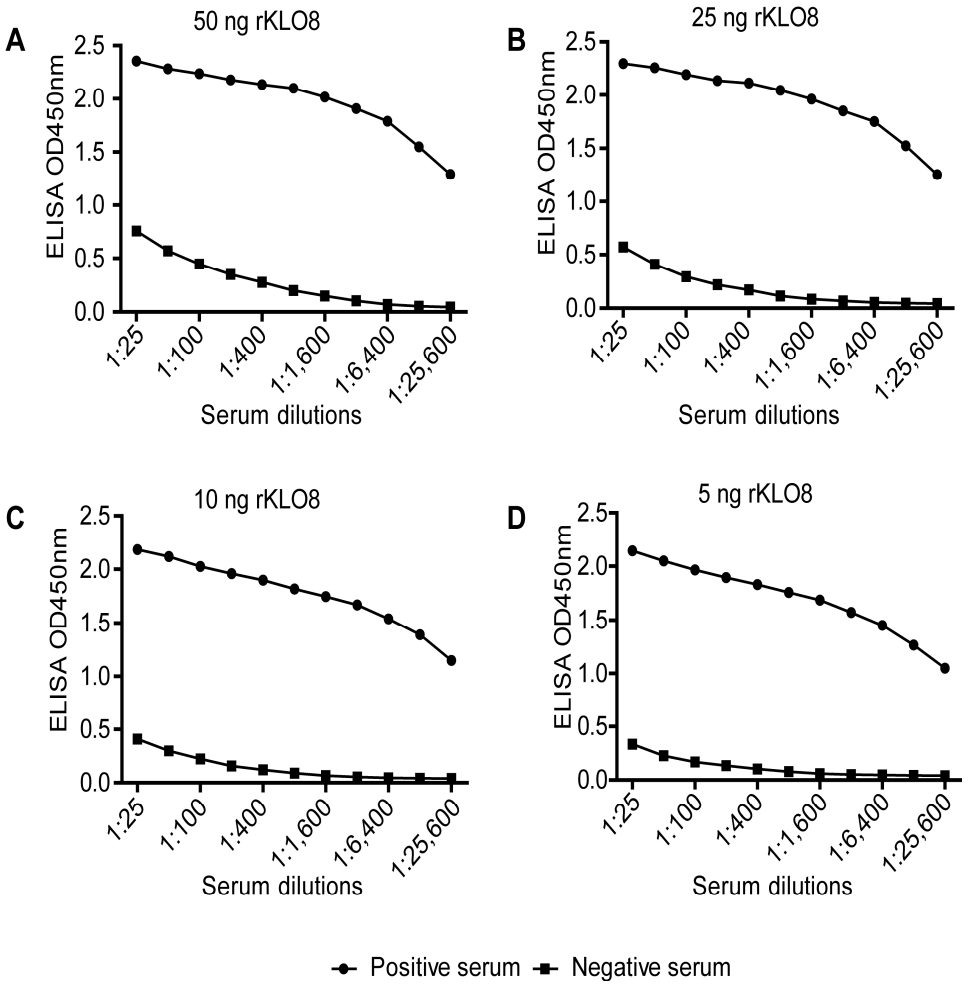

Fig. 3.5 Establishment of an indirect IgG ELISA for detection of *Leishmania*-specific antibodies. For selection of the optimal ELISA conditions, 10 pooled VL or healthy control sera were titrated at serial twofold dilutions (1:25-1:25,600) and tested against different concentrations of the recombinant protein rKLO8. (A) 50 ng/100µl, (B) 25 ng/100µl, (C) 10 ng/100µl, (D) 5 ng/100µl. Sera were tested in duplicates and means were taken.

3.1.5 Performance of rKLO8 and rK39 recombinant proteins for detection of IgG antibodies in Sudanese VL patients:

The original assumption was that antibodies of VL patients from Sudan will better react with homologous proteins of *L. donovani* than the heterologous one of *L. chagasi* (K39). Thus, reactivity of the two recombinant proteins rKLO8 and rK39 were evaluated in ELISA using individual human VL (n=106) and control (n=77) sera from Sudan. The recombinant protein rK39, obtained from Rekom Biotech, was expressed as 6 x His-tagged fusion protein in *E. coli*. To ensure similar conditions, rKLO8 was also expressed as 6 x His-tagged protein in *E. coli*. Sera of patients were diluted at 1:800 and tested on a protein concentration of 5 ng/well.

Quantitative analysis of antibodies in sera of VL to both recombinant proteins demonstrated significantly higher antibody levels than those of control subjects ($P<0.0001$) (**Fig. 3.6A**) although absorbance values among the patients` sera varied depending on the recombinant proteins. In general, sera tested on rKLO8 yielded higher OD values than on rK39 with a mean value of 1.12 ± 0.97 for rKLO8 and 0.93 ± 0.77 for rK39. In addition, sensitivity of rKLO8 was also increased with 92.5% (98/106) for rKLO8 versus 86.8% (92/106) for rK39. Notably, none of the healthy or diseased controls (n=77) showed cross-reaction with either of the recombinant proteins.

Sera of VL that were negative on rK39 or rKLO8 (n=14) were then retested and compared to control sera (n=77) at 1:100 serum dilution. As shown in **Fig. 3.6B**, retesting on rKLO8 yielded increased positive detection of VL patients (12/14) compared to rK39 (10/14) at cut-off values of 0.41 and 0.32 for rKLO8 and rK39, respectively. In addition, control sera tested on rKLO8 revealed less cross reactivity as compared to rK39. Both proteins showed cross reactivity with 3 sera from malaria patients and in addition rK39 showed false positivity with one healthy endemic individual. These results demonstrate the increased reactivity of rKLO8 compared to rK39 in Sudan. Although the difference was not significant, it is likely that rKLO8 provides enhanced sensitivity for detection of *Leishmania*-specific antibodies in Sudan. Therefore, a rapid test based on rKLO8 may be more sensitive for the detection of VL in Sudan and other East African countries where similar strains of *L. donovani* are endemic.

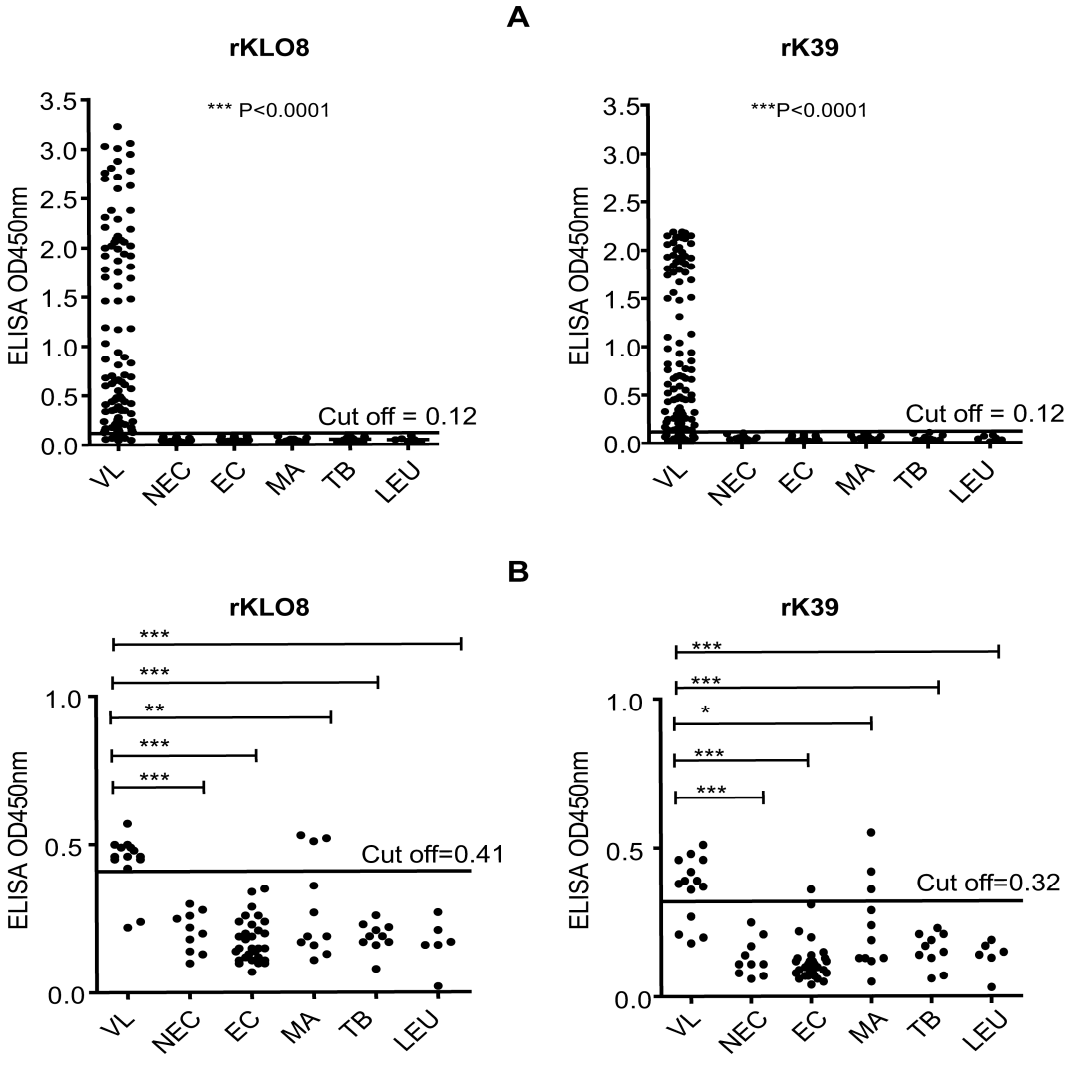

Fig. 3.6 Comparative reactivity of VL and control sera with rKLO8 and rK39 recombinant proteins. The rKLO8 and rK39 proteins were used and compared in ELISA using protein concentrations of 5ng/100µl in 0.1M sodium carbonate. A panel of sera from VL patients and controls were tested. Visceral leishmaniasis, VL (n=106), non-VL controls (n=77) including non-endemic healthy controls (NEC; n=20), endemic healthy controls (EC; n=30), malaria (MA; n=11), tuberculosis (TB; n=10), or leukaemia (LEU; n=6). (A) Sera were tested at dilutions of 1:800 and a cut off value (0.12) was established as means + 3 SD of 30 healthy controls from Sudan. (B) VL sera (n=14) with negative results at 1:800 were retested at a serum dilution of 1:100 and compared with the controls described in A. Cut off values were recalculated using 20 non-endemic healthy sera and found to be 0.41 and 0.32 for rKLO8 and rK39, respectively. Statistical analysis was performed using one way ANOVA nonparametric test.

3.1.6 Diagnostic performance of rKLO8- and rK39 ELISA and two commercial kits for diagnosis of visceral leishmaniais in Sudan:

Using the same panel of VL and control sera, results of rKLO8- and rK39 ELISA were next compared with two commercial diagnostic kits, the rK39 rapid test and a freeze-dried version of DAT. As shown in **Table 3.3**, overall sensitivity of rKLO8 (98.1%) was higher than that of rK39 (96.2%) when measured by ELISA. In comparison, the rK39 rapid test yielded sensitivity of 81.1% and DAT of 94.3%. With respect to specificity, rKLO8 ELISA showed slightly increased performance (96.1%) compared to the rK39 ELISA (94.8%) but lower than DAT (100%) and rK39 rapid test (98.7%). Accordingly, PPVs and NPVs were 97.2% and 97.4% for rKLO8 ELISA, 96.2% and 94.8% for rK39 ELISA, 98.9% and 79.2.9% for rK39 rapid test and 100% and 92.8% for DAT, respectively.

Interestingly, results of the four tests showed some discrepancies. Although tested positive in rKLO8 ELISA, 6 (5.7%) sera of the confirmed VL patients were negative (1:<1600) in DAT (**Fig. 3.7A**). In addition, sera of 6 patients had weak DAT titres (1:3200-1:6400). On the other hand, while being positive in DAT, 4 (3.8%) or 2 (1.9%) sera of VL patients were not detected by rK39 or rKLO8, respectively. Those 4 cases were also negative in the rapid test. However, VL sera with positive or negative DAT results reacted similarly with rKLO8 (**Fig. 3.7B**), suggesting that rKLO8 and DAT react with different antibody specificities and thus monitoring different immune reactivities. Thus, a combination of both, rKLO8 ELISA and DAT provided 100% sensitivity for detection of VL. In addition, rKLO8 ELISA detected all VL sera that were positive in rK39 rapid test (**Fig. 3.7C**), but sera negative in the rapid test displayed still low antibody reactivity when tested with rKLO8 ($p<0.0001$) (**Fig. 3.7D**).

Table 3.3 Diagnostic performance of rKLO8- and rK39 ELISA, rK39 rapid test (RT) and DAT for visceral leishmaniasis in Sudan:

Test	TP	FN	TN	FP	Sensitivity (n=106)	Specificity (n=77)	PPV	NPV
					Estimated values at 95% CI			
rKLO8 ELISA	104	2	74	3	98.1%	96.1%	97.2%	97.4%
rK39 ELISA	102	4	73	4	96.2%	94.8%	97.2%	94.8%
rK39 RT	86	20	76	1	81.1%	98.7%	98.9%	79.2%
DAT	100	6	77	0	94.3%	100%	100%	92.8%

Abbreviations: TP, true positive; FN, false negative; TN, true negative; FP, false positive; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; DAT, direct agglutination test; ITMA, Institute of Tropical Medicine Antwerp. ELISA values were calculated combining results obtained at serum dilutions of 1:800 and 1:100. Detection of *Leishmania* in lymph node smears was used as reference. Specificity was calculated using 77 confirmed negative sera, including healthy controls, malaria -, TB-, and leukaemia patients.

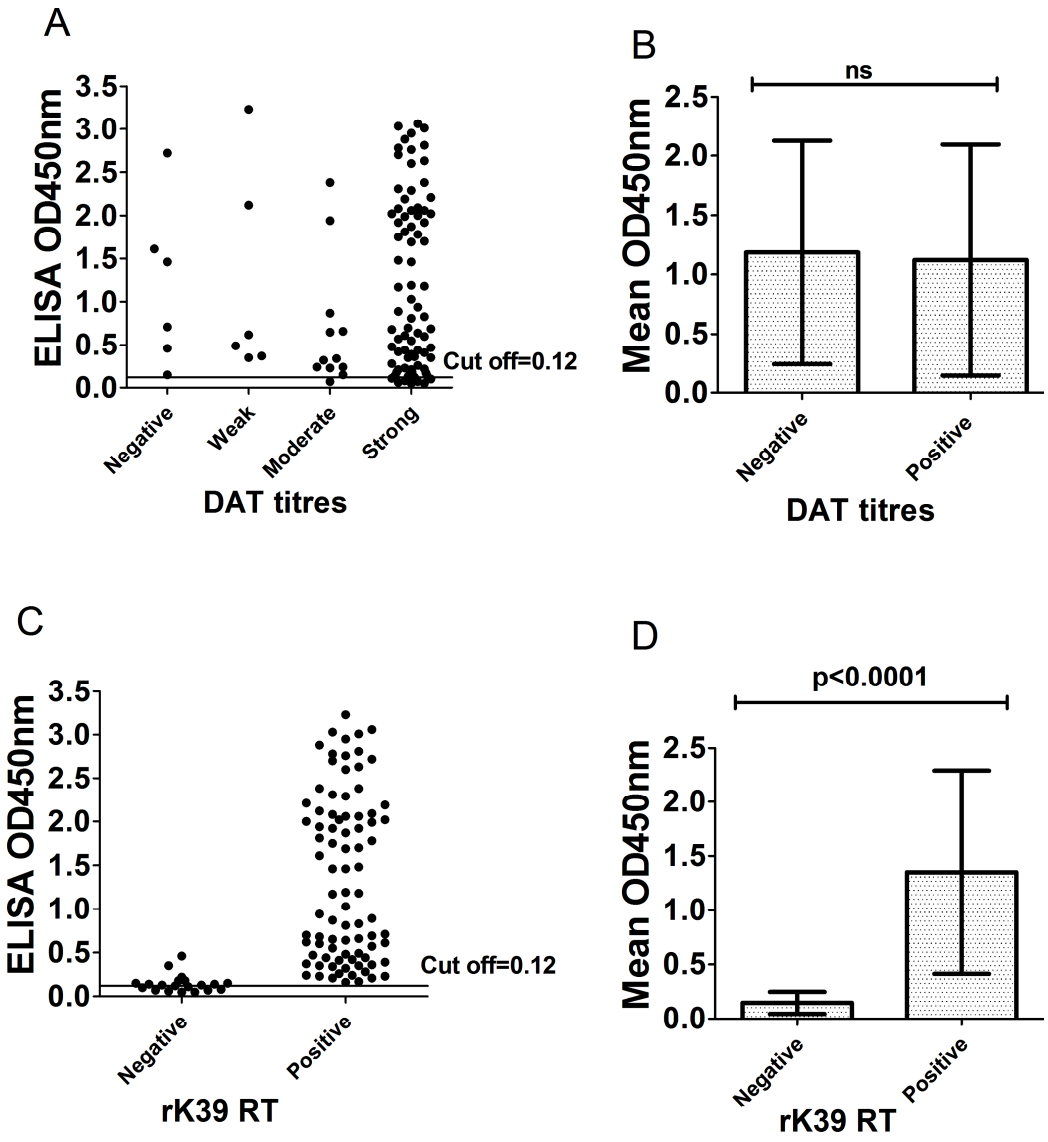

Fig. 3.7 Reactivities of VL sera in rKLO8 ELISA, DAT and rK39 rapid test (RT). ODs for 106 VL sera diluted 1:800 were measured in rKLO8 ELISA and compared with DAT antibody titres or rapid test results. (A) Sera were divided into 4 groups based on DAT titres; negative, 1:≤1600; weak, 1:3200-1:6400; moderate, 1:12800-1:25600; strong, 1:≥51200. (B) Mean ODs of VL sera with negative or positive DAT titres. Results are expressed as mean ± SD. (C) OD values of VL sera with negative or positive rapid test results. (D) Mean ELISA OD values of VL sera with positive or negative rapid test results. Dots represent values for individual sera and horizontal lines represent cut-off values. ns, not significant.

3.2 Performance of various serodiagnostic tests in three major endemic regions of visceral leishmaniais:

3.2.1 Comparison of rKLO8 and rK39 for detection of *Leishmania*-specific antibodies:

Since rKLO8 and rK39 show differences in reactivity with sera of VL from Sudan, we determined the performance of both proteins in other VL endemic regions. Antibody responses against recombinant proteins were measured and compared in ELISA using sera of VL and controls originated from India and France and were compared with those from Sudan. Protein concentrations of 5 ng and 1:800 serum dilutions were used. Under these conditions, *Leishmania*-specific antibodies could be detected specifically in sera of VL from India and France, as well.

Interestingly, individual OD values varied considerably among patients from the different geographical regions (**Fig. 3.8**). In contrast to patients' sera from Sudan and France, most sera of VL from India showed similarly high OD values. Out of 26 VL sera from India, 25 (96.2%) showed OD values of more than 0.6 (5-fold cut of value) when tested in both rKLO8- and rK39 ELISA. These values were 33/50 (66%) and 30/50 (60%) in sera of patients from Sudan with rKLO8 and rK39, respectively. Similar results were seen when both proteins were tested with sera from French VL and VL/HIV patients, yielding 18/26 (69.2%) and 6/11 (54.5%) positivity, respectively. Interestingly, 7 patients' sera from Sudan and India showed higher ODs of > 2.15 to rKLO8 than rK39 (**Fig. 3.8A & B**). Such values however were not observed with sera from French patients (**Fig. 3.8C**). In general, sera of VL from India showed significantly increased reactivity to rKLO8 and rK39 as compared to the patient groups from Sudan ($p<0.0001$) or France ($p<0.0037$) (**Fig. 3.9**).

Among sera of symptomatic suspects (VLS), 9/11 (81.8%) from India and 10/13 (76.9%) from France showed positive signals in the two ELISAs and thus were considered potential positives (**Fig. 3.8A & 3.8C**). Unexpectedly, rKLO8 detected more asymptomatic cases from France than rK39; 7/25 (28%) and 3/25 (12%) (**Fig. 3.8C**).

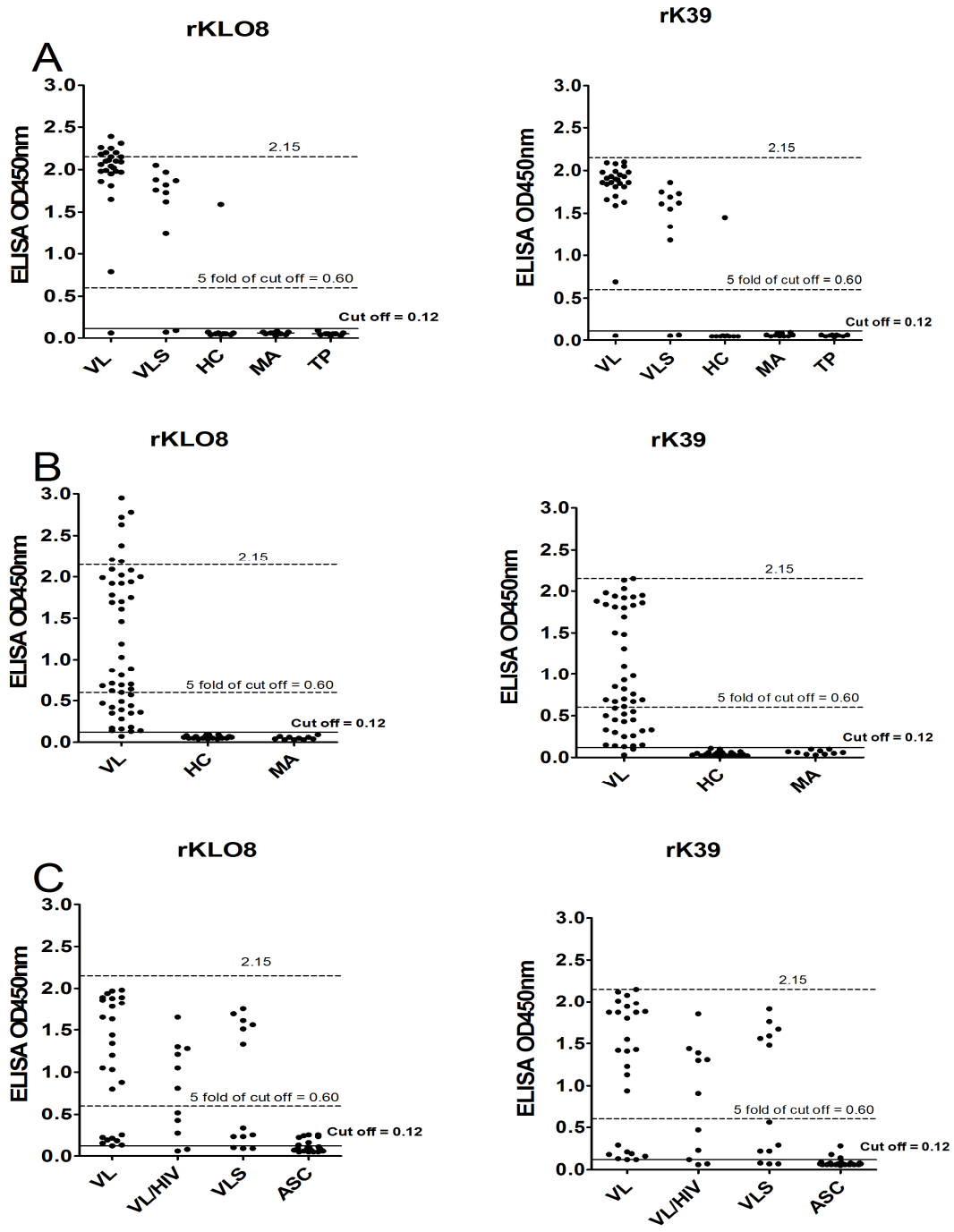

Fig. 3.8. Detection of *Leishmania*-specific antibodies in sera from various regions using rKLO8 and rK39. Protein concentrations of 5 ng/100µl rKLO8 or rK39 were tested using sera of patients and controls from India (A), Sudan (B) and France (C). Sera were tested at dilutions of 1:800. A cut off value of 0.12 was used. VL, visceral leishmaniasis; VLS, VL suspects; HC, healthy control; MA, malaria; TP, toxoplasmosis; VL/HIV, VL and HIV, ASC, asymptomatic cases. The black horizontal lines represent cut off values. OD values of 0.6 (5-fold cut off value) and 2.15 are shown in dotted lines.

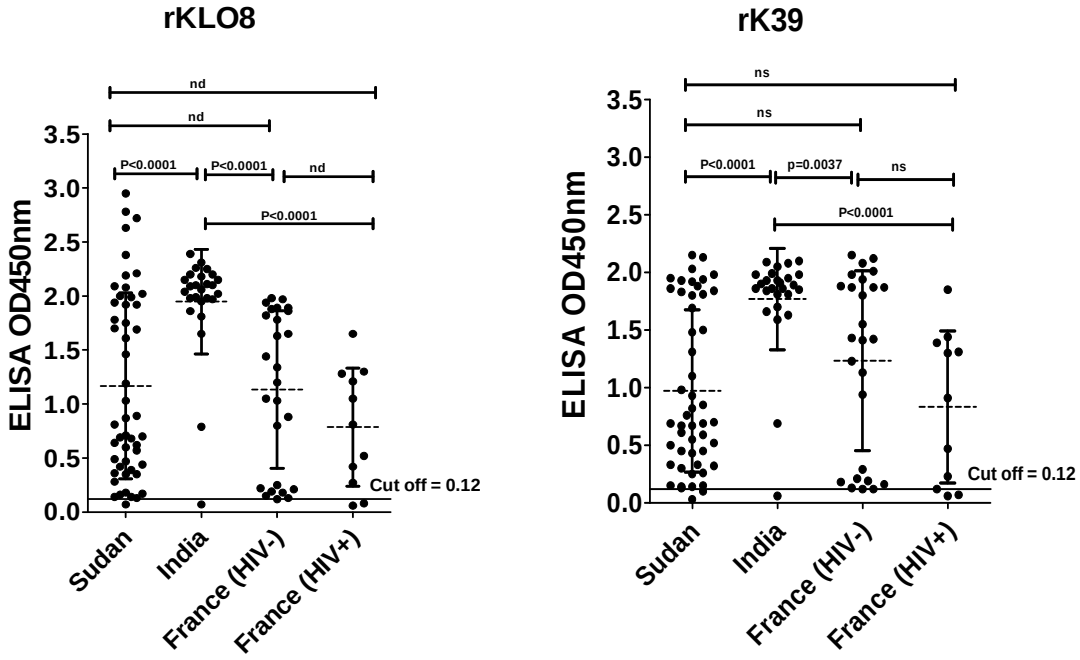

Fig. 3.9. Comparative reactivity of VL sera from various regions in rKLO8- and rK39 ELISA. OD values of VL sera from Sudan (n=50), India (n=26), France with negative (HIV-, n=26) or positive (HIV+, n=11) HIV were diluted 1:800 and tested in rKLO8- or rK39 ELISA. Means OD values were compared using unpaired student *t-test*. Dots represent values for individual sera and horizontal lines represent cut-off values (0.12). ns, not significant.

3.2.2 Performance of rKLO8- and rK39 ELISA in various endemic regions:

Diagnostic performance of rKLO8- and rK39 ELISA were estimated and compared for each endemic region. Sensitivity, specificity, PPV and NPV were calculated using results of confirmed VL and negative control groups. Both tests showed high diagnostic performance in the three regions (**Tables 3.4 - 3.6**). Sensitivity was similar in India (96.2%) and France (100%) but different in Sudan, 98% (49/50) for rKLO8 ELISA and 96% (48/50) for rK39 ELISA. Only one serum sample from Sudanese VL showed a false-negative result in rKLO8 and two with rK39. Among VL/HIV sera from France, sensitivity was markedly decreased to 81.8%. Specificity values were similarly high in Sudan (100%) and India (96.6%). One serum of a healthy individual from an endemic area in India showed a strong

reactivity (OD > 1.0) with both proteins (**Fig. 3.8A**). No cross reactivity was seen with sera from malaria and toxoplasmosis patients. Thus, PPVs of the two tests were high in Sudan and India with 100% and 96.2% for rKLO8 and rK39, respectively. NPVs were 97.6% and 95.2% in Sudan but similar (96.6%) in India. Together, these results demonstrate the high sensitivity of rKLO8- and rK39 ELISA for diagnosis of VL in immunocompetent subjects, with rKLO8 displaying increased reactivity in Sudan and India.

3.2.3 Performance of rapid tests based on rK39 and rKE16 proteins:

Qualitative detection of *Leishmania*-specific antibodies was assessed using two commercial rapid tests (RTs) based on rK39 and rKE16. Diagnostic performance for each of the two tests was calculated on basis of results obtained on sera from various endemic regions (**Tables 3.4 - 3.6**). Interestingly, only in India both tests showed similar high sensitivity of 96.2%. Sensitivity was low in Sudan and France, ranging from 88% to 88.5% (rK39 RT) and 64% to 73.1% (rKE16 RT), respectively. In case of VL patients co-infected with HIV (VL/HIV) sensitivities were further reduced; 81.8% for rK39 RT and 63.6% for rKE16 RT. Specificity of rKE16 RT was better than rK39 RT in Sudan (100% and 97.5%) and similar (96.6%) in India. One serum of a malaria patient from Sudan was tested positive with rK39 RT as well as a healthy subject from India showed also cross-reaction in the two tests. Therefore, PPVs were 97.8% for rK39 RT and 100% for rKE16 RT in Sudan and 96.2% for both tests in India. NPVs were similar in India (96.6%) and 86.7% and 83.3% in Sudan for rK39 RT and rKE16 RT, respectively.

With regard to symptomatic suspects (VLS), 81.8% (9/11) of the serum samples from India were tested positive in both tests. In France, rapid tests of rK39 detected more cases with 10/13 and 7/13 positive results for rK39 and rKE16, respectively. With respect to asymptomatic patients, 3 sera showed positive reaction in rK39 RT but tested negative in rKE16 RT. These results show that rapid tests based on rK39 and rKE16 have a high sensitivity only in India, highlighting the need for developing better rapid tests for VL in Sudan and France.

3.2.4 Performance of DAT for detection of *Leishmania*-specific antibodies:

Semi-quantitative analysis of *Leishmania*-specific antibodies was determined with DAT, which uses *L. donovani* strain 1S (from Sudan). Antibody titres were determined and compared in sera of VL from three regions. In general, patients from Sudan and India revealed increased antibody titres as compared to patients from France (**Table 3.7**). DAT titres greater than or equal to 1:51200 were observed in 86% and 92.3% of the sera from Sudan and India, respectively. Sera of patients from France showed relatively weak DAT titres, strong antibodies were found in 57.7% of patients with VL and in 36.4% co-infected with HIV. A high proportion (45.5%) of the later group revealed DAT titres of 1:100-1:800. Such low titres were not observed in sera from Sudan but only in one serum of VL from India.

Performance of DAT in various endemic regions was shown in **Tables 3.4 - 3.6**. Sensitivity was high in Sudan (94%) and India (96.3%) as compared to France (88.5% for VL and 54.5% for VL/HIV sera). No cross-reactivity was found in sera of healthy or diseased controls from Sudan. One healthy subject from India was tested positive with strong titre (1:>102400), resulting in 100% specificity for Sudan and 96.6% in India. Accordingly, PPV was better in Sudan (100%) than in India (96%) and NPVs were similar in the two regions (93% and 93.3%). Among symptomatic suspects (VLS), 81.8% of serum samples from India and 76.9% from France demonstrated positive results. In contrast, out of 25 asymptomatic cases from France, only one serum was tested positive. Altogether, these results clearly show that the DAT from TMIA is suitable for serodiagnosis of VL both in Sudan and India.

Table 3.4: Performance of diagnostic tests for visceral leishmaniais in Sudan:

Diagnostic Test	Performance index (%) at 95% CI			
	Sensitivity	Specificity	PPV	NPV
rKLO8 ELISA	98%	100%	100%	97.6%
rK39 ELISA	96%	100%	100%	95.2%
rK39 RT	88%	97.5%	97.8%	86.7%
rKE16 RT	64%	100%	100%	67%
DAT	94%	100%	100%	93%

RT, rapid test; DAT, direct agglutination test; PPV, positive predictive value; NPV, negative predictive value.

Table 3.5: Performance of diagnostic tests for visceral leishmaniais in India:

Diagnostic test	Performance index (%) 95% CI			
	Sensitivity	Specificity	PPV	NPV
rKLO8 ELISA	96.2%	96.6%	96.2%	96.6%
rK39 ELISA	96.2%	96.6%	96.2%	96.6%
rK39 RT	96.2%	96.6%	96.2%	96.6%
rKE16 RT	96.2%	96.6%	96.2%	96.6%
DAT	92.3%	96.6%	96%	93.3%

See Table 3.4 for abbreviations.

Table 3.6: Sensitivity of diagnostic tests for visceral leishmaniais in HIV-negative (VL) or HIV-positive (VL/HIV) cases from France:

Diagnostic test	Sensitivity (%) 95% CI	
	VL	VL/HIV
rKLO8 ELISA	100%	81.8%
rK39 ELISA	100%	81.8%
rK39 RT	88.5%	81.8%
rKE16 RT	73.1%	63.6%
DAT	88.5%	54.5%

Sensitivity was calculated separately for the two groups, VL and VL/HIV. RT, rapid test; DAT, direct agglutination test; PPV, positive predictive value; NPV, negative predictive value.

Table 3.7: DAT antibody titres in sera of visceral leishmaniasis from the three endemic regions:

Origin of sera (no.)		Reciprocal DAT titres number positive (%)				
		100-800	1600	3200-6400	12800-25600	≥51200
Sudan (n=50)		0 (0%)	3 (6%)	2 (4%)	2 (4%)	43 (86%)
India (n=26)		1 (3.8%)	1 (3.8%)	0 (0%)	0 (0%)	24 (92.3%)
France	HIV –ve (n=26)	3 (11.5%)	0 (0%)	3 (11.5%)	5 (19.2%)	15 (57.7%)
	HIV +ve (n=11)	5 (45.5%)	0 (0%)	1 (9.1%)	1 (9.1%)	4 (36.4%)

Data were expressed as number positive (%). DAT titres; 1: 100-800, negative; 1:1600, marginal; 1:3200-1:6400, weak; 1:12800-1:25600, moderate; 1:≥51200, strong.

3.3 rKLO8 ELISA for serodiagnosis of canine visceral leishmaniasis (CVL):

3.3.1 Detection of *Leishmania*-specific antibodies in sera of symptomatic and control dogs:

The rKLO8 ELISA was further evaluated for detection of *Leishmania*-specific antibodies in sera of symptomatic and negative control dogs. The symptomatic group included 9 sera of parasitologically confirmed CVL cases from South Portugal and 21 sera of unconfirmed symptomatic cases (SC) from Portugal and Brazil. Control samples included 20 sera of healthy dogs from a known endemic area of CVL in Croatia. This group was used to determine the cut off value and found to be 0.12. rKLO8 ELISA was optimised using the same protein concentration (5 ng/well) and serum dilutions (1:800) as described for human sera. At these conditions, the differences between positive and negative sera were optimal. rKLO8 ELISA was compared to results of DAT which was performed at the time of diagnosis. As shown in **Table 3.8**, both tests were highly sensitive in positive samples, detecting all sera of confirmed CVL (100% sensitivity). In addition, high proportion of unconfirmed symptomatic cases tested positive in both tests, 7/18 (38.9%) and 2/3 (66.7%) with rKLO8 ELISA and 6/18 (33.3%) and 2/3 (66.7%) with DAT, respectively. No cross reactivity was found with the negative control group, demonstrating 100% specificity. These findings indicate that rKLO8 has the potential for detection of *Leishmania*-specific antibodies in canine visceral leishmaniais.

Table 3.8: Detection of *Leishmania*-specific antibodies in sera of dogs using rKLO8 ELISA and DAT:

Clinical condition (sera no.)	Origin of sera	% of samples positive (no. positive/total)	
		rKLO8 ELISA	DAT*
CVL (n=9)	Évora, South Portugal	100% (9/9)	100% (9/9)
SC (n=18)	Évora, South Portugal	38.9% (7/18)	33.3% (6/18)
SC (n=3)	Caicó Paraíba, Brasil	66.7% (2/3)	66.7% (2/3)
HC (n=20)**	Croatia	0 (0/20)	nd

CVL, canine visceral leishmaniais; SC, symptomatic cases with negative smear results; HC, healthy controls with negative immunofluorescence antibody test (IFAT); DAT, direct agglutination test; nd, not done.

3.3.2 Diagnosing *Leishmania infantum* infections in asymptomatic dogs:

Since *L. infantum* may causes subclinical infection in dogs, performance of rKLO8 ELISA for serodiagnosis of asymptomatic CVL was also assessed. Immunoreactivities of 130 sera of asymptomatic dogs from two endemic areas in Brazil and Croatia were assessed and compared to control sera. This group included 72 sera previously classified as having *Leishmania*-specific antibodies in immunofluorescent antibody test (IFAT) ($\geq$1:80) and 45 sera with negative IFAT titres ($\leq$1:80). Twenty sera of the healthy dogs from Croatia were included as controls. As shown in **Fig. 3.10**, positive ELISA signals (ODs $\geq$ 0.12) were obtained with most of asymptomatic sera from Brazil (9/13, 69.2%) and all IFAT positive sera from Croatia (72/72, 100%). As expected, signals were significantly higher than the controls ($p \leq 0.0001$). Importantly, *Leishmania*-specific antibodies were also detected in 12/45 (26.7%) sera with negative IFAT titres. These results indicate that rKLO8 ELISA is suitable for serodiagnosis of *L. infantum* infections in asymptomatic dogs in areas where CVL is endemic.

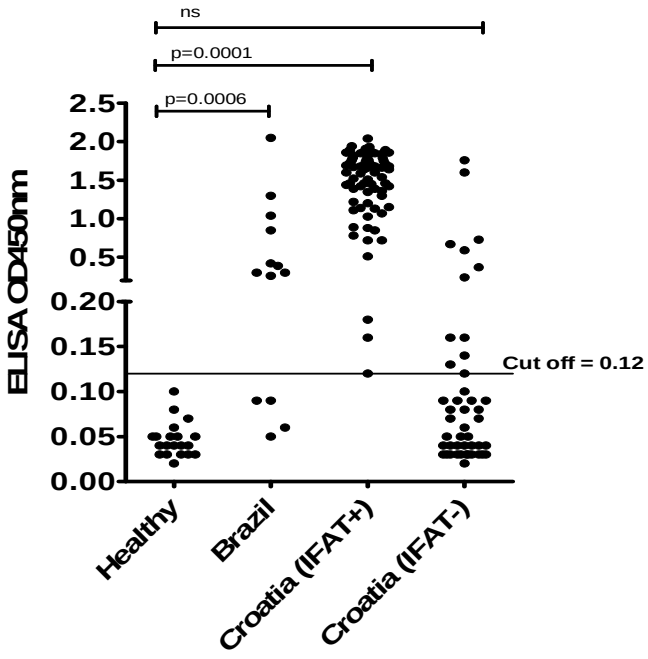

Fig. 3.10. Detection of *Leishmania-specific* antibodies in sera of asymptomatic dogs. rKLO8 ELISA was assessed using sera of asymptomatic dogs from different endemic regions for CVL, 13 from Brazil, 72 with immunofluorescent antibody test positive (IFAT+) titres and 45 with IFAT negative titres (IFAT-) form Croatia and 20 sera of healthy dogs. A cut off value of 0.12 was established as means of 20 healthy dogs from Croatia pus 3SD. The black horizontal line represent cut off value. Statistical analysis was performed by unpaired student *t test*. ns, not significant.

4. DISCUSSION:

Although proved to be extremely useful for detection of VL in human and animals, all of the existing serological tests reveal discrepant diagnostic outcome in comparison with parasitological procedures (Zijlstra et al., 1992). The variable performance of these tests in various endemic regions has prompted research to focus on the genetic difference between *Leishmania* parasites that might have contributed to the different outcome of these tests. These research efforts have further helped in learning about variability and heterogeneity of *Leishmania* antigens and have subsequently stimulated development of better diagnostic tests.

In this study, we investigated whether sera of VL patients react better with antigens of *Leishmania* donovani originating from the same endemic area (homologous antigen). This might explain the difficulties associated with VL diagnosis in certain geographical areas such as in Sudan. To address this problem, a new protein antigen of *L. donovani* from Sudan was expressed and used to evaluate homologous and heterologous antibody reactions of VL sera from human and dogs.

4.1 rKLO8, a novel antigen for diagnosis of VL in Sudan:

VL in Sudan is considered a major health problem in poor communities with limited resources where most patients don't have access to standard health care facilities. Therefore, availability of a simple and fast diagnostic test is of crucial importance. Because of their simplicity and low cost, lymph node aspiration and direct agglutination test (DAT) are currently the main tests used for VL diagnosis in the Sudan (Abass et al., 2007; Babiker et al 2007; Gorski et al., 2010; Musa et al 2010).

Despite the availability of several recombinant proteins for VL diagnosis, the existing rapid tests are mainly based on kinesin proteins, i.e. rK39 of *L. infantum* (synonymous *L. chagasi*) and rKE16 of *L. donovani* (Burns et al., 1993; Sivakumar et al., 2006). While these tests are quite effective in Brazil and India,

their use in East Africa is not satisfactory (Abass et al., 2006; Ritmeijer et al 2006; Cunningham et al 2012). Unfortunately, there seems to be little interest in developing improved diagnostic tools for VL, as the disease is endemic in most developing countries of the world (Mabey et al., 2004). Improving VL diagnosis in these countries requires identification and testing of new antigens from autochthonous strains of *L. donovani*.

In this study, a new recombinant protein of *L. donovani* from Sudan, designated rKLO8, showed significant homology with kinesin proteins of *Leishmania.* As expected, rKLO8 showed very high sequence identity with the LdK39 protein of *L. donovani* strain 1S from Sudan that has never been used for diagnostic purposes. Coding sequence of rKLO8 contains a conserved tandem repeat of 117 bp, encoding a protein with 39 amino acids each. Proteins with such tandem repeats are more likely to be targets of immune responses and more immunogenic than non-tandem repeat proteins (Cowman et al., 1985; Burns et al., 1993; Goto et al., 2006; Folgueira et al., 2010). It is worth mentioning that tandem repeat scores of rKLO8, rK39 and rKE16 were greater than 500, a cut off value to eliminate genes with small repeat domains (Goto et al., 2010). But rKE16 has relatively low score compared to rKLO8 and rK39, implying thus less conserved domains and subsequently weaker immunogenicity.

Importantly, immunodominant epitopes of the 3 common *Leishmania* antigens show marked variations (**Fig. 3.3**). One can therefore assume that antigenic variation will most likely influence antibody reactivity in different regions which explains why use of rK39 and rKE16 was not optimal to provide reliable diagnosis in different endemic regions. Based on this assumption, the rKE16 (antigen from an Indian strain of *L. donovani*) showed better performance for VL detection in India than in Brazil and East African countries (Cunningham et al., 2012).

Antigenic variation due to diversity of *L. donovani* sub-species has been proposed by others to be the cause for the low sensitivity in VL diagnosis based on rK39 (Sivakumar et al., 2008; Cunningham et al., 2012; Bhattacharyya et al., 2013). Other authors have shown significant increase in the level of antibody

titres when homologous antigens of *Leishmania* were used in DAT compared to heterologous antigens (Garcez et al., 1997). Similar findings have been shown in ELISA using homologous *L. chagasi* or heterologous *L. braziliensis* antigens for detection of antibodies in symptomatic and asymptomatic dogs in Brazil (Ribeiro et al., 2011). However, it cannot be generalized that use of homologous antigens will always result in improved diagnostic sensitivity, as sera of VL from Bangladesh reacted equally well with rK39 and rKRP42 derived from *L. donovani* from Bangladesh despite the marked heterogeneity of the two proteins (Tagaki et al., 2007).

4.2 Performance of rKLO8 for VL diagnosis in Sudan:

Diagnostic methods with high sensitivity for VL diagnosis are highly needed in Sudan to replace low sensitive tests based on the kinesin of *L. infantum* (rK39). Since rKLO8 differs from other *Leishmania* antigens, we investigated the influence of amino acid heterogeneity of the recombinant proteins on reactivity with VL sera from Sudan. The results clearly indicate that rKLO8 protein have increased reactivity with sera of Sudanese VL patients compared to rK39. This improvement is of particular importance for detection of VL at early or subclinical phases that are characterized by weak antibody responses. The decreased immune responses to rK39 observed in some of the VL patients from Sudan (Pattabhi et al., 2010) may explain the low sensitivity of rK39-based diagnostic tests in this region. This observation seems to be in accordance with our findings that VL sera with negative rK39 strip test results showed significantly low immune responses to rKLO8 (**Fig.5C and 5D**). The influence of antibody titres on diagnostic test results has been recently shown with rK39. Quinnell et al., (2013) have reported a strong positive association between positive signal in rK39 ELISA and positivity of rK39 rapid test. Results also show that the rKLO8 ELISA is more sensitive than DAT (94.3%) and rK39 strip test (81.1%), confirming the low sensitivity of rK39 strip test in Sudan. Due to its increased reactivity, rKLO8 provides enhanced sensitivity for sera VL with low antibody titres. Thus, formulation of this antigen in a rapid test may improve field diagnosis of VL as compared to available tests.

An ideal diagnostic test should identify all positive sera without cross reacting with negative sera. The data show that none of the serological test used was able to detect all VL cases from Sudan, demonstrating the complexity of diagnosis. Only the combination of rKLO8 ELISA and DAT resulted in 100% diagnostic sensitivity. Combining results of different tests has been suggested to overcome the problem of low sensitivity in East Africa (Cunningham et al., 2012). As sera of VL patients may have different level of specificities to recombinant proteins, combination of these antigens in a single test would further improve the diagnosis. Several authors suggested that use of synthetic recombinant antigens expressing multiple immunodominant B- cell epitopes of *L. infantum* is essential for improving diagnostic performance (Soto et al., 1998; Boarino et al., 2005).

We have to be aware that recently infected individuals have elevated IgM responses but not yet mounted an IgG response. Sera of such patients will expected to reveal false negative results, if tested in an ELISA based on IgG antibody detection. This could partially explain the results of some patients that were tested negative in the rKLO8 ELISA and rapid test despite thrir strong positivity in DAT, which detects different antibody (sub-) classes. More difficult to interpret are those 6 confirmed VL cases which were negative in DAT despite having detectable antibody responses to rKLO8. Again, antibody specificities and parasite diversity could play a role. As a consequence, specific immune responses to *Leishmania* antigens can be lost completely if tested against parasites isolated from different endemic areas (Forgber et al., 2006). In addition, the same authors have shown that there are no uniformly dominant antigens targeted by the immune responses of different individuals. Variation of antibody reactivity in individual sera against recombinant proteins has also been confirmed in animal infection models where antigens of *L. infantum* were differently recognised by sera of hamster infected with the same parasite (Folgueira et al., 2010).

Low antibody titres of VL sera could be attributed to co-infection with HIV, which complicates VL diagnosis. Patients with *Leishmania*/HIV co-infections are known to have reduced immune responses and therefore false negative results can be expected (Piarroux et al., 1994; Herrera et al., 1995; Ezra et al., 2010). Nevertheless, because sera were obtained already time ago, it was not possible

to obtain additional clinical data associated with false negative results. Further, differences in antibody levels due to age and nutritional status of patients may also influence immune responses in VL (Cunningham et al., 2012).

Prevalence of malaria and other diseases is common in areas also known to be endemic for VL (de Beer et al., 1991; van den Bogaart et al., 2012). Studies have reported prevalence of VL and malaria co-infections in various countries such as Sudan, Uganda, Bangladesh and India (cited in van den Bogaart et al., 2012). Therefore, a good test system needs robust discrimination between VL and co-infections. The results of this study indicate that serum dilutions of 1:800 provide optimal specificity and sensitivity for rKLO8. Sera of malaria patients did not give positive signal in rKLO8 ELISA. This finding is concordant with our previous finding from Sudan, where 100% specificity of VL detection has been shown with sera tested at high dilutions of 1:1600 (Abass et al., 2006). Other authors have reported cross reactivity to rK39 with sera of malaria and healthy controls from endemic and non-endemic areas in Sudan (Veeken et al 2003; Romero et al 2009; Pattabhi et al., 2010). Cross reactivity may be explained by the homology of *Leishmania* proteins and proteins of other prokaryote (Goto et al 2008). However, in the original publication describing rK39, sera of malaria were not evaluated (Burns et al 1993). On the other hand, Carvalho and colleagues have not shown cross reactions to rK39 using sera of malaria patients from Brazil (Carvalho et al 2003). In general, results with low antibody titres should be interpreted with caution. Notably, the DAT kit showed no cross reaction with any of the control sera tested and thus provides the best specificity.

4.3 Performance of serodiagnostic tests in three major endemic regions of VL:

VL diagnosis is complicated by fact that the disease is caused by various *Leishmania* sub-species in different endemic regions, with known genetic differences (Dey et al., 2007; Sivakumar et al., 2008; Hamad et al., 2010; Srivastava et al., 2011b; Zackay et al., 2013; Bhattacharyya et al., 2013). Despite this knowledge, diagnostic tests which are often based on a single antigen are used in different endemic countries, with the consequence of suboptimal diagnosis in some regions. Therefore, it is essential to develop appropriate

species-specific diagnostic tests for different regions. Control of the disease in these regions will largely depend on the availability of accurate detection systems.

In this study, performance of rKLO8- and rK39 ELISA was compared with two rapid tests (rK39 and rKE16) and DAT in 3 endemic regions for VL. In general, the performance of rKLO8- and rK39 ELISA was excellent in all regions. Optimal sensitivity and specificity have been previously reported for the rK39 ELISA in several countries (see Chappuis et al., 2007). The strong immunogenicity of rKLO8 and rK39 allows detection of *Leishmania*-specific antibodies at dilutions as high as 1:800 with 100% specificity, with no cross-reactivity against malaria which was otherwise observed in Sudan at a lower dilution of 1:100. A sensitivity of 81.8% for VL diagnosis was achieved even in HIV immunocompromised patients from France. Unfortunately, the geographical distribution of VL and HIV is known to overlap in many areas (Alver et al., 1997b). Co-infection should therefore be taken into account when interpreting results of serological tests (Piarroux et al., 1994; Medrano et al, 1998).

VL sera from the various endemic regions demonstrated different levels of reactivity towards rKLO8 and rK39 antigens. Nearly all sera of patients from India showed high antibody titres to both recombinant proteins as compared to patients from Sudan and France (**Fig. 3.8**). On the other hand, some of the sera from Sudan and India reacted more strongly with rKLO8 as with the rK39. It was not surprising that sera of Indian VL patients have strong antibody titres as patients infected with homologous *L. donovani* strains are expected to develop strong responses. Strains of *L. donovani* in India are known to have high homology compared to strains in East African countries where a marked heterogeneity has been reported (Kuhls et al., 2007; Alam et al., 2009; Hamad et al., 2010). In agreement with our results, other authors have detected strong antibody responses to rK39 in sera of VL from India (Kumar et al., 2001). Variability of *L. donovani* strains from East Africa might be due to spread of parasites from other regions where different parasite populations are endemic or as a result of diversification of autochthonous parasites that have been endemic for long time in the same area (Gelanew et al., 2011).

The results also confirm the variable sensitivity of rK39- and rKE16 rapid tests (RTs) in several VL endemic regions (Chappuis et al., 2007; Cunningham et al., 2012). Although both tests showed high specificity, their sensitivity is only moderate in the VL sera with high antibody titres from India, suggesting that high titers can compensate for the heterogeneity of rK39 antigen, at least in India. Subsequently, VL sera with low antibody titres such as those from Sudan and France can only be detect with low sensitivity. This is supported by the previous findings wherein Sudanese VL sera with negative rK39 RT results had significantly low antibody responses to rKLO8 and rK39. The fact that rK39 RT performs very well in India but not in Sudan and France, in combination with the high sensitivity of rK39 ELISA in Sudan and France indicates that antibody levels rather than test formats, determine the sensitivity for VL diagnosis. The influence of antibody level on VL diagnosis is clearly demonstrated by the high DAT sensitivity reported in India. Possibly, the use of DAT antigen prepared from a Sudanese strain of *L. donovani* (1S) contributed to its high sensitivity in Sudan. Similarly, DAT sensitivity was clearly increased when applying antigens of *L. donovani* originating from the respective areas (Harith et al., 1995). However, a DAT kit also based on *L. donovani* 1S strain from Sudan, showed high sensitivity (94-98.5%) in India, Nepal, Ethiopia and Kenya but lower sensitivity in Sudan (85.7%) (Cañavate et al., 2011).

The results reflected again the complexity of VL diagnosis in the different regions as none of the available tests detected all positives and negative sera from the various regions. As discussed before, results of serological tests should be interpreted with caution and combination of different test results seems to be important (Boelaert et al., 2008; Cunningham et al., 2012). It is also important to note that detection of *Leishmania* in spleen or bone marrow aspirations missed several sera of symptomatic cases that showed positive reactivity in all serological tests. This also indicates that misdiagnosis of VL can be expected even by applying this gold standard test. Due to the absence of an absolute diagnostic reference method, it is not uncommon that clinical suspected VL cases are confirmed serologically (Harith et al., 1989; Chowdhury et al., 1993; Pizzuto et al., 2001; Raguenaud et al., 2007).

4.4 Application of rKLO8 ELISA for serodiagnosis of canine VL:

Dogs play an essential role in the transmission cycle of VL (Ashford, 1996). Therefore, effective control strategy of the disease in human should be based on controlling canine VL (CVL), ideally through identification and treatment or elimination of infected dogs (Palatnil-de-Sousa et al., 2001; Coura-Vital et al., 2011). Since parasitological examination is invasive and insensitive, serodiagnosis is routinely applied for identification of infected dogs (Schallig et al., 2004; Otranto et al., 2009; Quinnell et al., 2013).

In this study, the performance of rKLO8 ELISA and DAT was also assessed for detection of CVL using sera of parasitologically confirmed cases and healthy controls. Although, only few sera were tested, results demonstrate that both rKLO8 ELISA and DAT are equally sensitive and specific for detection of CVL, implying 100% sensitivity and specificity. However, these findings need further confirmation with larger numbers of dogs. It is worth mentioning that some sera of symptomatic dogs with negative parasitological results tested positives in both rKLO8 ELISA and DAT. Based on clinical symptoms and high prevalence of the disease in those regions, our results seem to be acceptable. Considering its high sensitivity and absence of cross reactivity, rKLO8 ELISA represents a supplementary diagnostic procedure for CVL.

Previous studies on dogs with CVL have demonstrated different diagnostic sensitivity levels for the most commonly applied serological tests (Mettler et al., 2005; Otranto et al., 2009; Quinnell et al., 2013). This difference can also be attributed to genetic variability between *Leishmania* sub-species prevalent in these areas. Also as earlier discussed in human, specificity of host antibody responses against individual antigens could have played a role (Falqueto et al., 2009). Other authors have shown variable sensitivity of serodiagnosis during different stages of the disease in natural infected dogs, being lower at early infection and higher later (Quinnell et al., 2001).

Co-infections of *Leishmania* parasites and other vector-borne diseases in dogs are common in subtropical and tropical regions. As a result, co-infections may manipulate classical clinical presentation and thereby complicate diagnosis.

Cross reactivity of canine sera with other diseases can give rise to reduced specificity. But, serodiagnosis when carefully defined is more sensitive than parasitology. A study of cross reactivity in serum samples from dogs infected with other parasitic diseases (such as *Trypanosoma cruzi* and *Ehrlichia canis*) showed that ELISA and IFAT have a higher degree of cross reactivity than DAT (Mancianti et al., 1996; Ferreira et al., 2007). The high specificity of DAT has also been shown in sera of dogs infected with *Babesia* or *Leptospira* (Oskam et al., 1996).

The role of dog as the major reservoir for VL necessitates the application of accurate tests to identify asymptomatic cases. If not detected, such cases can be permanent source of the parasite for phlebotomine sandfly vectors (Molina et al., 1994). We think that the limited sensitivity of the current serodiagnostic tests for detection of asymptomatic carriers is a major limitation for controlling VL. Currently, serological methods such IFAT and DAT are commonly applied in epidemiological surveys of CVL. Although IFAT is widely used in Mediterranean countries, its low sensitivity and subjective interpretation of results are among the important limitations (Evans et al., 1990; Reed, 1996; Mettler et al., 2005; Silva et al., 2001).

The results demonstrate that rKLO8 ELISA is better than IFAT for testing of asymptomatic *L. infantum* infected dogs. rKLO8 ELISA detected all IFAT positive asymptomatic sera (100%) and most IFAT negative sera (69.2%). This is particularly important as 50% of seropositive dogs can be asymptomatic although harbouring the parasites and never develop clinical disease through out their life (Moreno and Alvar, 2002; Mohebali et al., 2005). These cases may be related to high exposure to the parasite and development of protective immunity (Pinelli et al., 1994). In fact, using of serological tests for CVL depend on the purpose. A high sensitivity is required to identify asymptomatic infected dogs as potential carriers of the parasite. Thus, the higher sensitivity of rKLO8 ELISA compared to IFAT would be an advantage, suggesting the possibility for detection of early cases with low antibody titres. Indeed, a dog with seropositivity should be considered an individual which has to be monitored through follow-up examinations (Talmi-Frank et al., 2006).

4.5 Conclusion and outlook for future research:

Diagnosis of VL is a complex problem because none of the available tests has absolute reliability in the different regions. Marginal antibody titres may result from co-infections such as HIV or malaria. Therefore, a good test system needs robust discrimination between VL and potential co-infections. The non-availability of an absolutely reliable diagnostic test and the presence of substantial numbers of asymptomatic VL cases constitute major limitations in effectively controlling the disease. The available tests can neither differentiate between active and past infections nor between symptomatic and asymptomatic infections. Performance of serodiagnosis is dependent on antibody level in individual VL patients as high antibody titres can compensate for the heterogeneity of antigens. The sensitivity for VL diagnosis can be improved by using antigens of autochthonous *Leishmania* parasites. The increased reactivity of Sudanese VL sera with rKLO8 makes this antigen a potential candidate for improving diagnosis of VL in Sudan.

Based on the conclusion, further studies are needed in the following areas:

i. Since, rKLO8 evidenced potential for detection of VL in cases with low antibody titres, it will be important to evaluate its performance in a rapid test format for field application.

ii. Because of the marked heterogeneity among stains of *L. donovani* in East Africa and specificity of host immune responses, further analysis of the genetic variations in immunodominant epitopes will enable improving diagnosis of VL. This can be achieved by developing a synthetic protein construct expressing multiple immunodominant epitopes.

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6- APPENDIX:

1- *Leishmania donovani* strain LO8 immunodominant kinesin-related protein KLO8 gene, partial cds

GenBank: KC788285.1
FASTA Graphics

Go to:

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LOCUS       KC788285                883 bp    DNA     linear   INV 25-MAY-2013
DEFINITION  Leishmania donovani strain LO8 immunodominant kinesin-related
            protein KLO8 gene, partial cds.
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SOURCE      Leishmania donovani
  ORGANISM  Leishmania donovani
            Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
            Leishmaniinae; Leishmania.
REFERENCE   1  (bases 1 to 883)
  AUTHORS   Abass,E., Bollig,N., Reinhard,K., Camara,B., Mansour,D.,
            Visekruna,A., Lohoff,M. and Steinhoff,U.
  TITLE     rKLO8, a novel Leishmania donovani - derived recombinant
            immunodominant protein for sensitive detection of visceral
            Leishmaniasis in Sudan
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 883)
  AUTHORS   Abass,E., Bollig,N., Lohoff,M. and Steinhoff,U.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-MAR-2013) Institute for Medical Microbiology and
            Hygiene, University of Marburg, Hansmeerwein strasse2, Marburg,
            Hessen 35043, Germany
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2- Immunodominant kinesin-related protein KLO8, partial [*Leishmania donovani*]

GenBank: AGL98402.1

FASTA Graphics

Go to:

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VERSION     AGL98402.1  GI:505582991
DBSOURCE    accession KC788285.1
KEYWORDS    .
SOURCE      Leishmania donovani
  ORGANISM  Leishmania donovani
            Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
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REFERENCE   1  (residues 1 to 294)
  AUTHORS   Abass,E., Bollig,N., Reinhard,K., Camara,B., Mansour,D.,
            Visekruna,A., Lohoff,M. and Steinhoff,U.
  TITLE     rKLO8, a novel Leishmania donovani - derived recombinant
            immunodominant protein for sensitive detection of visceral
            Leishmaniasis in Sudan
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 294)
  AUTHORS   Abass,E., Bollig,N., Lohoff,M. and Steinhoff,U.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-MAR-2013) Institute for Medical Microbiology and
            Hygiene, University of Marburg, Hansmeerwein strasse2, Marburg,
            Hessen 35043, Germany
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//
```

3- Sequence alignment of immunodominant repeats of KLO8:

```
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Repeat 2     LEQQLRDSEERAAELASQLEATAAAKSSAEQDRENTRAA 39
Repeat 3     LEQQLRDSEERAAELASQLESTTAAKTSAEQDRENTRAT 39
Repeat 4     LEQQLRDSEERAAELASQLESTTAAKMSAEQDRENTRAA 39
Repeat 5     LEQQLLESEERAGELASQLESTTAAKMSAEQDRENTRAA 39
Repeat 6     LEQQLRDSEERAAELASQLESTTAAKMSAEQDRENTRAT 39
                   :     .       : :               :
```

Tandem repeats of KLO8 were performed to show variability in amino acid composition within the repeats. Analysis was performed using ClustalW2-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Conserved amino acids in various repeats were highlighted in black and different residues were left unmarked.

7- CURRICULUM VITAE:

Page removed, contained personal information.

8- PUBLICATIONS:

1- **Abass E**, Piarroux R, Walden P, Lohoff M, Steinhoff U. Performance of various serodiagnostic tests in three major endemic regions of visceral leishmaniais (under submission).

2- **Abass E**, Bollig N, Reinhard K, Camara B, Mansour D, Visekruna A, Lohoff M, Steinhoff U (2013) rKLO8, a Novel *Leishmania donovani*–Derived Recombinant Immunodominant Protein for Sensitive Detection of Visceral Leishmaniasis in Sudan. *PLoS Neg Trop Dis* 7(7): e2322.

3- Bollig N, Brüstle A, Kellner K, Ackermann W, **Abass E**, Raifer H, Camara B, Brendel C, Giel G, Bothur E, Huber M, Paul C, Elli A, Kroczek R, Nurieva R, Dong C, Jacob R, Mak T and Lohoff M (2012) Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proc Natl Acad Sci U S A*. 109(22):8664-9.

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5- Mansour D, **Abass E**, Mutasim M, Mahamoud AE, and Harith AE (2007) Use of a Newly Developed β-Mercaptoethanol Enzyme-Linked Immunosorbent Assay to Diagnose Visceral Leishmaniasis in Patients in Eastern Sudan. *Clin Vacc Immunol* 14: 1592-1595.

6- **Abass E**, Mansour D, Harith AE (2007) Demonstration of agglutinating anti-Leishmania donovani antibodies in lymph node aspirate for confirmation of kala-azar serodiagnosis*. J Med Microbiol* 56: 1256-1258.

7- **Abass E**, Mansour D, Elmotasim M, Hussein M, Harith AE (2006) Beta mercaptoethanol modified ELISA for diagnosis of visceral leishmaniasis. *J Med Microbiol* 55, 1193-1196.

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visceral leishmaniasis at rural level in eastern Sudan. *J Med Microbiol*. 55. 1343-1347.

9- Harith AE, Mutasim M, Mansour D, **Abass E**, Harold Arvidson (2003) Use of glycerol as an alternative to freeze drying for long term preservation of antigen for direct agglutination. *Trop Med Intern Health* 8, No. 11, 1025 – 1029.

Abstracts:

Abass E, Elhussein M (2009) Visceral leishmaniais and HIV co-infection in patients referred to Biomedical Research Laboratory of Ahfad University during 2003-2007. *Annals of Medicine and Healthcare Research*. Proceedings of the 2009 International Online Medical Conference.

Patent:

Diagnosis of Leishmania infection. European patent, Application No./Patent No. 13151858.1 - 1406. Date of filling: 18.01.2013.

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