EPIDEMIOLOGY OF ASYMPTOMATIC VISCERAL LEISHMANIASIS IN ENDEMIC REGIONS OF NEPAL USING NESTED-PCR ENDORSED BY SEROLOGY

M. Sc. Thesis
(2013)

Submitted to

CENTRAL DEPARTMENT OF BIOTECHNOLOGY
Tribhuvan University
Kirtipur, Kathmandu, Nepal

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Date: Dec 01, 2013

Recommendation

This is to certify that the research work entitled "**Title Title Title Title Title Title Title Title** " has been carried out by **Mr. Pratap Khadka** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. I, hereby, recommend this thesis for final evaluation.

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Dedicated to

my beloved parents, father Kumar Bahadur Khadka &

mother Radha Khadka, brother & sisters,

who have supported, motivated & inspired all the way since

the beginning of my studies.

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Pratap Khadka

Glossary Acronyms

ADP	Adenosine Diphosphate
AIDS	Acquired Immune Deficiency Syndrome
AmB	Amphotericin B
AP	Alkaline Phosphatase
Aq antigen	Aqueous antigen
ATP	Adenosine Triphosphate
BC	Buffy coat
BCG	Bacille Calmette-Guerin
BCIP-NBT	5-Bromo-4-Chloro-3-Indoyl phosphate – Nitro Blue Tetrazolium chloride
BHC	Benzene Hexachloride
BMA	Bone Marrow Aspirate
bp	Base Pair
BSA	Bovine serum albumin
CBB	Coomssie Brilliant Blue
CCR	Chemokine Receptor
CD	Cluster of differentiation
CDBT	Central Department of Biotechnology
CFR	Case Fatality Rate
CL	Cutaneous Leishmaniasis
CMI	Cell Mediated Immunity
cRPMI	Complete Rosewell Park Memorial Institute
CSA	Crude Soluble Antigen
CSB	Conserved Sequence Block
DAT	Direct Agglutination Test
DC	Dendritic Cell
DDT	Dichloro-diphenyl-trichloroethane

DNA	Deoxyribonucleic Acid
DTH	Delayed Type Hypersensitivity
EDCD	Epidemiology and Disease Control Division
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FD	Freeze-Dried
FML	Fucose-Mannose Ligand
gp63	Glycoprotein 63
HIV	Human Immuno Deficiency Virus
HRP	Horseradish Peroxidase
Hsp	Heat Shock protein
ICT	Immuno Chromatographic Strip Test
IFAT	Indirect Fluorescence Antibody Test
Ig	Immunoglobulin
IL	Interleukin
INF-ϒ	Interferon-gamma
IRD	Institute of Research for Development
ITMA	Institute of Tropical Medicine in Antwerp
ITN	Insecticide Impregnated Net
ITS	Internal Transcribed Spacer
IVM	Integrated Vector Management
KAtex	Latex Agglutination Test
kDa	Kilodalton
kDNA	Kinetoplast DNA
LACK	*Leishmania* Analogue of the Receptor Kinase C
LD body	Leishman-Donovan body
Ld-ESM	*L. donovani* Excretory, Secretory and Metabolic antigens
LPG	Lipophosphoglycan
LST	Leishmanin Skin Test

MCL	Mucocutaneious Leishmaniasis
NF	Nuclear Factor
NK	Natural Killer
NO	Nitirc Oxide
n-PCR	Nested-Polymerase Chain Reaction
OD	Optical Density
PAGE	Polyacrylamide Gel electrophoresis
PAMPs	Pathogen-Associated Molecular Patterns
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PGS	Phosphate-Glucose-Saccharose
PKDL	Post Kala-azar Dermal Leishmaniasis
PMNs	Polymorphonuclear Neutrophils
PSG	Promastigote Secretory Gel
PVDF	Polyvinylidene Difluoride
Rf	Retardation factor
RFLP	Restriction Fragment Length Polymorphism
rK39	recombinant Kinesin 39 amino acid
RNA	Ribonucleic Acid
rORFF	recombinant Open Reading Frame
RP	Research participant
RT	Room Temperature
RT-LAMP	Reverse Transcriptase-Loop-Mediated Isothermal Amplification
SA	Soluble Antigen
Sb^V	Pentavalent Antimony
SDS	Sodium Dodecyl Sulphate
SEAR	South East Asia Region
SSCP	Single Strand Conformational Polymorphism
SSU rRNA	Small Subunit Ribosomal RNA

STIDH	Sukraraj Tropical and Infectious Disease Hospital
TAE buffer	Tris-Acetate Electrophoresis buffer
TDR	Tropical Disease Research, WHO
TDW	Triple Distilled Water
TH1/TH2	T helper 1 / T helper 2
VDC	Village Development Committee
VL	Visceral Leishmaniasis
WB	Western Blotting
WHO	World Health Organization

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Abstract

Epidemiology of asymptomatic visceral leishmaniasis in endemic regions of Nepal using Nested-PCR endorsed by Serology

Visceral leishmaniasis (VL) is a neglected tropical disease affecting socially marginalized and poorest communities. Nearly 8 million people in the 13 endemic districts of Nepal are at risk and annual incidence of 43 to 55 per 100,000 is associated with the lack of efficient diagnostic options, expensive chemotherapeutics, no antileishmanial vaccine, inaccessibility of health amenities to poor communities, futile controlling measures, insufficient surveillance of the disease, etc. VL can manifest asymptomatic forms to obvious disease. Symptoms of VL are fever for more than 2 weeks with splenomegaly, anemia, weight loss and sometimes darkening of the skin. Asymptomatic infections of VL acts as a reservoir in transmission of VL and such cases ranged from 1:2.4 to 18:1 in various foci of the world. Till date there are very few research done in diagnosing and estimating the asymptomatic VL population. The present investigation studied asymptomatic VL, associated with PCR positivity, in the three districts of terai by n-PCR. Additionally, serological tests such as direct agglutination test (DAT) and immunoblotting were also exemplary. The nested PCR employed amplified 680 bp fragment from the variable region of abundantly found kDNA minicircles (10, 000 minicircles) and the limit of the detection is as little as $1/10^{th}$ of one *Leishmania* parasite. After completion of n-PCR the research participants were analyzed into different analytical grounds. In the field based and hospital based strategies 7.55% (n=53) and 10.64% (n=47) of research participants were found to be VL positive respectively. Concerning endemicity, only 11.39% (n=79) from endemic region were positive while all RP belonging to non-endemic and buffered endemic were negative to the disease. Age wise distribution of data exhibited the incidence of VL as highest to 25% in 41-50 year old group and lowest 2.78% youngs of 21-30 year old. Similarly, gender wise division disclosed more females (9.43% in 53) being affected than males (8.5% in 47). On reviewing all the samples in n-PCR ground, 9% (n=100) were detected positive as asymptomatic case in this study. DAT assay carried out using aq. DAT antigen, prepared using Nepalese strain parasite (JKP01/2011) for the first time in Nepal, was agglutinated by the sera but had lower 44.44% sensitivity. Furthermore, immunoblotting of polypeptides on nitrocellulose membrane and interaction with sera displayed 16kDa, 18kDa, 22kDa, 65kDa and 72kDa distinct polypeptides bands reported as a better diagnostic marker of VL and 16kDa and 18kDa as marker of asymptomatic cases.

Key words: Asymptomatic visceral leishmaniasis, *Leishmania donovani*, Nested PCR, DAT, Western blotting

Chapter I

INTRODUCTION

1.1 A brief history

The history of leishmaniasis dates back to 7th century BC with the detailed description of Old World Cutaneous Leishmaniasis, also known as oriental sore. The story had been found inscribed in the 10th century (Manson-Bahr, 1986). On the Babylonian tablet from the 7th century BC, leisons similar to cutaneous leishmaniasis have been accounted, some of which may have been derived from earlier text from 1500 to 2500 BC. Muslim physicians including Avicenna in the 10th century AD gave comprehensive descriptions of a type of skin lesion, termed as Balkh sore, from northern Afghanistan (Manson-Bahr, 1986; Cox, 2002; Stanford, 2012). Cutaneous Leishmaniasis and Mucocutaneous Leishmaniasis (Espundia: found to be described as "white leprosy") caused disfiguring conditions that have been recognized in sculptures since the 5th century and in the literatures of the Spanish missionaries in the 16th century (Lainson, 1996).

Visceral leishmaniasis (VL) also known as Kala-azar was first noted in Jessore (now in Bangladesh) in India in 1825 (Elliott, 1863; Cox, 2002) though the disease was not identified at that time. In the year following 1858 an epidemic of quinine-resistant fever was reported in the district Burdwan (Gibson, 1983) and called the disease as Burdwan disease. The cause remained unidentified and several eminent clinicians including Sir, Ronald Ross were convinced that the disease was a virulent form of malaria (Ross, 1899; Cox, 2002), until a Scottish army doctor, William Leishman, and the Professor of Physiology at Madras University, Charles Donovan, independently discovered the parasite in the spleen of patients (Hoare, 1938; Kean et al., 1978). Then Sir Ronald Ross named the parasite as *Leishmania*. The disease, visceral leishmaniasis, later found spread in many countries of the Old World (Asian and European/Mediterranean countries) and New World (American countries). New and Old World Leishmaniasis were thought to be same, until 1911 Gaspar Vianna found a new species of parasite named *Leishmania braziliensis* different from those in Africa and India (Vianna, 1911; Cox, 2002).

In Nepalese scenario, the Kala-azar epidemics of 19th century has not been documented, since Nepal was isolated from rest of the world up to 1953 (Rijal, 2006). Napier in 1926 reported that VL cases ventured to Nepal from Calcutta (Napier, 1926) but was indistinct if those were Nepalese residing in India or travelling to India for treatment (Rijal, 2006). The first documented evidence of Kala-azar was made in 1953 by Indian scientist Raghavan, who after a survey for vector borne disease in 1949 claimed that Kala-azar to be endemic in

the southern terai (Shrestha and Pant, 1994). In Nepal, VL was officially recorded in 1980 from Dhanusha district (Bista, 1998; Rijal et al., 2010). Henceforth, it is endemic in 13 districts in central and eastern Terai lowlands bordering North Bihar (Pandey et al., 2011) with an estimated 8 million people at risk (Rijal et al., 2010; Pun et al., 2011). Recently, the disease has been reported even from the non-endemic districts like Doti and Bardiya of the county (Pandey et al., 2011).

1.2 *Leishmania* parasite

1.2.1 Systematic position

Kingdom:	Protista (Haeckel, 1866)
Subkingdom:	Protozoa (Goldfuss, 1818)
Phylum:	Sarcomastigophora (Honlgber and Balomuth, 1903)
Subphylum:	Mastigophora (Diesing, 1943)
Class:	Zoomastigophora (Calkins, 1909)
Order:	Kinetoplastidae (Honigberg, 1963; Vickerman, 1976)
Suborder:	Trypanosomatina (Kent, 1880)
Family:	Trypanosomatidae (Doflein, 1901)
Genus:	*Leishmania* (Ross, 1903)

1.2.2 Morphology and Ultra Structure of the parasite

The *Leishmania* are unicellular and dimorphic protozoan parasite existing into two different hosts, extracellular flagellated promastigotes in the gut and coelomic fluid of haematophagus sandfly and intracellular aflagellated amastigotes in the vertebrate host (Mishra et al., 2009).

(i) Promastigote form

Parasites residing in the intermediate host, the sandfly (*Phlebotomus argentipes*) are the leptomonad form. They are 15-20×3.5µm flagellated spindle shaped protozoans and tapering at the tail end (Herwaldt, 1999). The anterior end possesses single long paraxial rod like tubular latticed structure called flagellum measuring 15-28µm in length (Fig. 1.1A and Fig. 1.2) (Lainson and Shaw, 1987; Herwaldt, 1999).

On the basis of their location on the sandfly gut, promastigotes can be distinguished into following morphologies *viz.* procyclic, nectomonad, haptomonad and metacyclic promastigotes. The procyclic promastigote is the transformed form of amastigote in the posterior midgut of the sandfly triggered by biological environment of increase p^H (7.2) and

decrease temperature (26^{o}C) within hours of ingestion (Bates, 1994; Ashford and Bates, 1998; Kamhawi, 2006). This is followed by transformation into nectomonad (elongated 15-20µm), haptomonad (attached to stomodael valve) and metacyclic (highly motile, long flagella, 5-8µm) promastigote (in digestive tract) form consecutively (Lainson and Shaw, 1987; Lang et al., 1991; Bates, 1994; Herwaldt, 1999; Manandhar, 2008). Promastigote form has centrally located nucleus and transversely placed kinetoplast towards the anterior end. The kinetoplast is the unique features of these protozoan parasites with presence of DNA containing granule located within the single mitochondrion and associated with the flagellar base. They multiply asexually by binary fission and are morphologically similar to those grown in *in vitro.* Their mode of nutrition is absorptive and reproduction is asexual binary fission.

(ii) Amastigote form

Amastigote form, also known as leishmanial form, resides in the parasitophorous vacuoles in the macrophages of the reticuloendothelial cells (liver, bone marrow, spleen and lymph nodes) of vertebrate host (Fig. 1.1B and Fig. 1.2). They are round and club shaped without flagella measuring 2-4µm in diameter (Herwaldt, 1999). The centrally located round/oval nucleus and transversely placed small rod shaped kinetoplast provides a distinctive feature. Kinetoplast is a dense mass of mitochondrial DNA and composed of several thousand circular DNA molecule linked together in a catenated network (Shlomai, 1994). These DNA network constitute mitochondrial genome containing 25-250 maxicircles of approximately 30 kb and 5000-10,000 minicircles of about 2 kb (Chen et al., 1995; Chen et al., 1995). In amastigotes (Fig. 1.1C) a flagellar pocket present on the surface of the membrane contains a non-functional flagellum not extending beyond the cell body. According to Webster and Russell, 1993 flagellar pocket serve as the site of endocytosis and exocytosis which is further supported by the presence of Golgi complex in vicinity (Webster and Russell, 1993). The cytoplasm contains both rough and smooth endoplasmic reticulum. Lysosomes are also found in the cytoplasm together with an organelle unique to kinetoplastids, the glycosome (Opperdoes, 1990).

(iii) Transformation of forms

All *Leishmania* parasites are of a flagellated promastigote form in the gut of sandflies (Phlebotamine) and a non-flagellated intermediate amastigote in the mammalian host (Jaffe and McMahon-Pratt, 1983). The interaction of the metacyclic promastigote with skin macrophages initiates the developmental cycle of parasite. After uptake and internalization of metacycylic promastigotes in a phagosome; fusion with lysosome proceeds as normal and the parasite inhabits in secondary lysosome or phagolysosome. During this process, the

metacyclic promastigote transforms into an amastigote within 24-48 hours and continues to grow and divide within the phagolysosomal compartment. Within the transition from the sandfly to the mammalian host, the promastigote face two major environmental changes: a temperature shift to 35-37^{O}C and p^{H} to 4.5-5.5. Changes in the environment accompany the transformation of promastigote into obligate intracellular amastigote with loss of flagellum. Closing the flagellar pocket, drastic reduction in size, biochemical modifications and changes in gene expression are also attributed by environment change (Molynex and Killick Kendrick, 1987; Hommel, 1999).

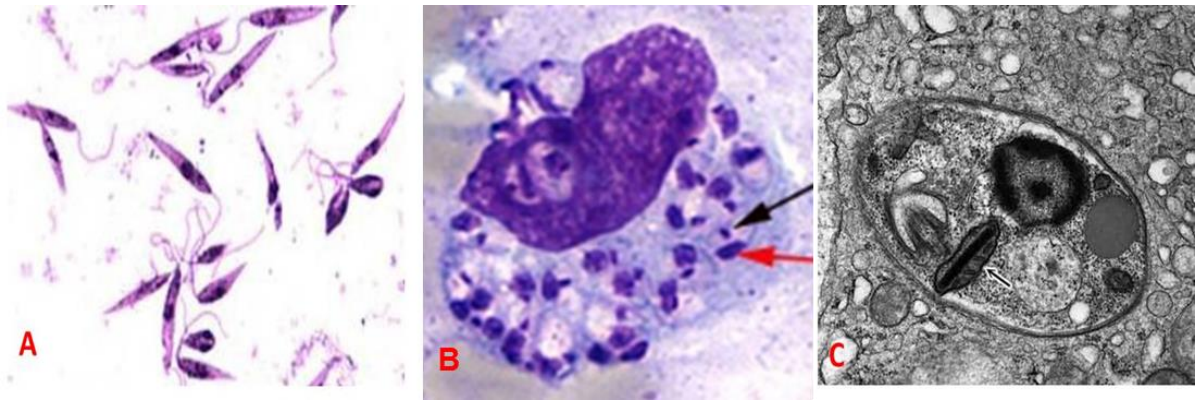


Fig. 1.1 Giemsa stained promastigotes (A) Promastigotes (B) an intracellular amastigotes: nucleus (red arrow), rod-shaped kinetoplast (black arrow) (C) ultra-structure of amastigote [Source: CDC website, Parasites Leishmaniasis (Manandhar, 2008; Manandhar, 2011)]

1.2.3 Life cycle of Leishmania

Leishmania spp have complex life cycle with two hosts where they adopt different morphologies. When a female sandfly (Fig. 1.10) feed on blood meal of *Leishmania*-infected mammalian host, it pools the amastigote along with. This process is assisted by the cutting action of mouth parts of sandfly because sandflies are pool feeders, meaning they insert their saw-like mouth parts into their skin and agitate them to create a small wound into which the blood flows form superficial capillaries (Lane, 1993). The change in the conditions moving from the mammalian host to the sandfly midgut (decrease in temperature and increase in p^{H}) triggers development of the parasite in the vector (Bates and Rogers, 2004; Kamhawi, 2006). . The non flagellated amastigotes convert to flagellated promastigotes which keep on dividing by binary fission and transform into procyclic promastigotes in posterior mid-gut of the sandfly. Procyclic promastigotes continue to divide by binary fission and transform to nectomonad forms. Approximately 3 days after blood feeding, the peritrophic membrane of the gut contacting these parasites begins to breakdown and promastigotes are set free. They then move forward to the anterior midgut of the host. After 5 days in the anterior mid-gut, the nectomonads transform to heptomonad and attach to the stomodeal valve. From 5th day

onwards, highly motile metacyclic stage parasites emerge out. They are found in the lumen of the anterior midgut or foregut or both. During next blood feeding, metacyclic forms of these leishmanial parasites enter the human host via proboscis. Within the human host, the amastigotes continue to grow and divide by binary fission within the phagolysosomal compartment. The heavily populated phagolysosomes burst, releasing amastigotes in blood circulation. From the blood they reach to liver, spleen and bone marrow. These parasites again will be taken by sandfly to be transmitted to new host and cycle thus repeats (Fig. 1.3).

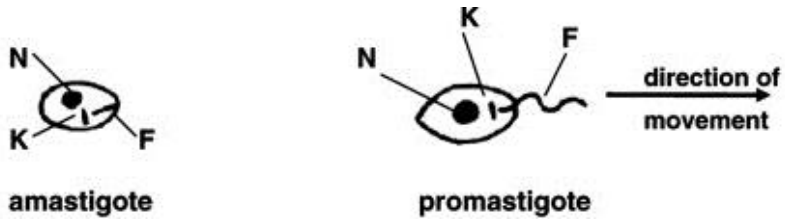


Fig.1.2 Morphology of amastigotes and promastigotes. Each form has a nucleus (N), kinetoplast (K) and flagellum (F) (Bates, 2007).

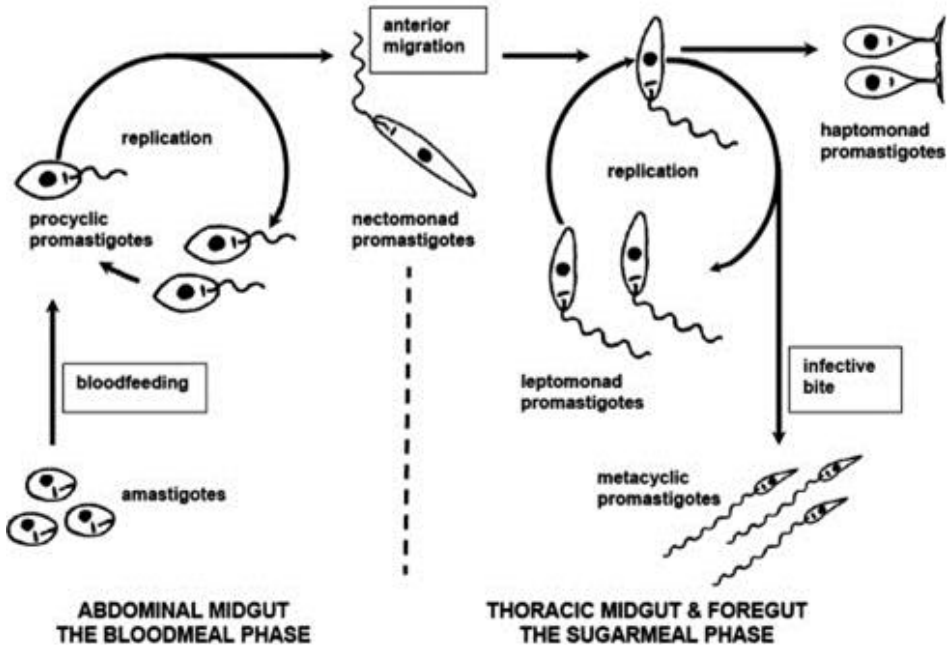


Fig.1.3 Development of *Leishmania* species in the sand fly vector. The developmental sequence of the five major promastigote forms: procyclic promastigotes, nectomonad promastigotes, leptomonad promastigotes, haptomonad promastigotes and metacyclic promastigotes (Bates, 2007).

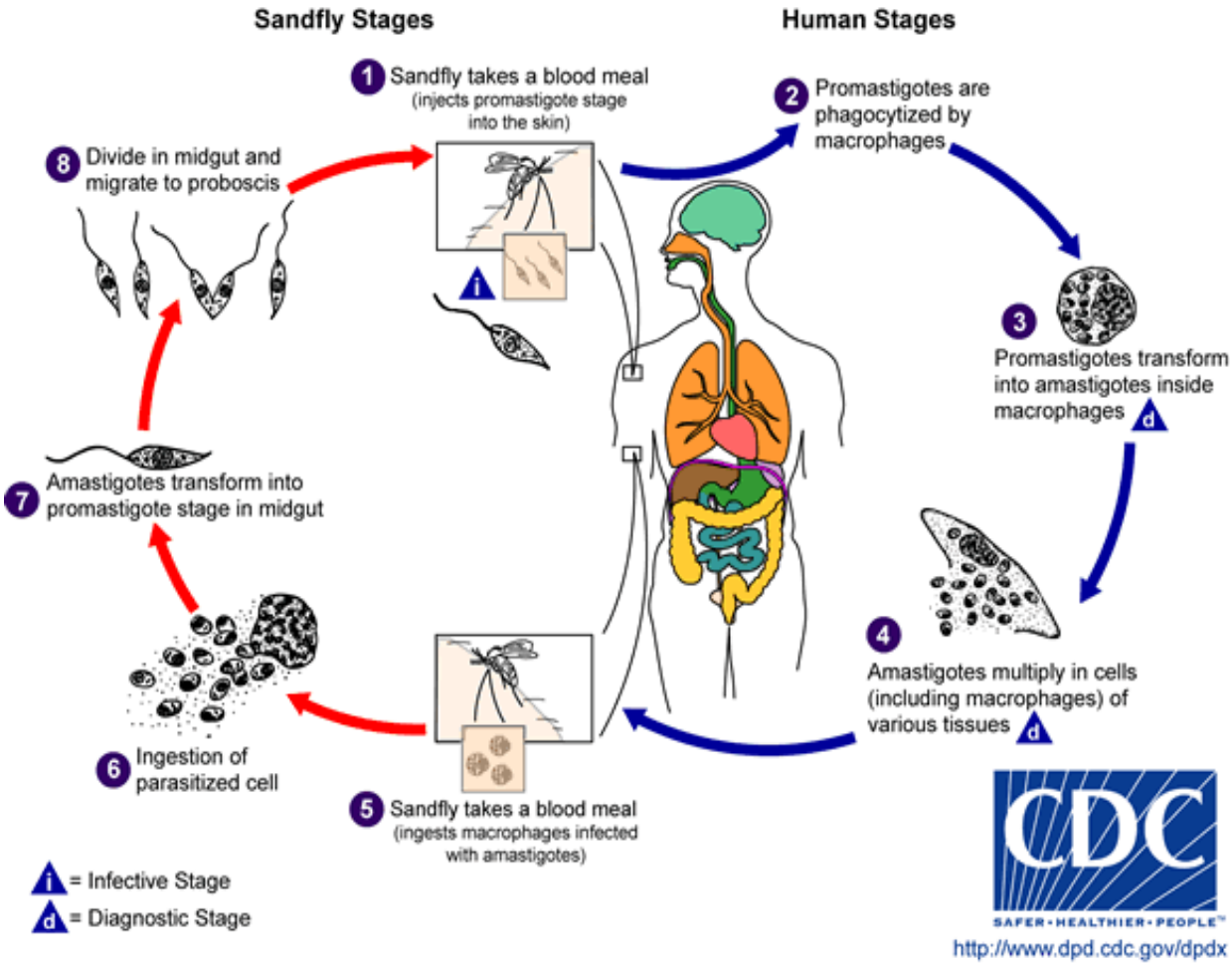


Fig.1.4 Life cycle of *Leishmania donovani* (Source: CDC website, laboratory identification of parasite)

1.3 Pathogenesis and Clinical spectrum of Leishmaniasis

1.3.1 Visceral Leishmaniasis (VL)

Visceral leishmaniasis (VL) is the most severe form of the leishmaniasis caused by the obligate protozoan parasites of the genus *Leishmania.* In the Indian subcontinent and Africa *L. donovani* is the causative agent, whereas *L. infantum* in the Mediterranean region, Southwest and Central Asia and *L. chagasi* in South Amaerica (Herwaldt, 1999; Chappuis et al., 2007; Mishra et al., 2009). VL is also known as Dumdum fever or Kala-azar (referring to hyperpigmentation with fever during the disease). An alternative derivation is from 'Kal' – meaning to death or signifying a fatal illness. The parasites infect the reticulo-endothelial cells of the visceral organs such as liver, spleen (hence 'visceral') and bone marrow, and if left untreated, is almost fatal (Desjeux, 2004; Malla and Mahajan, 2006).

WHO definition of VL

WHO has established a definition of VL as; a case of visceral leishmaniasis (VL) is a person showing clinical signs (prolonged irregular fever, splenomegaly and weight loss) with serological (at peripheral geographical level) and/or (when feasible at central level) parasitological confirmation of the diagnosis. In the malarious areas, VL must be suspected when fever lasts for more than 2 weeks and no response has been achieved with anti-malarial drugs (assuming drug-resistant malaria has also been considered) (WHO., 2001; Chappuis et al., 2006).

The outcome of the VL infection manifests asymptomatic forms (80-90%) to obvious disease with subclinical or oligosymptomatic forms. The incubation period generally lasting between 2 and 6 months (Chappuis et al., 2007). The clinical manifestations of visceral leishmaniasis starts generally within 3 months with fever, weakness, night sweats, anorexia, and weight loss that progress over weeks to months. Other prominent manifestations are splenomegaly, hepatomegaly, polyclonal-hypergammaglobulinemia (IgG and IgM), hypoalbulinemia, leucopenia, thrombocytopenia, lymphadenopathy, severe cachexia, and pancytopenia (Chappuis et al., 2007). If untreated the disease advance with profound cachexia, hyperpigmentation, multisystem disease, thrombocytopenia, susceptibility to secondary infection like malaria, pneumonia, tuberculosis, amoebic or bacillary dysentery.

1.3.2 Post Kala-azar Dermal Leishmaniasis (PKDL)

PKDL also known as Post Kala-azar dermatosis , is a sequel of visceral leishmaniasis even after successful treatment or incomplete treatment or inadequate immune response or genetic predisposition (Mondal and Khan, 2011). *L. donovani* is the principal causative agent of PKDL even though a few cases from *L. infantum* or *L. chagasi* have been reported. A cutaneous condition that is characterized by macular, de-pigmented eruptions found mainly on the face, arms, upper part of the trunk and high levels of interleukin (IL-10) in the blood and skin are peculiar to PKDL (Fig. 1.5 C) (Gasim et al., 1998). Chronic PKDL lesions are considered to play an important role in disease transmission between outbreaks (Addy and Nandy, 1992).

PKDL occurs in about 10-20% in Indian subcontinent, although the number is much higher (50%) in Sudan (Ramesh and Mukherjee, 1995; Zijlstra et al., 2000). A recent retrospective cohort study (2000-2010) showed 5.4% (37/680) presented active skin lesions suspect of PKDL with 2.4% confirmed PKDL in south-eastern region of Nepal (Uranw et al., 2011).

1.3.3 Cutaneous Leishmaniasis (CL)

Cutaneous leishmaniasis, also known as 'Oriental sore' 'Aleppo boil', 'Bagdad boil', 'Delhi boil', etc. is the most common form of leishmaniasis caused by *Lieshmania tropica*, *L. major* and *L. aethiopica* in old world and *Lieshmania mexicana* species complex specially *L. mexicana*, *L. amazonesis* and *L. venzuelensis* and some *Viannia* subgenus of *Leishmania* in new world (Herwaldt, 1999) (Table 1.1). CL usually produces papulae in skin which are followed by ulceration with raised borders at the site of the vector bite. After few weeks to months, these papulae break with bloody pus (Fig. 1.5 D). There may be a large number of lesions-sometimes up to 200- which can cause serious disability. When the ulcers heal (from 3-4 weeks to 12 months), they invariably leave permanent scars, which are often the cause of serious social chauvinism. According to WHO CL represents 50-75% of all new cases of leishmaniasis worldwide (WHO., 1998). It has been estimated that 1.5 million new cases of cutaneous leishmaniasis occur annually and more than 90% of the total cases affect individuals of Brazil, Iran, Syria, Saudi Arabia, Peru, Afghanistan and Sudan. Four cases of cutaneous leishmaniasis in the year 2006 have been reported from Nepal (Pandey et al., 2006). Clinically important CL are: (i) Localized cutaneous leishmaniasis: it causes skin ulcers that heal very slowly. The nodular leisons are limited in extent and number. (ii) Diffuse cutaneous leishmaniasis: in this leishmaniasis, cutaneous nodules and plaques do not ulcerate but can sometimes spread over the entire body (Fig. 1.5 F). (iii) Recidivans or Lupoid or Tuberculoid leishmaniasis: is a chronic case of CL that may last for years leading to destructive disfiguring(WHO, 2010)

1.3.4 Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous leishmaniasis is the most feared form of cutaneous leishmaniasis because it produces destructive and disfiguring lesions. The infection is confined to mucous secreting organs such as nose, throat, anus and larynx (Fig. 1.5 E). After initial skin lesions which heal slowly but spontaneously, chronic ulcers appear after months or years with destruction of underlying tissues (e.g. Nasal-cartilage). Death might result from severe respiratory tract infections due to massive destruction of the pharynx (ElHassan et al., 1995). MCL is frequently found in New World caused by *L. braziliensis* and *L. panamensis* (Herwaldt, 1999). Nearly 90% of all MCL cases are reported in just three countries: Bolivia, Peru and Brazil.

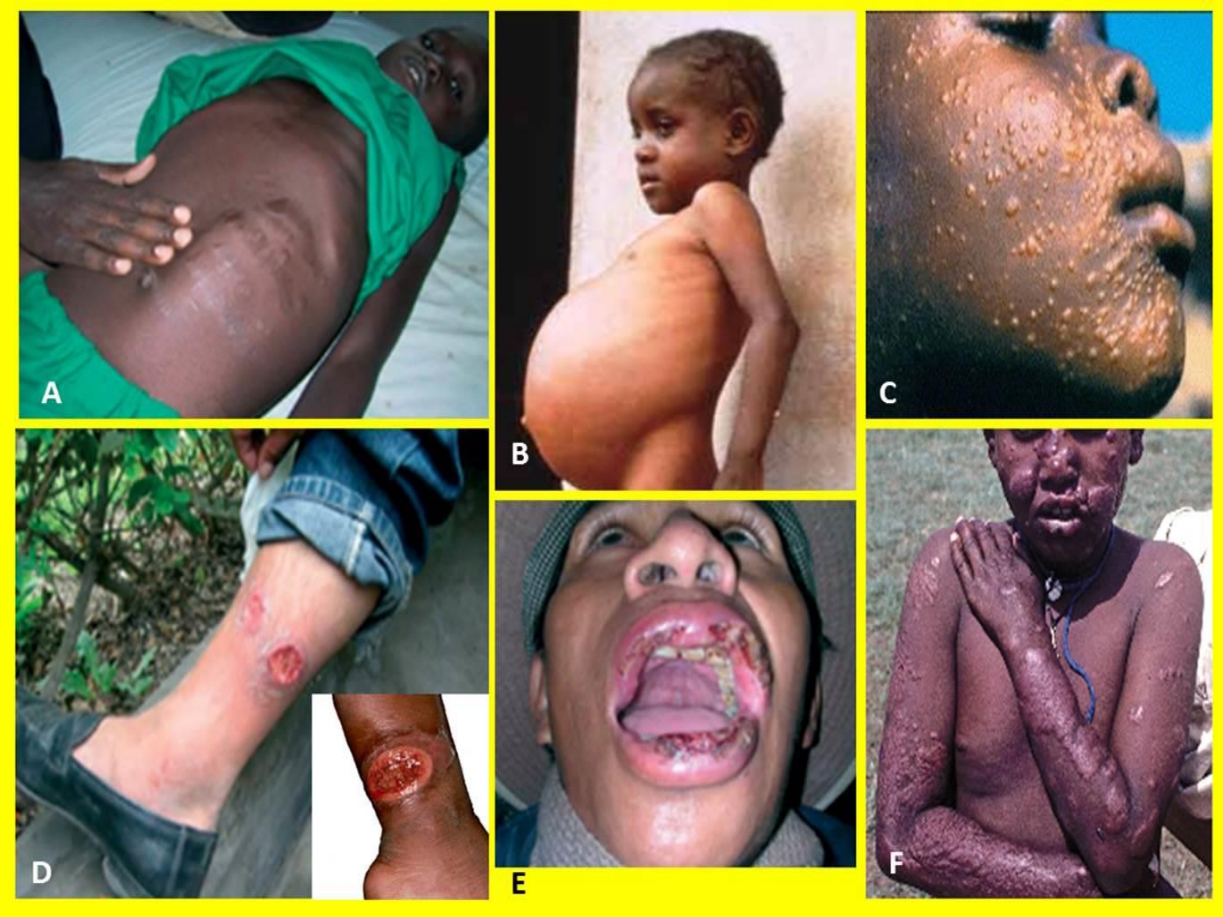


Fig. 1.5 Clinical spectrum of Leishmaniasis.(A & B) Visceral leishmaniasis (C) Post kala-azar dermal leishmaniasis (PKDL) (D) Cutaneous leishmaniasis and (E) Mucocutaneous leishmaniasis. (F) Diffuse cutaneous leishmaniasis (Zijlstra et al., 2003; Chappuis et al., 2007; Manandhar, 2008).

1.3.5 Leishmania-HIV co-infection

Visceral leishmaniasis is emerging as an important opportunistic infection associated with HIV infected patients. The first case of leishmaniasis associated with HIV-infection was reported in 1985, in southern Europe (Alvar et al., 2008; WHO, 2010). Nearly 90% cases of co-infection are reported from Southwestern Europe and are expanding to Eastern Africa and Indian subcontinent (Pintado et al., 2001; Desjeux and Alvar, 2003). To date 35 countries have reported cases of co-infection (Alvar et al., 2008). In Nepal, approximately 6% of VL patients were recorded to be infected with HIV co-infection (Gurubacharya et al., 2006).

HIV infection increases the risk of developing VL by 100 to 2,320 times in areas of endemicity, reduces the likelihood of therapeutic response and greatly increases the

probability of relapse (Rosenthal et al., 1995; Pintado and Lopez-Velez, 2001). Both *Leishmania* and HIV are known to infect and multiply in monocytes/macrophages having latent infection potentiality. Co-infection of VL with HIV patients facilitates uncontrolled parasite growth and spread of amastigotes to atypical and unusual sites outside reticuloendothelial system such as adrenal gland, gastrointestinal tract, CSF, pleural fluid, larynx, myocardium and tongue (Alvar et al., 1997). Clinical studies have shown high peripheral parasitemia (Bossolasco et al., 2003) indicative of potential reservoir or source of infection among the intravenous drug users through shared syringes (Alvar et al., 1997).

Together in the progression of leishmaniasis and HIV the balance between TH1 and TH2 cells get alter by the cytokine profile shift from TH1 to TH2 cells (Reiner and Locksley, 1993; Clerici and Shearer, 1994). The deactivation of macrophage function, depression of TH1 cells activity, inhibition of phagosome-lysosome fusion and enhancement of TH2 cells activity are the influence produced by HIV that favor amastigote multiplication (Holaday et al., 1993; Wolday et al., 1999). It has been observed that *Leishmania* infection is related to high virus load and accelerated progression of HIV, through increased expression of specific chemokine receptor ($CCR5^+$, $CD3^+$) on T cells: major co-receptor for HIV entry in target cells (Reynes et al., 2001; Nigro et al., 2007). *Leishmania* also modulates the life cycle of HIV-1 through elevation of TNF-α and interleukin-1a (IL-1a) functioning in an autocrine/paracrine manner to up regulate virus gene expression mediated through induction of NF-ƙβ (Zhao et al., 2004; Zhao et al., 2004).

1.4 Epidemiology and Geographical distribution of Leishmaniasis

1.4.1 Scenario of Leishmaniasis in the world

According to Desjeux, nearly 350 million people are at risk of leishmaniasis with global prevalence of 12 million with 2 million new cases per annum (WHO, 2013). VL accounts for 0.5 million new cases and over 90% of these cases occur in Brazil, Bangladesh, India, Nepal & Sudan (Fig. 1.6) (Desjeux, 1996; Chappuis et al., 2007). Harhay *et al* and Matlashewski *et al* reported 51,000 and 59,000 deaths respectively due to VL alone (Harhay et al., 2011; Matlashewski et al., 2011). Economic burden is further surged by the global morbidity of 2,090,000 Disability-Adjusted Life Years (DALYS) from leishmaniasis (Evans and Kedzierski, 2012). Recently 88 countries are facing the problems of leishmaniasis, out of which 82% are developing countries. Similarly, CL found in focal areas of nearly 88 countries, account for 1.5 million new cases and 90% of cases in 8 countries *viz.* Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, and Syria (Old World) and Brazil and Peru (New World) (Fig. 1.7) (Mishra et al., 2007; WHO, 2013). Currently, the number of reported cases and geographical areas

have increased and this has ignited concern regarding the contribution that global warming might have on this observation (Desjeux, 2001).

Table 1.1 Geographical distributions of Leishmaniasis and their pathogenic species, vectors and reservoir.

Clinical manifestations	Geographical distribution	Pathogenic species	Vector	Reservoir
VL	Northest India, Nepal, Bangladesh, Burma	*L. donovani* (Asia)	*Phlebotomus argentipes*	Human
	Mditerrnean basin, Middle East, China, Central Asia	*L. infantum*	*P. perniciosus, P. ariasi*	Dogs, Foxes, Jackals
	Sudan, Kenya, Horn of Africa	*L. donovani* (Africa)	*P. orientalis, P. martini*	Rodents, Cannines, Humans
	Central America, Northern South America, esp Brazil, Venzuela	*L. chagasi*	*Lutzomyia longipalpis*	Foxes, Dogs, Opossums
CL	Semi deserts in Middle East, North India, Pakistan, North Africa, Central Asia	*L. major*	*P. papatassi*	Gerbils
	Sub-Saharan Savanna, Sudan	*L. major*	*P. duboscqi*	Rodents
	Towns in Middle East, Mediterranean basin, central Asia	*L. torpica*	*P. sergenti*	Humans
	Highlands of Kenya, Ethiopia	*L. aethiopica*	*P. longipes, P. pedifer*	Hyraxes
	Yuctan, Belize, Guatemala	*L. mexicana*	*L. olmeca*	Forest rodents
	Tropica forests of South America	*L. amazonensis*	*L. flaviscutellata*	Forest rodents
MCL	Tropical forest of South and Central America	*L. braziliensis*	*Lutzomyia spp., L. umbratilis*	Forest rodents, peridomestic animals
	Guyana, Surinam	*L. guyanensis*	*L. umbratilis*	Sloths, Arboreal anteaters
	Panama, Costa Rica, Colombia	*L. panamensis*	*L. trapidoi*	Sloths
	West Andes of Peru, Argentine highlands	*L. peruviana*	*L. verrucarum, L. peruenis*	Dogs

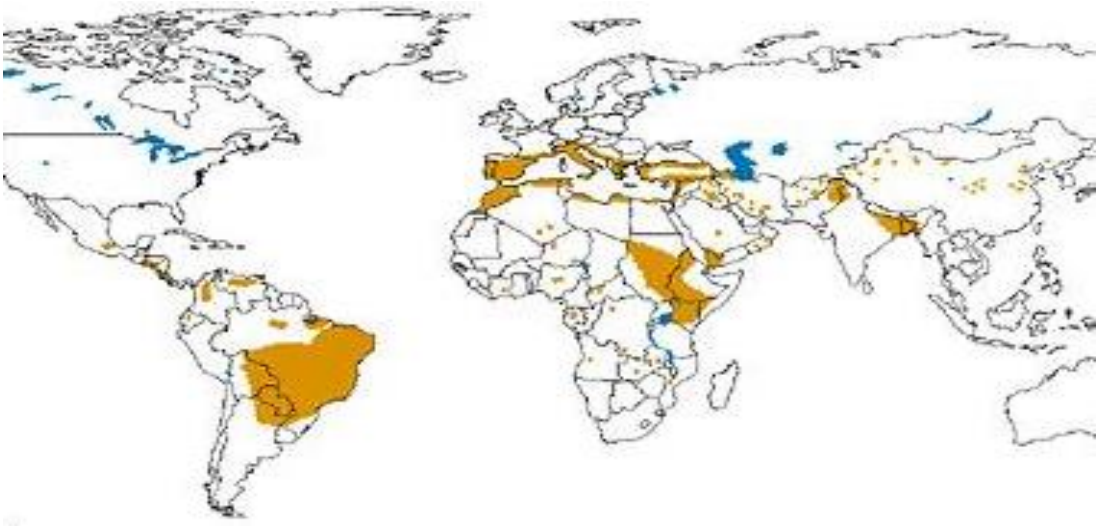


Fig.1.6 Geographical distribution of visceral leishmaniasis (VL) worldwide [Source: World Health Organization, (WHO, 2013)]. The majority of (>90%) of VL cases occur in just six countries – India, Nepal, Bangladesh, Sudan, Brazil and Ethiopia.

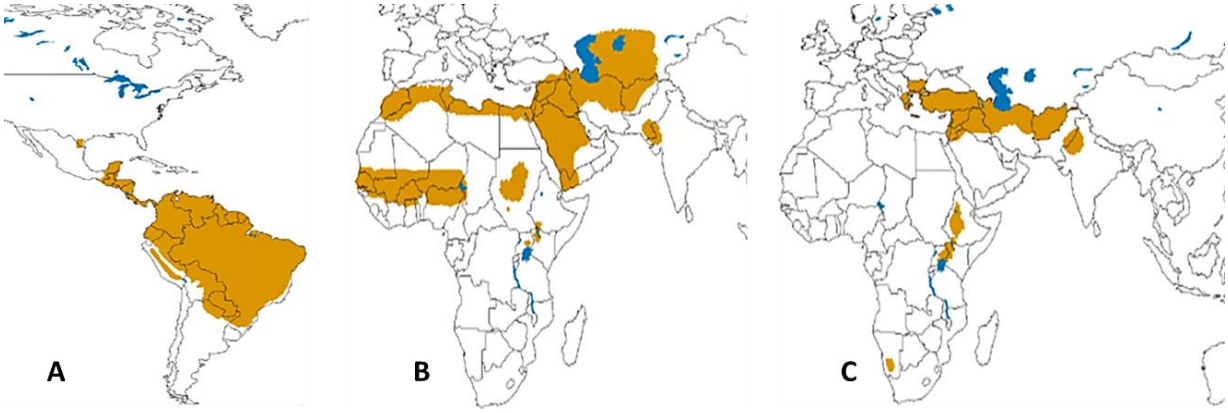


Fig.1.7 Geographical distribution of (A) cutaneous and mucocutaneous leishmaniasis in the New world (B) Old world cutaneous leishmaniasis due to *L. tropica* and related speies and *L. aethiopica*. (D) due to *L. major* [Source: World Health Organization, (WHO, 2013)].

1.4.2 Scenario of Leishmaniasis in Nepal

VL is a disease of socioeconomically vulnerable population living in under developed countries with high prevalence. In Nepal VL was postulated to be endemic in southern terai of Nepal by an Indian scientist Raghavan in 1953. During 1960's and 1970's VL ceased mainly due to countrywide malaria eradication activities with DDT spraying. Since DDT was banned to be used, VL cases started reemerging and were first recorded in 1980 (Bista, 1998) with the incidence rate of 1.5 per 100,000 population and case fatality rate of 5.88

percent (EDCD, 2010). The annual VL incidence in Nepal is 43-55 per 100,000 populations (Rijal, 2006; Hirve et al., 2010). In Nepal, 13 districts from southeastern Terai region are endemic zone of VL and more than 5.7 million people are at risk with average 1341 cases annually (Pun et al., 2011). Although eastern Terai belt of Nepal is endemic region for VL, it is increasingly reported from not endemic regions like Khotang, Bardiya and Doti (Joshi et al., 2006; Pandey et al., 2009). Not only VL, four cases of cutaneous leishmaniasis in the year 2006 has also been reported from Nepal (Pandey et al., 2006). The expansion of disease co-relates with the urbanization and vector's adaption to the socio-demographic factors like migration, formation of new vegetative land destroying forest or urban setting or others (Werneck, 2008).

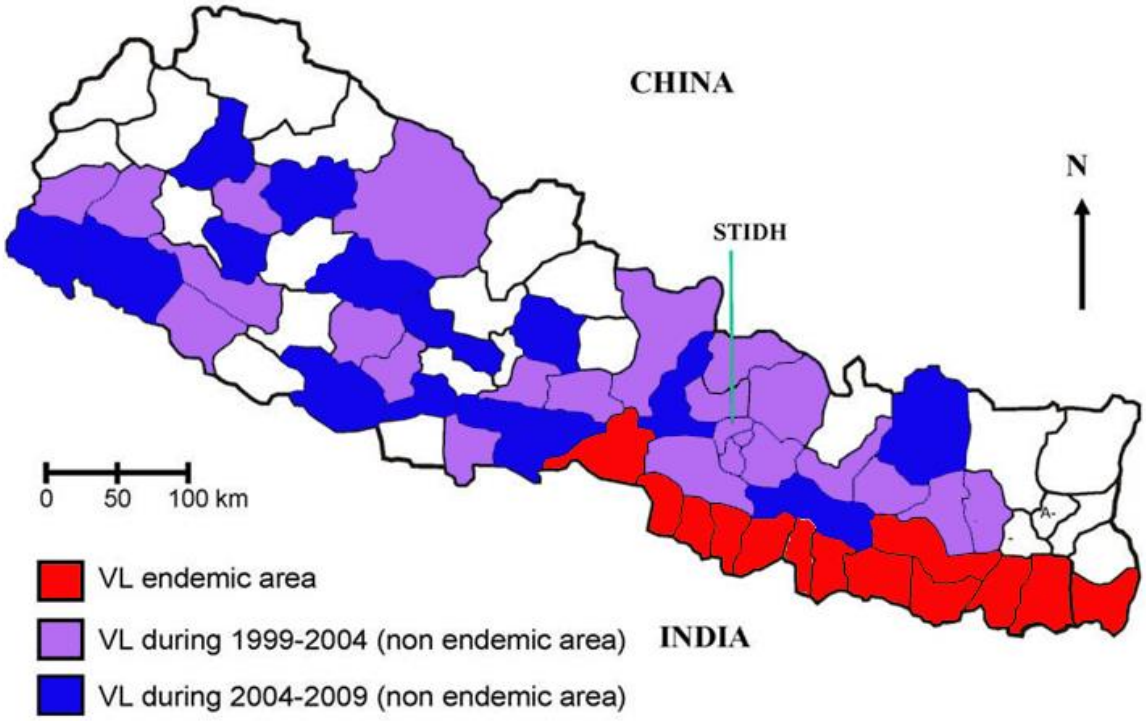


Fig.1.8 District map showing geographical distribution of VL in Nepal, April 1999 to March 2009. VL endemic area: 13 Districts of eastern Terai namely - Jhapa, Morang, Sunsari, Saptari, Siraha, Udayapur, Dhanusha, Mohattari, Sarlahi, Rautahat, Bara, Parsa and Chitwan. [Source: (Pun et al., 2011)] STIDH: Sukraraj Tropical and Infectious Disease Hospital

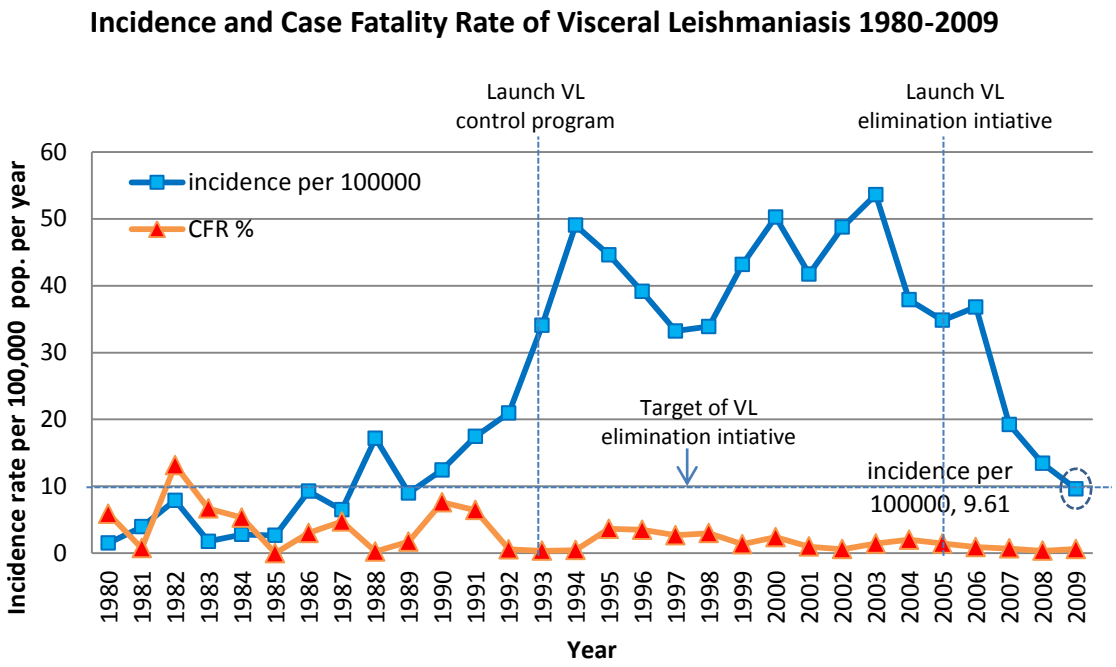


Fig.1.9 Evolution of VL reported yearly incidences in Nepal from 1980 to 2009 [Source: Annual Reports, Ministry of Health and Population, Nepal (EDCD, 2010)].

1.5 Vector of Leishmaniasis

Sandflies, the vectors of *Leishmania* parasites, are insects of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Killick-Kendrick et al., 1985; Sharma and Singh, 2008). Out of 600 species of sandfly, only 30 species have been implicated in the transmission of leishmaniasis (Shaw, 1994; Desjeux, 1996; Ashford, 1997). *Phlebotomus argentipes* is responsible for kala-azar transmission in Indian subcontinent (Swaminath et al., 1942; Sharma and Singh, 2008). In 1921, it has been experimentally proved that sandflies of genus Phlebotomus are responsible for transmission of the disease (Sergent et al., 1921).

Fig.1.10 *Phlebotomus argentipes* (A) biting and (B) feeding (C) engorged after feeding on human hand (Wilson).

Sandflies live in dark, damp places, and are relatively weak fliers, with a range of only 50 meter from the breeding site. Unlike mosquitoes, they fly silently and their small size (2-3mm long) allows them to penetrate mosquito nets. Most sandfly vectors are active from dusk to dawn, with a peak biting immediately after sunset and have diurnal resting sites: e.g., houses, cellars, stables, caves, fissures in walls, rocks or soil, dense vegetation, tree holes, burrows of rodents and other mammals, bird's nests, etc. The vector is highly endophilic and sufficiently anthrophilic ensuring adequate reservoir/host contact throughout the year. In Nepal, vector density was observed to start rising from April, peaking in May and then a decline from September to a negligible level in December (Das, 1998). Insecticides like DDT, BHC, Dieldrin and Malthion, are effective in sandfly control.

1.6 Parasite reservoir

In the zoonotic VL there is an animal reservoir (Dogs, Foxes, Jackals, Rodents, Opossums, Gerbils, Sloths) where the parasite is maintained and a man being an occasional host, while in an anthroponotic form the parasite is exclusively maintained in a man-vector-man cycle in the absence of any animal reservoir (Desjeux, 1996). Human is the principal reservoir for anthroponotic VL that is common in Indian subcontinent and Sudan (Sharma and Singh, 2008). Asymptomatic cases described in various foci of the world can acts as a potential reservoir of the parasite (Sharma et al., 2000). PKDL has been implicated as reservoir for initiating (Addy and Nandy, 1992) and sustaining epidemics (Rai and Sundar, 1996).

1.7 Transmission

Sandfly is only the vector known to transmit infection. It may obtain parasite either directly from the infected skin or by ingesting the blood of the infected host. There are various factors such as proximity of residence to sandfly breeding and resting sites (Werneck et al., 2003), type of housing, occupation and extent of exposure to sandfly bites, resistance to infection, virulent of the parasite species, zoonotic or anthroponotic reservoirs, density, seasonality, longevity and flight range of sandfly populations and the vectorial capacity (defined as the number of infective bites delivered per human per annum (Degu, 2006)) influences the transmission of the disease. The incubation period for transmission is generally 2-6 months but can also vary form 10 days to 2 years. For this reason sometimes it is impossible to determine the actual transmission period for infection (Rai and Sundar, 1996). Occasionally, sandflies are not involved in transmission, instead, VL can be transmitted by contact of amastigotes to the blood through shared needles, transfusion, trans-placental spread or organ transplantation and CL by inadvertent needle stick containing infected materials (Cruz et al., 2002; Pagliano et al., 2005).

1.8 Diagnosis

VL diagnosis and treatment follow-up pose a challenge to physicians, since clinical features of VL mimic several diseases like febrile illness, malaria, typhoid, tuberculosis, etc. Hence, precise diagnosis of VL should be decisive as the treatment is associated with significant toxicity. Invasive and risky techniques involving demonstration of the parasite in stained preparations from splenic and bone marrow aspirate is still the gold standard for VL diagnosis but is irrelevant for asymptomatic carrier and have issues related to skills of technician. The widely accepted diagnostic tool for VL detection is rK39 strip test which is simple, economic, and easy to perform and have significant sensitivity and specificity. But this strip test cannot discriminate symptomatic and asymptomatic individuals and shows positive test even in cured individuals. Other different diagnostic options available are culture, DAT, Immunoblot, ELISA, LST, Katex IFAT, PCR (targeting rRNA & miniexon genes, kDNA minicircles repetitive nuclear DNA sequences) and more but their field applicability need to be established.

1.9 Treatment

1.9.1 Chemotherapy

Chemotherapy is the only effective measure to control leishmaniasis worldwide. Date back from quinine resistant infection, search for specific drugs and their trials for treatment of VL have developed various synthetic chemical compounds like pentavalent antimonials, pentamidine, amphotericin B, parmomycin, miltefosine, sitamaquine and others for the treatment of leishmaniasis.

1.9.1.1 Current Parenteral Chemotherapy

Presently there are about 25 compounds and formulations showing antileishmanial effects. Most of the drugs have one or more limitations like unaffordable cost, difficulty in administration, toxicity and the development of resistance in the parasite. The development of resistance is most alarming against the antimonial compounds and poses a major impediment in successful therapy of the disease (Mbongo et al., 1998).

Pentavalent antimonials (Sb^V)

These are non-covalent chelates of Sb^V with improved solubility and uptake property as compared to trivalent antimonials. World Health Organization recommends a dose of 15-20mg Sb^v/kg of body weight per day for 21-28 days, injected intramuscularly or intravenously.

Pentavalent antimony is a prodrug that can be spontaneously reduced to a toxic trivalent form by Glutathione S-transferase and the parasite specific trypanothione (Franco et al., 1995). A number of potential targets for pentavalent antimonials are: glycolysis (inhibition of ADP phosphorylation followed by depletion of ATP level in *Leishmania*) and beta-oxidation of fatty acid (Berman et al., 1987). In addition, pentavalent antimony inhibits *Leishmania* purine transporters and DNA topoisomerase I (Wyllie et al., 2004).

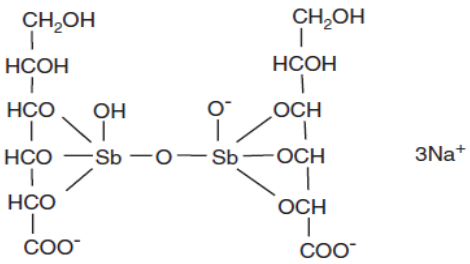


Fig.1.11 Chemical Structure of Sb^V

Pentamidine (Aromatic diamidines)

Pentamidine belongs to the class of aromatic diamidines. The recommended regimen of pentamidine consists of 4 mg/kg thrice a week continued for 3-4 week and is administered either intravenously or intramuscularly.

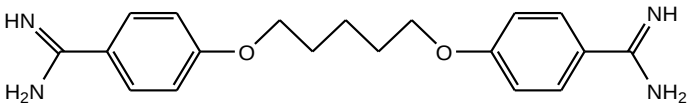


Fig.1.12 Chemical Structure of Pentamidine

It acts on the parasite genome by hindering replication and transcription at the mitochondrion level. Polyamines are substituted at nuclei acid binding sites, which preferentially bind to kinetoplast DNA (Mishra et al., 2007).

Amphotericin B (Polyene antibiotics)

Amphotericin B (AmB), a polyene macrolide, is strongly recommended as a second line of drug for the treatment of leishmaniasis. Originally it is produced from strains of *Streptomyces nodosus* (Gold et al., 1956). AmB is poorly absorbed by the gastrointestinal tract, so the preferred route is intravenous with a dosage regimen of 1mg/kg either daily for 20 days.

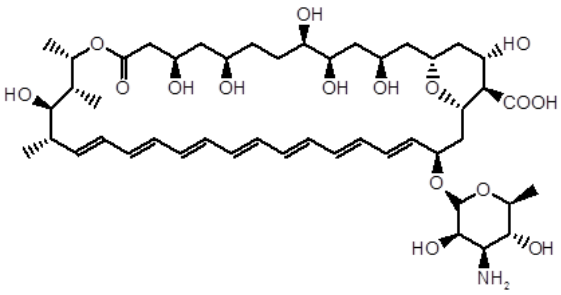


Fig.1.13 Chemical Structure of Amphotericin B

AmB binds with greater affinity (in parasitic cell membrane) to ergosterol forming intimate binary complex. This binary complex and AmB induced lipid peroxidation of cell membrane leads to cellular dysfunction and eventually cell lysis (Brajtburg et al., 1985). Prolong

hospital stay, IV infusion, serious toxicities (renal dysfunction, hypokalemia, hepatic dysfunction one marrow suppression and myocarditis) and high cost are the major limiting factors of amphotericin B (Chappuis et al., 2007).

Drug Delivery Systems of Amphotericin B: Amphotericin B has serious toxicities, explained by its binding to lipoproteins and internalization of Amphotericin B-lipoprotein complex. Thus, in order to increase therapeutic index of the drug and to reduce toxicity, lipid formulations of Amphotericin B were developed. AmB lipid formulations involve either phospholipids or detergents and the resultant associations are referred to as "liposomes" and "mixed micelles", respectively. Liposomal AmB is currently marketed in 3 forms: Liposomal Amphotericin B (Ambisome); Amphotericin B Colloidal Dispersion (Amphocil or Amphotec); Amphotericin B Lipid Complex (Abelect) (Brajtburg and Bolard, 1996).

Liposomal Amphotericin B

Among the three lipid formulations of AmB, only Ambisome is a true "liposomal" preparation. It consists of unilamellar lipid vesicles, averaging 60-70 nm in size. These are made up of phospholipid, having saturated fatty acid side chains of hydrogenated soy phosphatidylcholine and disteraroylphosphatidylglycerol, stabilized by cholesterol. These lipid formulations reduced systemic side effects and course of therapy but are exorbitantly costly.

Parmomycin (Aminosidine)

Paromomycin, an aminocyclitol-aminoglycoside produced by *Streptomyces riomosus*, was isolated in 1956. Preferred route is intramuscular at a dose of 12-16mg/kg/day for 21 days (Balana-Fouce et al., 1998).

Paromomycin inhibits protein synthesis by binding to the 30S ribosomal subunit, interfering with initiation of protein synthesis by fixing the 30S-50S ribosomal complex at the start codon of mRNA, leading of accumulation of abnormal initiation complex (Sundar and Chakravarty, 2008). In addition, paromomycin also induces destabilization of the membrane.

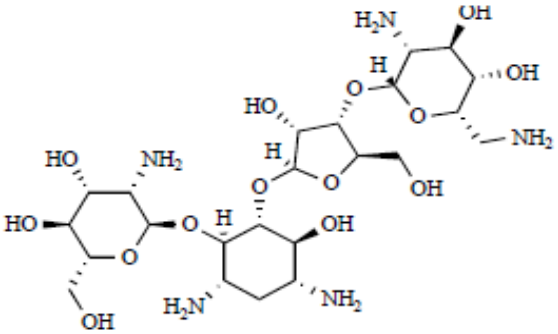


Fig.1.14 Chemical Structure of Paromomycin

1.9.1.2 Oral Chemotherapy

Miltefosine (Alkyllysophospholipid)

Miltefosine affects the cell signaling pathways and membrane synthesis holding excellent antileishmanial activity (Croft et al., 1996). Recommended regimen for miltefosine is 2.5mg/kg/day for 28 days given orally.

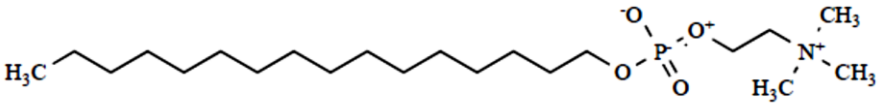


Fig.1.15 Chemical Structure of Miltefosine.

Antiprotozoal activity of miltefosine are: damage to the flagellar membrane (Santa-Rita et al., 2000), perturbation of alkyl-phospholipid metabolism and glycosylphosphatidylinositol anchor biosynthesis (Lux et al., 1996), interference with ether-lipid remodeling through the inhibition of alkyl-lyso-phosphatidylcholine specific acyltransferase (Lux et al., 2000) and inhibition of the de novo synthesis of phosphatidylcholine (Lira et al., 2001). It is reported that miltefosine induces a apoptosis type of death in *Leishmania donovani* (Paris et al., 2004). Furthermore, Na^+, K^+-ATPase has been reported to be inhibited by miltefosine (Berkovic et al., 1992). Gastrointestinal disturbances and renal toxicity and teratogenic are associated effects of Miltefosine (Sundar and Chatterjee, 2006).

Sitamaquine (Primaquine analogue)

Sitamaquine, an 8-aminoquinoline analogue, is responsible for changes in the amastigote's outer membrane in the flagellar pocket, mitochondria, kinetoplast and cytoplasm. Furthermore, it inhibits the relaxation and decatenation reactions catalyzed by type I and II DNA topoisomerases respectively (Mishra et al., 2007). The recommended dose of sitamaquine is 2mg/kg/day for 28day (Jha et al., 2005).

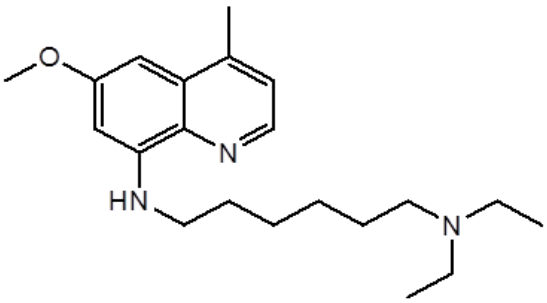


Fig.1.16 Chemical Structure of Sitamaquine

1.9.2 Other agents

Several antifungal azoles (ketoconazole, itraconazole and fluconazole) are potent inhibitors of 14-α-demethylase, an enzyme involved in sterol metabolism responsible for ergosterol biosynthesis. Purine analogues (Allopurinol, Mycophenolic acid and Formycin B) have been found to possess antileishmanial activity. Purine nucleoside phosphotransferases, unique to

Leishmania, are potent drug targets of purine analogues. Plant-derived products with leishmanicidal activity include quinones, alkaloids, terpenes, saponins, phenolic derivatives and other metabolites.

1.9.2.1 Cytokine therapy

Remedy of leishmaniasis appears to be dependent upon the development of an effective immune response. This process is suppressed by the infection itself, which down regulates the requisite signaling between macrophage and T cell such as the IL-12, the IFN-ϒ and the presentation of major histocompatibility complex. It was observed that inactivation of IFN-ϒ by anti-IFN-ϒ monoclonal antibodies led to progressive disease in BALB/c mice. Therefore, it is reasonable that IFN-ϒ can be used in human kala-azar to improve the treatment outcome. Amphotericin B in conjunction of IL-12 or IL-10 was more efficient and led to a reduction of the amphotericin dose (Murray et al., 2003).

1.9.2.2 Nanomedicine

The concept of nanomedicine was first proposed by late Nobel physicist, Richard P. Feynmann in late 1959, with his says “There are plenty of rooms at the bottom”. In respect to the therapeutic array, the foremost characteristic of nanonisation of drugs are: bioavailability and targeted drug delivery. As per Manandhar *et al* and Prajapati *et al* the use of nanoparticles and microspheres have increased the delivery of conventional amphotericin B against experiment VL (Manandhar et al., 2008; Prajapati et al., 2011).

1.10 Prevention and Control strategies

The preventive measures to control leishmaniasis depend chiefly on the detection of symptomatic, asymptomatic and oligosymptomatic individuals, treatment of those individuals and reduction in the transmission of the disease. Active case detection and prompt treatment is important to control the disease in the Indian subcontinent type of kala-azar, in which infected human itself acts as a reservoir. Primary available strategies to control the transmission of the infection to human are (i) Vector Control / reduction of sandfly population (ii) Prevention from exposure to infected sandfly (iii) Reduction of reservoir (Murray et al., 2005; Chappuis et al., 2007; Sharma and Singh, 2008).

1.10.1 Vector control

Reduction of sandfly population is better alternative to shrink the ratio of infected population. Integrated Vector Management (IVM) is globally accepted approach applied in

multi-disease cases like malaria, visceral leishmaniasis, dengue, Japanese encephalitis and lymphatic filiariasis. The population of sadfly can be reduced by spraying of chemical insecticides notably DDT (Dichloro-diphenyl-trichloroethane), BHC (Benzene hexachloride), Dieldrin and Malathion. House spraying with residual insecticides is recommended by the WHO (WHO, 1990) against endophilic sandfly species. Another innovative technique to control sandfly is the use of *Bacillus saphaericus*. In this technique, bait-fed adults were used to carry the bacterial control agent to larval habitats in animal burrows, resulting in larval mortality in burrows up to 10-30 m away from the baited solution (Claborn, 2010). Identification and destruction of sandfly breeding places or resting site also helps in the vector control program.

1.10.2 Prevention from exposure to sandflies

Adequate clothing, insecticide impregnated nets (ITN), window and door mesh, use of repellants, etc. have proved successful in prevention from exposure to sandflies. The use of insecticide impregnated bed nets in endemic region is an effective way to control VL which is currently running under Kala-Net project funded by European Union in Nepal and India. In most studies the insecticides used were synthetic pyrethroids (ermethrin, deltamethrin, and lambda-cyhalothrin) (Elnaiem et al., 1999).

1.10.3 Reduction of reservoir

The proximity to a VL infected persons is a major risk factor of VL, thus human are the only reservoir host for VL in Indian subcontinent (Bern et al., 2005). Therefore, active case and passive case detection and treatment of those found to be infected (including PKDL) is an important attempt to abolish the human reservoir and control the disease. In addition, health education is a key in controlling leishmaniasis.

1.11 Research Plan and Design

1.11.1 Research Hypothesis

1. There is remarkable number of asymptomatic cases in the endemic regions of Nepal.
2. Antibodies developed in patient against *L. donovani* express best agglutination with the aqueous particulate antigen of the indigenous parasite.
3. Antigenic fractions of cocktail *Leishmania donovani* parasite interact with antibodies developed in VL patient.

1.11.2 Research Objectives

General objectives

Study of asymptomatic population of Kala-azar in endemic settings of Nepal using Nested-PCR endorsed by DAT and Immunoblotting techniques.

Specific objectives

1. Extraction of DNA from buffy coat using Genomic DNA Mini Kit
2. Assess the quality and concentration of extracted DNA using Gel electrophoresis and Spectrophotometry
3. Employ the nested-PCR for confirmation of asymptomatic VL
4. Preparation of aqueous DAT antigen and cocktail CSA from *L. donovnai* culture
5. Diagnosis of asymptomatic VL by immunological tools (DAT and Immunoblot).
6. Validation of the marker for asymptomatic population

1.11.3 Research Plan

The work was planned to accomplish the claimed contemporary research objectives as disclosed in the following chart.

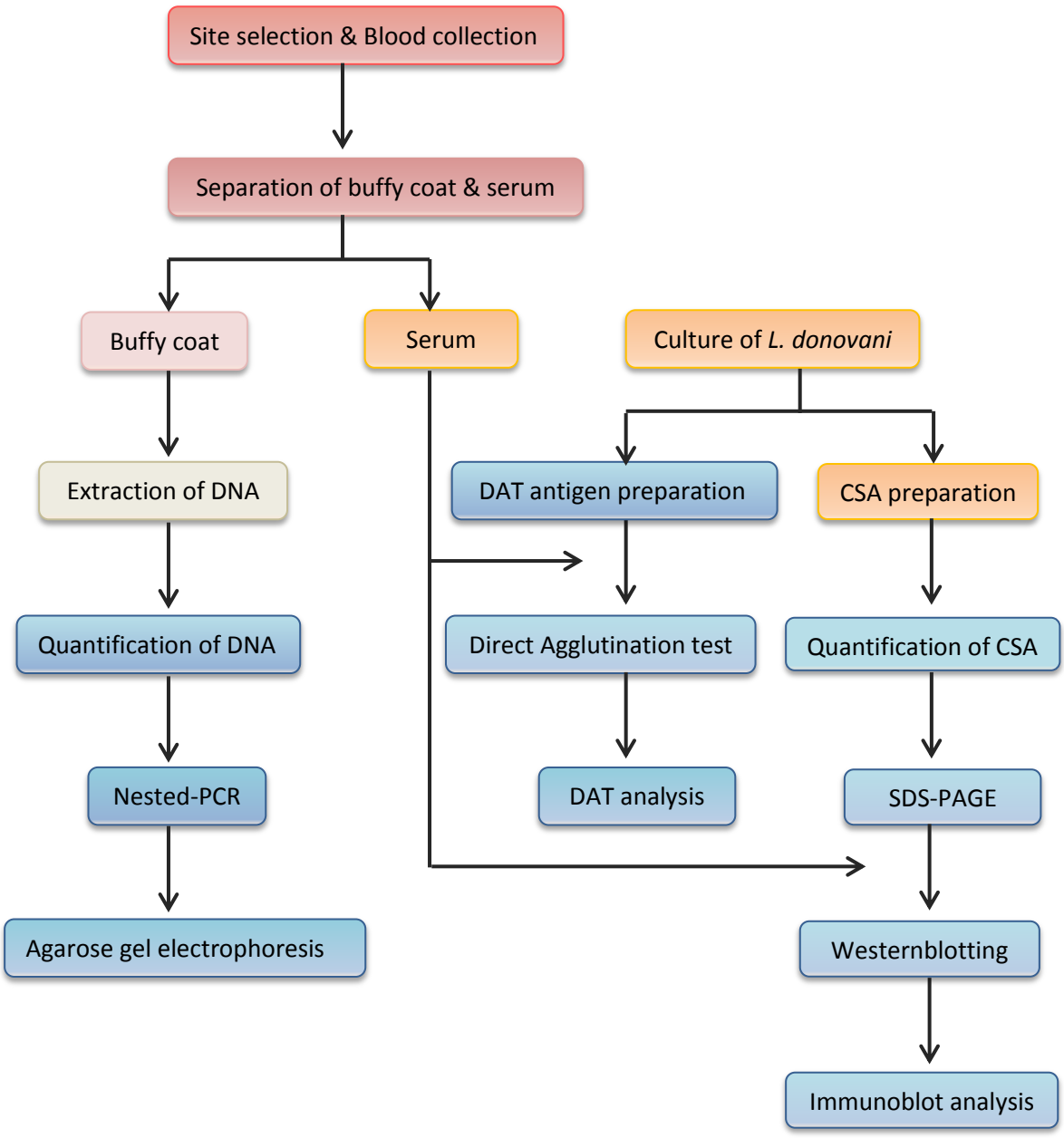


Fig. 1.17 Flow chart: Diagnosis of asymptomatic visceral lesishmaniasis using different diagnostic tools.

1.11.4 Rationale

Visceral leishmaniasis is one of the serious health threats to underdeveloped countries like Nepal and until the public health condition is improved, upliftment of national economy is not possible. Therefore, concerning authorities has to keep eyes open in elimination programs of such diseases. Since the identification of *L donovani* in 1903, continuous efforts are being continued to develop vaccine, diagnostic tools and medicine against this disease, however, till date the efforts are not completely succeeded. Nepal is one of the endemic zones for the VL diseases among five countries where 90% VL cases of the world are confined. The disease is threatening to 28% population i.e. more than 5 million peoples, residing in east and middle terai belt of Nepal (Pun et al., 2011). Hence, this disease is and has to be kept in top most priority for control as well as elimination. In order to control it, proper diagnosis with the best diagnostic tool has to be emphasized in parallel to treatment of infected individuals. Diagnostic tools developed may not work with equal efficiency in all regions and may require regional/national based tools. Until prompt diagnosis is done, the dissemination of the disease will erupt recurrently as the carrier will be freely available for transmission. Although, the diagnosis and treatment is on the way with emphasis, the subtle carrier role played by asymptomatic cases has yet to be studied in Nepalese context. The most efficient molecular tool is now widely used worldwide. Among the different PCR techniques, nPCR has been reported as the most efficient as it can detect $1/10^{th}$ one parasite burden (Noyes et al., 1998).

This research work has tried to put efforts in the field of validation and development of diagnostic tool for visceral leishmaniasis in the context of Nepal which is supportive to the national development towards the fields of science and technology, and is found very relevant to need of national issue. Thus this research justifies the relevancy based on following points:

- It is an epidemiological study of asymptomatic visceral leishmaniasis which is prevalent in our country as endemic in the world.
- This research work has used the most sensitive molecular tool, nested PCR for detection of parasitic load in healthy individuals residing in endemic regions.
- We have developed aqueous particulate antigen from the indigenous *L donovani* parasites for diagnostic tool, Direct Agglutination Test (DAT), however, require improvements.
- This research work became able to validate the marker for asymptomatic cases by Western blot technique.

Highlighting on above four aspects, this six-month-long research work has been found very worthy to me, Central Department of Biotechnology, Tribhuvan University and the nation.

Chapter II

Literature review

Leishmaniasis, the second highly infectious disease, is prioritized by international organizations like WHO, One World Health, Bill Gate and Foundation and European Union for better human health. After first official recording in 1980 from Dhanusha district (Bista, 1998; Rijal et al., 2010), Nepal has become endemic country among 88 countries of the world having prevalence of leishmaniasis. In addition, asymptomatic infections of VL, described in various foci of the world, play foremost role in transmission of the disease or may subsequently progress to clinical disease. At this point, prompt diagnosis of VL and asymptomatic VL followed by their treatment plays a vital role in understanding the epidemiology and control of the disease. This research work is focused on molecular and immunological diagnostic array. The review of most important diagnostic methods from past to present along with immunological perspectives and vaccine candidate of VL has been presented.

2.1 Diagnosis of Leishmaniasis

Visceral leishmaniasis, a second largest parasitic killer after malaria, is a neglected tropical disease transmitted by phlebotomine sandfly. The consequence of the VL infection manifests asymptomatic forms (apparently healthy) to obvious disease with subclinical or oligosymptomatic forms. The mean period from the onset of the symptoms to diagnosis (mean diagnostic lag period) of VL is 7.7±5.96 months (Sundar et al., 1991) during which the patient not only suffers but also continues to spread the disease. Since clinical features i.e. night sweats, weakness, prolonged fever, polyclonal-hypergammaglobulinemia, splenomegaly, hepatomegaly, lymphadenopathy, pancytopenia etc. of visceral leishmaniasis mimic several other diseases like febrile illness, malaria, tuberculosis, lymphoma, brucellosis, leukemia, histoplasmosis, bacterial endocarditis and others (Singh and Sivakumar, 2003), precise diagnosis of VL is decisive as the treatment is associated with significant toxicity. Early case detection followed by adequate treatment is central to the control of VL (Boelaert et al., 2000; Davies et al., 2003).

Diagnostic tests play a major role in the areas like patient management, screening of asymptomatic infections, surveillance including verification of elimination and epidemiological studies. An ideal diagnostic test should discriminate acute disease and asymptomatic infection, should have high sensitivity and specificity, simple and affordable. But unfortunately some

serological tests carry some significant disadvantages: the inability to differentiate between clinically active and asymptomatic infections and detect positive even long after cure. Molecular diagnostic tools like PCR and real-time PCR are quite sensitive and specific but are cumbersome to perform and have high cost. DNA-based tests are available in strip formats but these cannot be used in fields (Deborggraeve et al., 2008).

2.1.1 Microscopic demonstration of parasite

Demonstration of the amastigote form of the parasite by microscopic examination of tissue aspirates from spleen, bone marrow, liver biopsy, or lymph nodes and even from buffy coat of peripheral blood is the recommended and suitable method of VL diagnosis. Different stains like Giemsa, Leishman, Romanowsky and Diff Quick are used to stain parasites. In preparations stained with Giemsa or Leishman stain, amastigotes appear as round or oval bodies measuring 2 to 3 µm in length (found intracellular in monocytes and macrophages) and their cytoplasm appears pale blue, with a relatively large nucleus that stains red at right angle to a deep red or violet rod-like body called kinetoplast. The use of microscopy offers benefits of high specificity and is directly able to detect parasite density on a logarithmic scale from 0 (no parasites in 1,000 microscopic fields) to 6+ (>100 parasites per microscopic field) using 10X eye piece and 100x oil immersion lens (Table) (Chulay and Bryceson, 1983; WHO, 2008).This grading system is useful in measuring the speed of response to treatment and in distinguishing slow responders from non-responders (Chulay and Bryceson, 1983).

Table 2.1 Grading of *L. donovani* amastigotes in splenic aspirate smears (Chulay and Bryceson, 1983). *Using 10X eye piece & 100X oil immersion lens

Grade	Average parasite density
6+	>100 parasites/field*
5+	10-100 parasites/field
4+	1-10 parasites/field
3+	1-10 parasites/10 fields
2+	1-10 parasites/100 fields
1+	1-10 parasites/1,000 fields
0	0 parasites/1,000 fields

Parasitological diagnosis remains the gold standard in the diagnosis of leishmaniasis due to its high specificity and sensitivity (Herwaldt, 1999; WHO, 2008). Sensitivity and specificity ranges from study to study, parasite load and type of tissue aspirate i.e. lymph node, spleen, bone marrow, peripheral blood etc (Sundar and Rai, 2002). Splenic smears have the sensitivity of 93.1-98.7% (Zijlstra et al., 1992), bone marrow and the lymph node smears have lower sensitivity ranging from 52-85% (Ho et al., 1948) and 52-58% (Zijlstra et al., 1992) respectively

while peripheral blood smears have lowest sensitivity. Only at level III health institutions and special referral centers, parasitological examination through bone marrow or splenic aspirate is performed. The test is recommended only when i) rk39 test is negative but the suspicion of kala-azar is high, ii) patients diagnosed by rk39 but do not respond the first line drug, iii) patients with suspicion of relapse, iv) patients with kala-azar and HIV co-infection, v) quality assurance and vi) for monitoring of drug resistance (WHO, 2008). Although microscopy is highly sensitive and accurate test it is associated with life threatening complications (hemorrhage; painful and fatal) during splenic aspirate preparation and is irrelevant for asymptomatic carrier. Fatality of splenic test has been recorded as 2 out of 9,612 test performed over 10 years (Sundar and Rai, 2002), 1 out of 671 splenic aspirate in Kenya (Kager et al., 1983) and 3 out of 3,000 splenic aspirate in India (Thakur, 1997). Apart from these, highly skilled and experienced medical physician, well-equipped hospital, experienced laboratory technician and quality reagents are prohibitive in suitable diagnosis of VL.

2.1.2 Isolation of parasite

2.1.2.1 *In vitro* culture

In vitro culture of parasite is one of the diagnostic methods of leishmaniasis with 100% specificity. Mainly two types of media are in practice to culture parasite *viz.* monophasic and biphasic media. Schneider's insect medium, M199, Grace's medium are monophasic media while Novy-McNeal Nicolle medium (NNN medium) & Tobies medium are biphasic media. For the culture of parasite, one to two drops of tissue aspirate is inoculated in a tube containing 100µl media (cRPMI 1640) and allowed to grow the parasite in BOD incubator at 26^{O}C for 7 to 9 days. Finally, the culture is observed microscopically to conclude the result as positive (presence of parasite) or negative (absence of parasite). Parasites can also be cultured from blood sample, contains negligible amount of parasites, but takes longer period to develop to detectable level (Sundar and Rai, 2002).

In vitro parasite isolation still remains the reference method to indisputably identify the parasite at the species level, but it is expensive, cumbersome, and generally unhelpful in clinical practice because of the long duration of time (days to weeks) required to obtain a definitive result (Antinori et al., 2007). In addition, reports like difficulty to isolate *Leishmania braziliensis* and unsuccessful culture of *Leishmania infantum* in the standard NNN blood agar in Tunisia (Ben-Ismail et al., 1992) adds cons to the diagnostic knack of culture.

2.1.2.2 Animal inoculation

Demonstration of parasite after inoculating clinical aspirate to laboratory animals such as mice, golden hamster, guinea pigs is another conventional approach for diagnosis of leishmaniasis. Various routes like intradermal (nose skin, foot pad, belly skin), intraperitoneal, intrasplenic and intracardial are employed (Anjili et al., 1996) for inoculating clinical aspirate for animal model infection. Golden hamster is the animal of choice for maintaining *Leishmania donovani* complex (Sundar and Benjamin, 2003). The development of disease can be scrutinized through signs like hepatosplenomegaly, cutaneous or metastatic lesions and more. The diagnosis of inoculation of hamsters was achieved within 2-12 weeks, a mean of 34.5 days (Shatry et al., 1988). The absence of obvious signs does not justifies the absence of parasite in those inoculated models but further confirmation should be done by examining liver and spleen of animal model after sacrificing. Animal inoculation takes more months to diagnose the disease so, is not an appropriate test for diagnosis but is useful in studying different aspects like clinical manifestations, drug trial, vaccine development etc.

2.1.3 Immunodiagnosis

Immunodiagnosis of leishmaniasis is based on the presence of specific humoral immune response (Herwaldt, 1999) i.e. antibodies produced by B cells against parasite antigen. Different types of antibody detection tests (Napier's formol gel test, rK39, DAT, ELISA, ICT, IFA, WB etc.) and antigen detection test (KATex) are available. Immunodiagnostic test not only avoids invasive and risky techniques but also confronts simple, rapid and non-invasive methods of disease diagnosis. However, the accepted limits of *Leishmania* serologic testings are reduced sensitivity in patients with severe underlying immunosuppression, absence of discriminating power between active or past infections, and consequently, lack of value in monitoring the parasitological response to specific therapy (Antinori et al., 2007). In addition, a significant proportion of healthy individuals living in endemic areas with no history of VL are positive for anti-leishmanial antibodies owing to asymptomatic infections. The seroprevalence in healthy populations varies from <10% in low to moderate endemic areas (Koirala et al., 2004; Schenkel et al., 2006) to >30% in high-transmission foci or in household contacts (Ibrahim et al., 1999; Sundar et al., 2006).

2.1.3.1 Non-specific Test

Nonspecific methods, which depend upon the raised immunoglobulin levels, have been used in the diagnosis of VL for several decades. Some of these tests are Napier's formol gel or

aldehyde test and Chopra's antimony test.

Napier's observed that the addition of a few drops of formalin to the serum of kala-azar patients resulted in "egg white" solidification, known as formol gel test (Napier, 1922). Since globulins do not increase early in the course of the disease, they are of no value in patients presenting early. As they indicate raised globulin only, they can be positive in host of other conditions (Bray, 1985). Formol gel test is still used in East Africa and Asia because of its simplicity and low cost. However, as the sensitivity of this test is poor as low as 34% (Boelaert et al., 2004), some experts have recommended its use be discontinued (Sundar, 2003).

2.1.3.2 Antigen detection Test

Antigen detection tests, antibody based immunodiagnostic tests, are an outstanding method of diagnosing leishmaniasis. They are more specific than antibody based immunodiagnostic test, as they avoid cross-reactivity, correlate parasite load and can distinguish active from past infections (Vinayak et al., 1994; De Colmenares et al., 1995). The method is also useful where the antibody production is low as in AIDS (Sundar and Benjamin, 2003). De Colmenares et al from Spain have reported two polypeptide fractions of 72-75 kDa and 123 kDa in the urine of kala-azar patients. The sensitivity and specificity of 72-75 kDa fraction was 96% and 100%, respectively (De Colmenares et al., 1995). Additionally, these antigens were not detectable in the urine suggesting a good prognostic value.

The latex agglutination test (KATex), for the detection of urinary leishmanial antigen (low molecular weight & heat stable) in the urine of patients affected with VL, has been introduced by the Kalon Biological Ltd (Sarkari et al., 2002). This test has shown sensitivity of 68–100% and specificity of approximately 64–100% (El-Safi et al., 2003; Salam et al., 2011) but didn't produce encouraging result in East Africa and Indian Subcontinent (Boelaert et al., 2008). There are two practical limitations of latex agglutination test: the urine must be boiled to avoid false-positive reactions and it is difficult to distinguish weakly positive from negative results, which affects the reproducibility of the test (Rijal et al., 2004; Chappuis et al., 2006).

2.1.3.3 Indirect Fluorescence Antibody Test (IFAT)

Indirect fluorescent antibody (IFA) is one of the commonly used tests for anti-leishmanial antibody detection using fixed promastigotes (Singh, 2006). IFAT is based on detecting the antibodies that persist in the very early stage of infection but undetectable 6-9 months after cure (Singh, 2006; Srivastava et al., 2011). Titers above 1:20 are significant and above 1:128 are

diagnostic. However, there is a possibility of a cross-reaction with trypanosomal sera (Boelaert et al., 2004; Singh et al., 2005). The sensitivity of these tests varies extremely from as low as 28.4% to 100% and specificity 77% to 100% (Sinha and Sehgal, 1994; Boelaert et al., 2004). The requirement of sophisticated laboratory with fluorescence microscope, prohibitive cost etc. are limiting its applicability in the field (Sassi et al., 1999).

2.1.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA has been used as a potential serodiagnostic tool in almost all infectious diseases including leishmaniasis. The test is useful for laboratory analysis or field applications and to screen a large number of samples at a rapid pace. With the advances in automation, ELISA can be performed easily and is adaptable for use with various antigens such as whole cytoplasmic (soluble antigen, SA), purified antigens such as fucose-manose (Palatnik-de-Sousa et al., 1995), defined synthetic peptides and recombinant proteins as antigen (Maalej et al., 2003).

The sensitivity and specificity of ELISA is greatly influenced by the antigen used. Besides, the most commonly used soluble promastigote antigen, several antigenic molecules have been reported. An excretory, secretary and metabolic antigens released by *L. donovani* promastigotes (Ld-ESM) into a protein free medium was used for the serodiagnosis of VL by ELISA (Maalej et al., 2003). This antigen has been reported to be 100 percent specific and sensitive. An ELISA based test using recombinant rORFF protein was found highly sensitive and specific however, showed mild cross-reaction in 40% cases of confirmed CL from Turkey (Raj et al., 1999). Most promising results are shown by antigen rK39 with sensitivity and specificity of 100% and 96% respectively (Palatnik-de-Sousa et al., 1995). The antibody titers to this antigen directly correlate with the active disease and have potential in monitoring the chemotherapy and in predicting the clinical relapse (Kumar et al., 2001). In addition, rK39 ELISA has a high diagnostic and prognostic utility in HIV-infected patients (Houghton et al., 1998). Recently, the recombinant antigen (rKE16) from Indian isolate of *L. donovani* strain KE16, showed 100% sensitivity and specificity for diagnosis of kala-azar and PKDL (Sivakumar et al., 2006). It also showed 100% concordance with rK39 in sera from leishmaniasis patients from China, Pakistan, and Turkey (Sivakumar et al., 2006).

Dot ELISA (modified ELISA), done on nitrocellulose paper using soluble whole promastigotes protein as antigen, is field friendly test. It is more sensitive than ELISA (micro ELISA 97.6% & dot ELISA 98.4%)(Kumar et al., 2006).The need of sophisticated equipment, skilled manpower, electricity and high cost has prevented widespread application of this technique.

2.1.3.5 Immunochromatograpic strip test (ICT)

A promising ready-to-use immunochromatographic strip test is based on antigen impregnation on the strip and color development through antigen antibody interaction. The most celebrated immunochromatographic test is rK39 ICT. rK9, rK28, rK16 and rK26 are other ICT under trial.

rK9 and rk26 are cloned hydrophilic antigens of *Leishmania chagasi* that differ in the presence of 11 copies of a 14-amino-acid repeat in the open reading frame of K26 (Bhatia et al., 1999). They are less sensitive (78% for rK9 and 38% for rK26) and less specific (84% for rK9 and 80% for rK26) than rK39 strip test (Mohapatra et al., 2010). rKE16 strip test is based on a recombinant antigen (L.D.rKE16) obtained from a newly isolated Indian strain of *L. donovani* (MHOM/IN/KE16/1998) showed remarkable result (WHO, 2011). rK28 is another potential recombinant antigen prepared from a synthetic gene, k28, by fusing multiple tandem repeat sequences of the *L. donvoani* haspb1 and k39 kinesin genes to the complete open reading frame of haspb2, thereby increasing antigenic epitope density. rK28 polyprotein has great potential as a serodiagnostic tool having comparable efficacy with rK39 test (Pattabhi et al., 2010)

rk39 immunochromatographic strip test

The rk39 strip contains 39 amino acid repetitive immunodominant B-cell epitope of kinesin related antigen from *L. chagasi.* The protein is conserved in *L. donovani* complex and it is the product of 117bp gene with the protein size of 230kDa. The antibody titre against rK39 correlates the activity of the disease as antibody. The rK39 ICT is considered to be excellent method for field application, but it shows cross-reactivity with malaria, typhoid and tuberculosis (Chappuis et al., 2006). Different commercially available rK39 strips are DiaMed-IT LEISH (manufacturer Bio-Rad laboratories), Kalazar Detect^{TM} (InBios International Inc.) with good performance in three continents (WHO, 2011).

In rK39 antigen strip, the antigen is immobilized on a small rectangular piece of nitrocellulose membrane in band form, and goat anti-protein A is attached to the membrane below the antigen band. After finger prick, less than a drop of whole blood/serum is smeared at the tip of the strip, 4-5 droops of PBS is placed on a clean glass slide or tube and the lower end of the strip is allowed to soak in this solution for 10-15 minutes (Srivastava et al., 2011). If the antibody in patient's serum is present it will react with the conjugate (protein A colloidal gold) which is pre-dried on the assay strip. The mixture moves along the strip by capillary action and reacts with rK39 antigen band and gives a pink band. In positive patients two pinkish lines

appear in the middle of nitrocellulose membrane, the upper pinkish band serves as procedural control.

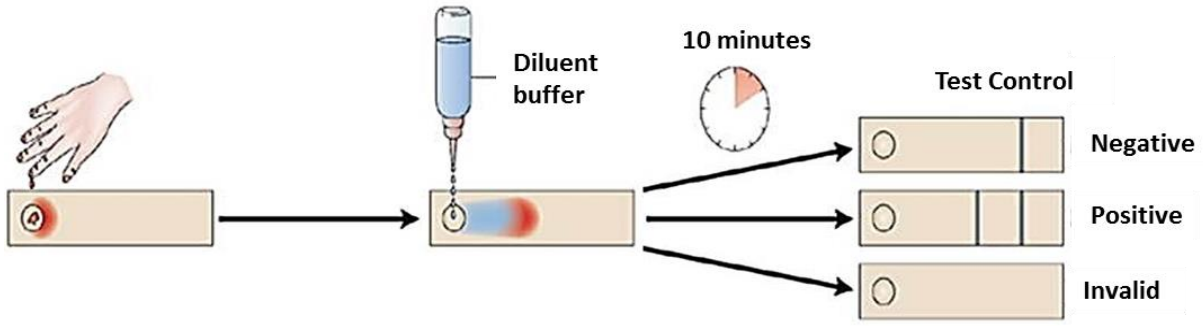


Fig.2.1 Immunochromatographic strip test using rk39 (Chappuis et al., 2007).

In the initial clinical evaluation 100% sensitivity and 98% specificity was observed (Sundar et al., 1998) and has been found highly sensitive and a reliable indicator of kala-azar (Houghton et al., 1998; Singh et al., 2002). In Nepal and India, rK39 ICT performed excellent diagnosis of the infection but showed low accuracy in East Africa. The hidden reasons may be Sudanese patients seem to develop lower titres of antibodies against rK39 than do Indian patients, although the format of the test might be a factor, as other brand of ICT performed better in this region (Chappuis et al., 2005; Ritmeijer et al., 2006).

Table 2.2 Pros and cons of 'rK39' test in the diagnosis of kala-azar (WHO, 2008).

Pros	Cons
Test can be performed with one drop of blood/serum	The test strips have to be stored at recommended temp. (20^{o}C-30^{o}) and humidity
It is simple, rapid & can be performed in any setting by a trained health worker	It is not recommended in patients who have HIV and kala-azar co-infections
It is reliable & compares well with confirmatory test so, no need to perform confirmatory test in all cases of kala-azar	The test cannot be used in patients who have a relapse or a reinfection
This test is also positive in cases of PKDL and is positive in 95-100% patients of kala-azar	This test is not recommended to determine the cure form kala-azar since the test continues to be positive even after the patient has clinically recovered.

2.1.3.6 Direct Agglutination Test (DAT)

Direct agglutination (DAT) for serodiagnosis of leishmaniasis was developed by A. E. Harith at the Royal Tropical Institute (KIT), Amsterdam, the Netherlands(Harith et al., 1986). The DAT is a semi-quantitative test that uses V-shaped microtiter plates in which increasing dilutions of

patient's serum or blood are mixed with Coomassie Brilliant blue stained, trypsinised *L. donovani* promastigotes fixed in formalin as an antigen (Harith et al., 1986; Harith et al., 1987). If specific antibodies are present, agglutination is visible after 18 hours with the naked eye. DAT can be carried out using plasma, serum, or even urine samples, making it suitable for both field and laboratory application (Bhattarai et al., 2009).

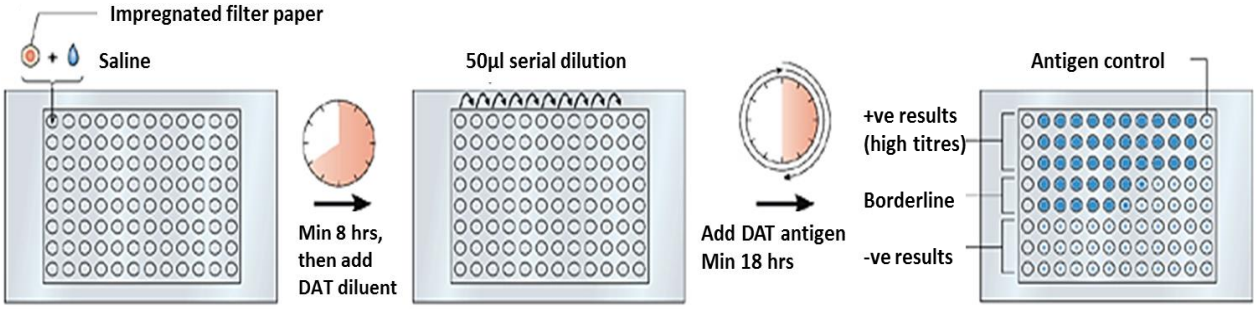


Fig.2.2 Direct agglutination test for visceral leishmaniasis (Chappuis et al., 2007).

Initially, aqueous DAT antigen was used for the test with diagnostic capacity very high in laboratory studies (Zijlstra et al., 1992). Performance of the DAT with the aqueous antigen (Aq antigen) can be improved by adding 0.8% of 0.1M 2-mercaptoethanol (2ME) to the sample diluent, while stability of the antigen increased by supplementing the suspension medium with 0.056M sodium citrate (el Harith et al., 1988). However, moderate reproducibility because of inter-observer and batch-to-batch variability as well as its sensitivity to temperature and shaking during transport lead to the development of freeze dried antigen. In 1995, (Meredith et al., 1995) of the KIT in Amsterdam developed a freeze-dried (FD) version of DAT antigen, and (Oskam et al., 1999) reported good diagnostic performance of this format. The major advantage of FD antigen are its better stability at higher temperatures and its longer shelf life, which make it much more suitable than aqueous version for use in the field. The sensitivity and specificity of freeze dried antigen is comparable to that of aqueous antigen is comparable to that of aqueous antigen i.e. 96% and 85% for freeze dried (FD) antigen and 97% and 87% for aqueous antigen (Sundar et al., 2006). DAT using FD antigen showed encouraging sensitivity (95%) and specificity (90%) in Indian subcontinent but not in East African countries (Boelaert et al., 2008). In several studies, detection of antibody response to *Leishmania donovani* amastigote antigen was reported as a better marker for diagnosis than antibody response to promastigote antigen (Malla et al., 2003; Singh et al., 2005). Today there are only two non-commercial suppliers of DAT antigen: the KIT in Amsterdam and ITMA in Antwerp.

DAT is a sensitive and specific test popular in Africa and research laboratories but is allied with cons like long incubation period, limited supply of qualitative antigen, fragility of aqueous

antigen, batch to batch to variability, multiple pipetting and well-trained staff to interpret the result correctly. A major drawback is DAT fails to differentiate symptomatic cases (patients) form asymptomatic cases and treated patients (Srivastava et al., 2011; Srividya et al., 2011). Also, 20-30% of healthy individuals residing in the endemic region give positive DAT reaction that could lead to misdiagnosis (Sundar et al., 2006). Like all antigen-antibody test DAT remains positive for a long period even after treatment so it cannot be used for relapse cases.

2.1.3.7 Immunoblotting

Western blot (also called protein immunoblot) is a widely accepted analytical technique originated in the laboratory of Towbin at the Friedrich Miescher Institute (Towbin et al., 1979). The name Western blot was given to the technique by W. Neal Burnette (Burnette, 1981). WB is used to detect specific proteins in the given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by 3D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein (Renart et al., 1979; Towbin et al., 1979). Similarly, the detailed antibody response against various leishmanial antigens can be detected using WB as described. In this test, the soluble protein extracted from log phase of promastigote culture of *L. donovani* is separated from SDS-PAGE, electroblotted onto a nitrocellulose or PVDF membrane and probed with primary antibody (serum sample). After a subsequent washing step, the membrane is incubated with an enzyme-conjugated secondary antibody that is reactive toward the primary. The activity of the enzyme, such as alkaline phosphatase (AP) and horseradish peroxidase (HRP), is necessary for signal generation. Finally, the membrane is washed again and incubated with an appropriate enzyme substrate, producing a recordable signal.

Western blotting has been shown to be much more suitable owing to its higher sensitivity and its analytic properties (Biglino et al., 2010). Immunoblotting provides broader information about the parasite antigenic profile (Kar, 1995) and is useful tool for prognosis & in suspected patients even if there is no splenomegaly (Kumar et al., 2002). The sensitivity (91%) and specificity (100%) of WB is significantly more than indirect immunofluorescence (52% and 79% respectively) and ELISA (62% and 71% respectively) and is being used in diagnosis of Leishmaniasis (Mary et al., 1992). A number of authors found WB as the most sensitive method in detecting asymptomatic carriers (le Fichoux et al., 1999; Costa et al., 2002; Riera et al., 2004). WB analysis also provides highly sensitive test for PKDL without cross reactivity to sera from other diseases like leprosy and vitiligo (Salotra et al., 1999). WB analysis of whole parasite

as a source of antigen is considered sensitive when low serum antibody titers are present (Riera et al., 2004) but often limited by the problems of cross-reactivity between species (Hoerauf et al., 1992; Kar, 1995). The common cross reaction for whole parasite as antigen is found with pathogens including *Trypanosoma cruzi*, mycobacterium, malarial parasites or amoebae, which are co-endemic with leishmaniasis in many parts of the world (Reed et al., 1987).

Immunoblotting profiles of leishmaniasis

Parasite antigen potentially useful for specific immunodiagnosis of VL includes proteins such as gp63 (Okong'o-Odera et al., 1993), p32 of *L. donovani*, *L. infantum* promastigotes (Tebourski et al., 1994). Purified proteins of 70 and 72 kDa from *L. donovani* promastigote (Jaffe and Zalis, 1988; Jaffe and Zalis, 1988), recombinant proteins such as rK39, a 39-amino acid repeat part of a 230 kDa protein predominant in *L. chagasi* amastigote (Burns et al., 1993) and recombinant gp63 antigens form *L. chagasi* and *L. donovani* (Shreffler et al., 1993) are reported as useful for VL diagnosis. The 74, 66, 65, 63kDa protein fraction has been reported as a better diagnostic marker for VL in Indian sub-continents (Singh et al., 1995; Kumar et al., 2002; Ravindran et al., 2004; Manandhar et al., 2008). The antibodies against 14, 16 kDa and / or 18kDa antigens are important for the diagnosis of asymptomatic infections (Mary et al., 1992; Sakru et al., 2007).

Drawbacks of immunoblot

Despite the popularity of WB, cross-reactivity between species (Hoerauf et al., 1992; Kar, 1995) using whole parasite as antigen, prohibitive cost, demanding skilled lab technician, time consuming are its shortcomings: confining it only to research laboratories for diagnosis of leishmaniasis.

2.1.3.8 Leishmanin Skin Test (LST)

The Leishmanin Skin test, also known as Montenegro test, is an important tool for the diagnosis and epidemiological surveys of CL. The first study of this test was carried out by Montenegro in 1926 in Brazil (Montenegro, 1926). This test is based on cell-mediated immunity that measures Delayed Type Hypersensitivity (DTH) reaction. LST uses phenol-killed whole parasite that is injected intradermal on the volar aspect of VL patient’s forearm. After

48-72 hours of injection, indurations greater than 5mm, measured by ball pointed pen technique (Sokal, 1975), represent the positive case.

LST is used as an indicator of the prevalence of cutaneous and mucocutaneous leishmaniasis in human and animal populations and successful cure of VL (during active kala-azar there will be no or negligible cell mediated immune response) (Singh, 2006). A Guatemala study of the LST showed that an antigen comprising *L. major* promastigotes gave a sensitivity of 85% and specificity of 100% in cutaneous leishmaniasis (Arana et al., 1999). . It has also been reported that LST positivity rate of 82% amongst cured individuals with VL (el-Safi et al., 1991) and similarly, in another study in Sudanese population, 80% developed a positive LST 6 months after successful treatment (Manson-Bahr, 1961) but no response in Indian active VL patient (Haldar et al., 1983).

As a diagnostic tool, the LST has its limitations in VL, because patients with active disease are in a state of anergy and unresponsive to the LST. The LST usually becomes positive after successful drug treatment of the disease within a few weeks to months (Zijlstra et al., 1994) or may become positive in asymptomatic patients who have presumably been exposed to infection (WHO, 1996). Additionally, no standardized antigen, not applicable to patients (using immunomodulators, corticosteroids, antihistamines or diagnosed with a systemic illness) etc. are its limitations.

2.1.3.9 Whole Blood Stimulation Assay

Whole blood stimulation assay is also based on the cell mediated immune (CMI) response that measures the INF-ϒ in plasma after the treatment of blood with leishmanial antigen. When the individuals primed *in vivo* with exogenous or endogenous antigen their lymphocytes in blood maintain an immunological memory for the priming antigen. The addition of antigen in vitro to blood taken from such primed individuals result in rapid re-stimulation of antigen specific effector/memory T cells and the release of the cytokine IFN-ϒ. Thus produced IFN-ϒ can be used as specific marker cytokine for a cell mediated or inflammatory immune response to that antigen. IFN-ϒ is produced by activated $CD4^+$ helper T cells (Th-1 type), activated $CD8^+$ cytotoxic T cells and ϒδ T cells in response to specific antigen or mitogen and by natural killer cells in response to IL-12 stimulation.

In whole blood stimulation assay fresh whole blood from suspected subject is treated with leishmanial antigens, mitogen (positive control) and PBS (negative control). After incubation at 37^oC for 18-22 hours in 5% CO_2, the blood cells release the different cytokines including IFN-ϒ

in plasma. Thus produced IFN-ϒ is quantified through ELISA. The cured and healthy control individuals will have high level of IFN-ϒ while low in patients as CMI is directly proportionate to the production of IFN-ϒ in case of VL. The specificity of this assay is based on the type of antigen used. A modified quantiferon assay was performed to detect the cell-mediated immune response against *L. infantum* in Turkey (Turgay et al., 2010).This test is much more reliable since the leishmanial antigen is allowed to interact with whole blood as *in vivo* and will maintain an immunological memory for the priming antigen (Manandhar, 2008). However, this test is not in practice, could be a good tool to evaluate the asymptomatic, cured and even prone cases of VL.

2.1.4 Molecular Diagnosis

Molecular diagnosis is progressively becoming pertinent to the diagnosis of infectious diseases. Information on DNA and RNA sequences are being extensively exploited for the development of PCR based assays. Amongst all the molecular advances gene amplification techniques have been most rewarding as far as diagnosis and diseases management is concerned. This is because gene amplification through PCR is extremely sensitive, rapid and ability to be performed with a broad range of clinical specimens (Tavares et al., 2003). Additionally, no more essential to undergo invasive methods (Bone marrow and splenic aspiration, lymph node biopsy, liver biopsy, etc.) or collect large volumes of blood samples are encouraging. Even a few drops of blood on filter paper may be sufficient (da Silva et al., 2004). Unlike the immunodiagnosis, no host specific reagents are required and the same reagents can be used for specimens from human, dogs or any other animal host (Schallig and Oskam, 2002). With the development in molecular techniques, detection or identification of the causative agent is directly possible from clinical samples like bone marrow, spleen aspirate, lymph node aspirates, skin biopsy, skin scrape/exudates, blood and even from archival specimens. In 1995, the use of PCR of PB samples to diagnose VL was first reported and employed PCR in patients with confirmed VL in India(Adhya et al., 1995; Nuzum et al., 1995), Brazil, Kenya (Nuzum et al., 1995; Schaefer et al., 1995), and Switzerland (Mathis and Deplazes, 1995).

PCR-based methods for detecting *Leishmania* species in clinical samples have been developed to amplify 18S-rRNA, small subunit rRNA (SSU rRNA), a repetitive genomic sequence of DNA, the miniexon (spliced ladder) gene repeat, the β-tubulin gene region, gp63 gene locus, internal transcribed spacer (ITS) regions; micro-satellite DNAs such as maxi- and minicircles of kinetoplast DNA (Wortmann et al., 2001; Gangneux et al., 2003; da Silva et al., 2004). These methods are of varying specificity: some will detect all *Leishmania* species while other

methods will identify the infecting *Leishmania* parasite to the species complex level. According to the target region of *Leishmania* genome and the type of sample like bone marrow, splenic aspirate, blood, or even conjunctive (in dogs) (Strauss-Ayali et al., 2004) the sensitivity and specificity varies. Sensitivity up to 99% using PB targeting kDNA (600 bp) by Ld1 primers was obtained by Maurya *et al* for kala-azar (Maurya et al., 2005) and most of PCR gives 100% specificity (Srivastava et al., 2011). However, in some other studies, the sensitivity of PCR of PB samples was unsatisfactory, ranging from as low as 54% (Katakura et al., 1998) to 70%–85% (Osman et al., 1997; Hu et al., 2000; da Silva et al., 2004). However, this discrepancies have been attributed to differences in the volume of blood tested in the PCR (Osman et al., 1997), to differences in the timing of sample collection (Adhya et al., 1995; Osman et al., 1997), or to differences pertaining to the species of *Leishmania* parasites, some of which may indeed circulate less frequently or at lower levels in the infected host (Osman et al., 1997). It has been reported that PCR assay could detect parasitemia before the appearance of any clinical signs or symptoms i.e. asymptomatic infections have been associated with high PCR positivity (Fakhar et al., 2008; Bhattarai et al., 2009). Recently, Martin-Sanchez *et al* using PCR-ELISA found 24 per cent asymptomatic individuals carrying *Leishmania* kDNA in their blood (Martin-Sanchez et al., 2004). The PCR is also appropriate where transfusion transmitted leishmaniasis is a potential threat in transfused patients in whom serology has limited role (Otero et al., 2000). The reported sensitivities and specificities of different PCR protocols in the diagnosis of visceral leishmaniasis are tabulated 2.3.

Real-time PCR (modification of conventional PCR) can be used qualitatively and quantitatively to determine the parasite load in given specimen (Wortmann et al., 2001; Monroy-Ostria and Sanchez-Tejeda, 2002). Recently, Bossolasco and coworkers examined 25 *Leishmania*-HIV-coinfected patients, who received liposomal amphotericin B and responded clinically, by real time PCR using SSU rRNA gene target (Bossolasco et al., 2003). This study showed that molecular diagnosis is very promising for monitoring therapy. New diagnostic tool named Reverse transcriptase-loop-mediated isothermal amplification (RT-LAMP), uses *Bst* DNA polymerase and six set of primers, has higher specificity and ten folds higher sensitivity than RT-PCR (Parida et al., 2004; Adams et al., 2010). The molecular diagnosis of leishmaniasis using PCR based array not only diagnose the disease but also helps in species, strain and genotype identification following modifications like enzymatic digestion of PCR products and resolution through various gradients such as agarose (PCR-RFLP) (Volpini et al., 2004), SDS-PAGE to check the single strand conformational polymorphism (SSCP) (el Tai et al., 2000), Southern hybridization (Schulz et al., 2003), multiplex PCR (Monroy-Ostria and Sanchez-Tejeda, 2002) or PCR sequencing (Schulz et al., 2003).

Table 2.3 Reported sensitivities & specificities of different PCR protocols in the diagnosis of VL.

Types of PCR (bp): *Leishmania* species	**Sensitivity of PCR, %**		**Specificity %**	**Study year**
	Whole blood	BMA		
MedRNA(180 bp): *L. donovani*	96.8	NR	100	(Adhya et al., 1995)
kDNA (nr): *L. donovani*	90[a]	NR	100	(Nuzum et al., 1995)
ssU-rRNA (603 bp): *Leishmania* species	72.7	100	100	(Mathis and Deplazes, 1995)
Repetitive nuclear sequence (140 bp): *L. infantum*	NR	84	99	(Piarroux et al., 1996)
ssu-rRNA (nr): *L. donovani*	98	98	100	(Costa et al., 1996)
kDNA (800 bp): *L. donovani*	92.5[b]	100	100	(Andresen et al., 1997)
ssU-rRNA (nr): Leishmania species	70[c]	100	100	(Osman et al., 1997)
n-PCR mini-exon gene (450 bp): *L. donovani*	NR[d]	83.3	100	(Katakura et al., 1998)
kDNA (204 bp): *L. donovani*	82.3	NR	100	(Singh et al., 1999)
ssU-rRNA (603 bp): *L. infantum*	97	100	100	(Lachaud et al., 2000)
ssU-rRNA (600 bp): *L. infantum*	75[e]	NR	NR	(Campino et al., 2000)
kDNA (297 bp): *L. donovani*	68.8	91	100	(Hu et al., 2000)
kDNA (792 bp): *L. donovani*	96	100	96	(Salotra et al., 2001)
ssU-rRNA (359 bp): *L. infantum*	100	100	100	(Pizzuto et al., 2001)
n-PCR (100 bp): L. infantum	100	100	100	(Fisa et al., 2002)
ssU-rRNA n-PCR (358 bp): *L. infantum*	95.4	100	100	(Cruz et al., 2002)
ssU-rRNA n-PCR (359 bp): *L. infantum*	100	100	100	(Cascio et al., 2002)
kDNA-Elisa (nr): *L. infantum*	100	67	100	(Martin-Sanchez et al., 2002)
kDNA (120 bp): *L. chagasi*	91	NR	100	(Disch et al., 2003)
nDNA (250 bp): *Leishmania* species	86	93	100	(Deniau et al., 2003)
ssU-rRNA real-time: *L. infantum*	100	NR	100	(Bossolasco et al., 2003)
kDNA (139 bp): *L. infantum*	NR[f]	NR	95	(Fissore et al., 2004)
kDNA (447 bp): *L. infantum*	NR	100	100	(Cortes et al., 2004)
ssU-rRNA (215 bp): *Leishmania* species	93.3 for Ic &100 for Id	NR	100	(Gatti et al., 2004)
kDNA (790 bp): *Leishmania* species	100	83	100	(Pal et al., 2004)
kDNA (120 bp): *L. chagasi*	84.7	100	100	(da Silva et al., 2004)
ssU-rRNA	73.2 (conventional PCR), 83.9 (ELISA PCR)	NR	87.2	(De Doncker et al., 2005)
kDNA (600 bp): L. donovani	99	NR	100	(Maurya et al., 2005)
ssU-rRNA n-PCR (358 bp): *L. infantum*	79	100	100	(Cruz et al., 2006)

Note: BMA, bone marrow aspirate; bp, base pair; Ic, immunocompetent patients; Id, immunodepressed patients; kDNA, kinetoplast DNA; MedRNA, multicopy mini-exon RNA; n-PCR, nested PCR; NR, not reported; ssU-rRNA, small subunit ribosomal RNA

[a] Sensitivity of PCR of whole blood specimens was 80% for Brazilian patients, 90% for Indian patients and 100% for Kenyan patients, [b] Sensitivity of PCR of lymph node samples was 100%, [c] Sensitivity of PCR of lymph node sample was 86.8%, [d] Sensitivity of PCR of buffy coat was 53.8%, [e] Overall sensitivity was 95.5%, [f] Sensitivity of PCR of serum was 97% [Source: (Antinori et al., 2007)]

2.1.4.1 Nested-PCR

Nested PCR is a modification of the polymerase chain reaction that uses two pairs of PCR primers to amplify a target DNA. The first pair of PCR primers (green) will amplify a fragment similar to a standard PCR. However, a second pair of primers (red) called nested primers (as they lie/are nested within the first fragment) binds inside the first PCR product to allow amplification of a second PCR product which is shorter than the first one. The advantage of nested PCR is that if the wrong PCR fragment (unwanted products of primer dimers, hairpins, and alternative primer target sequence) was amplified, the probability is quite low that the region would be amplified a second time by the second set of primers. Thus, Nested PCR is a very specific PCR amplification.

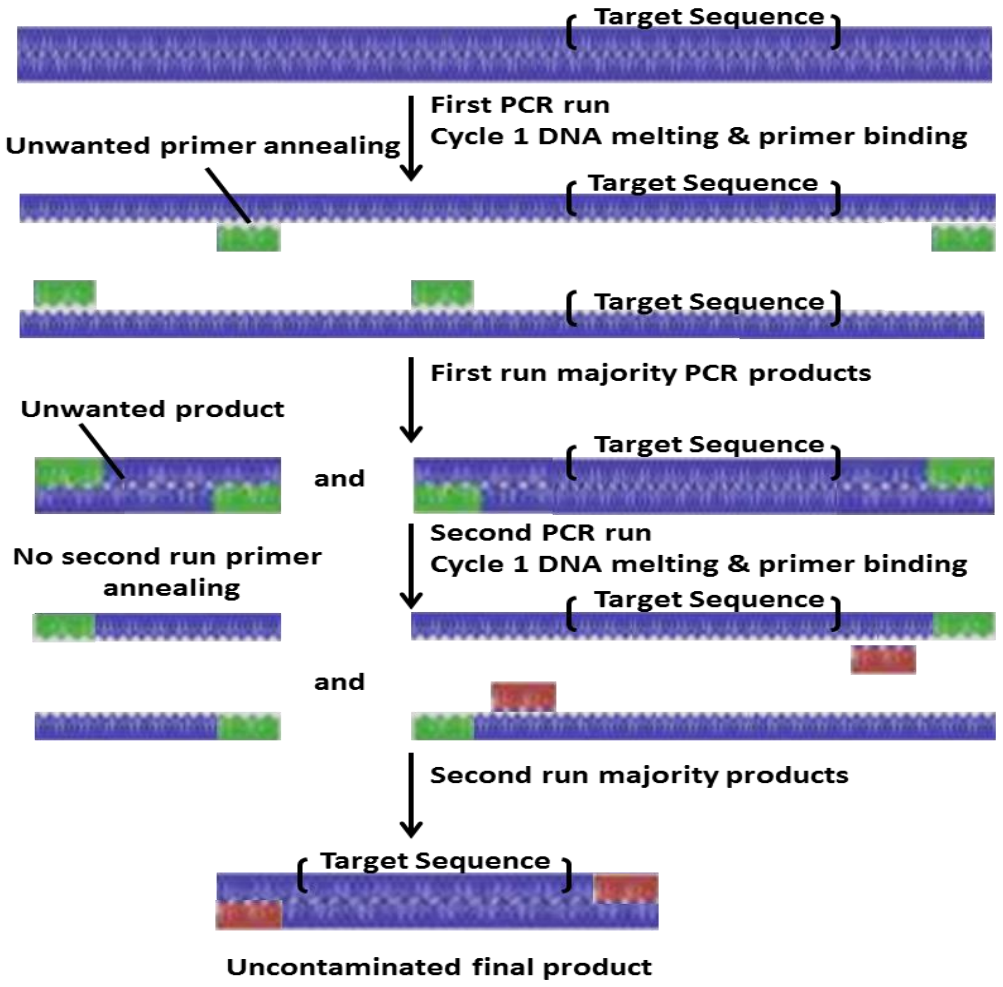


Fig.2.3 Diagram illustrating the process of nested PCR (Wheeler, 2005).

Leishmania are one of several genera within the family Trypanosomatidae and are characterized by the possession of kinetoplast kDNA, a unique form of mitochondrial DNA

(Singh et al., 2005). The kinetoplast, an organelle unique to the kinetoplastids, contains approximately 10,000 small circular DNAs known as kDNA minicircles, which are between 600 and 800 bp in size in members of the genus *Leishmania,* and maxicircle (20 t0 50 per associate) molecules (Simpson, 1986). Kinetoplast minicircles code for guide RNAs that are involved in editing the mitochondrial genes of trypanosomatids (Simpson, 1997). The 10,000 kinetoplast minicircles are distributed among about 10 different sequence classes. Within each minicircle class, sequences may vary by 1 or 2%. The number of minicircles in each class is very variable (Barker, 1987). The minicircle is divided into an approximately 120-bp conserved region and an approximately 600-bp variable region. The conserved region contains shorter blocks that are conserved throughout the genus *Leishmania* and in some other trypanosomatids as well. There are three highly conserved blocks (CSB): CSB1 (GGGCGT), CSB2 (CCCCGTTC), and CSB3 (GGGGTTGGTGTA) (with interspecies homology of 90 to 100%). CSB1 and CSB3 are almost identical in all species investigated to date, whereas CSB2 is less universal (Ray, 1989; Sheline and Ray, 1989). These conserved sequence blocks are ideal targets for PCR primers which can amplify all known minicircle classes from all *Leishmania* species (Barker, 1987; Ashford et al., 1995). The high copy number of the *Leishmania* minicircles makes them an ideal target for diagnostic tests. The heterogeneity of the variable region has been exploited to discriminate between strains of the same species. Digestion of the kinetoplast DNA with restriction enzymes yields fingerprint patterns that vary considerably within each *Leishmania* species. The fingerprint patterns themselves provide one of the most specific ways available to identify *Leishmania* strains (Angelici et al., 1989).

In the diagnosis of leishmaniasis, nested PCR has been developed to amplify the variable region of the abundantly found kinetoplast minicircles of all *Leishmania* species. The two sets of primers used are, external primers (targets CSB1 & CSB2) CSB2XF (C/GA/GTA/GCAGAAAC/TCCCGTTCA) and CSB1XR (ATTTTTCG/CGA/TTTT/CGCAGAACG) and internal primers (targets CSB3 & CSB1) LiR (TCGCAGAACGCCCCT) and 13Z (ACTGGGGGTTGGTGTAAAATAG)(Noyes et al., 1998). After completion of nested PCR, the PCR product is loaded on agarose gel to confirm the amplification of about 680bp fragment. As per the Noyes, Reyburn *et al* the nested PCR generated a strong signal from a minimum of 0.1 fg of *Leishmania* DNA. According to (Srivastava et al., 2010), the reported sensitivities and specificities of different targets are 82.3%-100% & 96%-100% for kDNA, 73.2%-100% & 87.%-100% for SSU-RNA, 100% & 100% for n-PCR (100bp) respectively. Cruz and coworkers reported that *Leishmania* specific nested PCR performed on blood samples was the technique of choice for diagnosis, monitoring the success of treatment and predicting relapses in patients with *Leishmania*–HIV coinfection (Cruz et al., 2002).

Limitations of molecular diagnosis

Albeit, PCR has certainly proved its merit in detecting *Leishmania* parasites being able to detect the equivalent of even less than one parasite per sample (Salotra et al., 2001), it has limitations. For example, PCR might pick dead parasitic DNA leading to wrong interpretation as ongoing infection, highly sophisticated technique, prohibitive and evaluation of several primers developed is needed before these can be employed for routine diagnosis.

2.2 Vaccine candidate

Most individuals who were once infected with *Leishmania* are resistant to clinical infections, when later exposed. An ideal antileishmanial vaccine need to possess following characteristic, such as; (ii) safety (ii) affordability, (iii) induction of robust CD4+ and CD8+ T cell responses and long term immunological memory, (iv) cross-species effectiveness against cutaneous and visceral forms, (v) stability at room temperature and (vi) effectiveness as a prophylactic and therapeutic vaccine (Kedzierski et al., 2009).

Different trials have been made on vaccine development with different approaches *viz.* whole parasite (killed or irradiated), soluble extracts, crude or pure surface antigen, synthetic peptides, amastigote antigen, plasmid DNA, recombinant antigens and immunostimulating complexes (Tewary et al., 2004; Basu et al., 2005). Different generations of vaccine against leishmaniasis are grouped into followings:

2.2.1 First generation vaccine

Live attenuated vaccine

Live-attenuated vaccines induce long lasting immunity to re-infection by maintaining a low level of asymptomatic infection but their reactivation in immunocompromised state leads to disease development. There are different processes for attenuation of live parasites *viz.* long term in vitro culture, using temperature sensitivity, ϒ-irradiation, chemical mutagenesis or by culturing under gentamycin pressure (Daneshvar et al., 2003).

Killed parasite vaccines

Studies in Iran with one dose of autoclaved *L. major* vaccine, was found to be safe and immunogenic (Sharifi et al., 1998; Momeni et al., 1999). Heat killed antigen produced higher degree of protection in mice with *L. donovani* infection showing significant parasite load

reduction (69% to 76% and 59% to 64% in the liver and spleen respectively) (Nagill et al., 2009).

2.2.2 Second generation vaccine

Vaccines of *Leishmania* fractions

Partially or fully purified *Leishmania* sub-fractions have been widely used in experimental models (Gradoni, 2001; Handman, 2001) owing to their excellent immunoprotective properties. Previous studies showed that vaccination with fucose-mannose ligand (FML) protects against experimental VL in several animal models such as mice and dog (Santos et al., 2002; Parra et al., 2007). Additionally, membrane antigens of *L. donovani* promastigotes (LAg), immuno-stimulatory sequence bearing non-coding plasmid DNA co-entrapped in liposomal (Soluble leishmanial antigen) SLA and (F2) fraction of *L. donovani* showed immuno-protective properties (Das and Ali, 2012).

Subunit protein vaccine

Several molecularly defined vaccine candidates have been evaluated against VL. LPG, gp63, nuclear histone protein (H2B), purified recombinant ORFF (Open Reading Frame) protein, ribosomal PO protein, *Leishmania* elongation factor 2 (LelF-2), amastigote cysteine proteases, hydrophilic acylated surface protein B1 (HASPB1), LEISH-F1(formerly Leish111f)(Coler et al., 2007) etc. are few of the subunit protein vaccines that have been tested against different forms of leishmaniasis with varying success.

Dendritic cell (DC)-based vaccines

DC-based antileishmanial vaccines comprise a novel yet nascent strategy for long lasting immunity. Generation of parasite-specific Th1 biased long term protection by plasmacytoid DC-based vaccination against *L. major* (Remer et al., 2007) provided the thrust for targeting DC vaccines against VL. Adoptive transfer of DCs pulsed *ex vivo* with soluble *L. donovani* Ags (SLDA) to naive mice induced the Ag-specific production of IFN-γ (Ahuja et al., 1999).

2.2.3 Third generation vaccine: DNA vaccine

DNA vaccination is the direct injection of plasmid DNA encoding foreign proteins that leads endogenous protein biosynthesis and cause specific immune response against it. Such vaccines elicit humoral, $CD4^+$ and $CD8^+$ T cells immune response and further modulated by addition of

cytokines and / or CpG oligonucleotides. gp63, *Leishmania* analog of the receptor kinase C (LACK) and ORFF are the most extensively studied DNA vaccine candidates against VL till date.

Genetically altered Live-attenuated vaccine

Vaccination with genetically modified *Leishmania* parasite is based on decoupling virulence for induction of protective immunity. This generally involves targeted disruption of virulence or essential leishmanial genes resulting in mutated parasites lacking virulence. These mutants closely mimic the natural course of infection through PAMPs (Pathogen-associated molecular patterns) required for adequate stimulation of innate immunity for long-term immune memory. Recently, *L. donovani* centrin null mutants (LdCen−/−) have been reported to protect BALB/c mice and Syrian hamsters against homologous as well as heterologous infectious challenge (Selvapandiyan et al., 2009).

2.3 Immunological Perspective of Leishmaniasis

Entrance of *Leishmania* promastigotes into the skin dermis of human starts natural *Leishmania* infection. In order to survive, the parasite must resist exposure to host serum component and destruction by innate immune cells present in or promptly recruited to the skin. The skin is a complex immunological organ in which multiple innate immune cells [Keratinocytes, Langerhans cells, Dendritic cells, Macrophages, Mast cells, Neutrophils polymorph nucleated (PMN) cells and NK cells] function to protect the host from pathogens. A substantial number of T cells, nearly twice that are present in the circulation (Clark et al., 2006), play an important role in the local response.

2.3.1 Humoral Immune response

Complement system is the major effector of the humoral branch of the immune system. During early transmission period, human complement exerts strong selective pressure on parasite survival and *Leishmania* parasite tends to enter a permissive monocyte rapidly to avoid lysis. Macrophage tissues and Langerhans cells take up *Leishmania* by phagocytosis using a variety of ligands on the parasite surface including Fc , C3b, Fibronectin receptors, gp63 and LPG (Russell and Talamas-Rohana, 1989). With the entry of promastigotes in serum, they are subjected to opsonization followed by binding with Fc or C3b receptors. This process lyse the parasite but metacyclic promastigotes are resistant [due to increased thickness and structural

modifications of LPG (Puentes et al., 1990)] and use the process to be internalized inside macrophages. Residing in phagolysosome of macrophage, parasite escapes the humoral immune response and consequently antibodies have no effect on the *Leishmania* and so host suffers (Cunningham et al., 2001).

Hypergammaglobulinemia (IgG and IgM) against various non-specific proteins and haptens are the remarkable features in Leishmaniasis (Awasthi et al., 2004). This hyperimmunoglobulin condition in VL is a result of an elaborated T cell activation induced by cytokines that switches B lymphocytes to several IgG isotypes leading humoral response. Similarly, the elevated levels of IgG, IgM and IgE (Ryan et al., 2002) are recorded in VL patients sera.

2.3.2 Cell mediated Immune response

Visceral leishmanaisis is characterized by suppression of cell mediated immune response. The first cellular immune response is elicited by Polymorphonuclear neutrophils (PMNs) which are short lived and go under spontaneous apoptosis (Squier et al., 1995). This apoptosis is delayed by *Leishmania* from 6-10 hours to 2-3 days (Aga et al., 2002). The Chemokine MIP-1α and MIP-1β secreted by the neutrophils recruit the monocytes and macrophages (Scapini et al., 2000). The macrophages ingest the infected PMNs and internalize the parasite to form parasitophorous vacuole. Then the macrophages are activated and kill the ingested *Leishmania* parasite presumably mediated by toxic metabolites of oxygen (Assreuy et al., 1994).

T cells, which can differentiate either in Th1 or Th2 type of effector cells, plays major role in generating specific & memory T cell response in intracellular parasitic infection. The *Leishmania* specific CD4+ T cells play a role to produce Th1 or Th2 cytokines for priming (Tanaka et al., 1993). The protective (Th1) and non-protective (Th2) immune response against *Leishmania* depends on the type of antigen recognized by the T cells (Sundar et al., 1997).

T reg cells are important in the pathogenesis of *Leishmania* infection (Aseffa et al., 2002; Hori et al., 2002). Treg cells are divided into adaptive T reg and natural T reg cells. The adaptive T reg cells that arise from CD25-Foxp3-T cells are antigen driven and can produce large amount of IL-10. They are more important than the natural T reg cells in the suppression of anti-leishmanial immunity in human VL. Interleukin-10 production by splenic $CD4^{+}$CD25-Foxp3-cell, associated with disease progression in *L. donovani* infected mice, has also been reported (Stager et al., 2006).

2.3.3 Cytokine response

The Th1/Th2 cells polarization with distinct pattern of cytokine profile are studied in murine models (genetically predisposed) infected with *L. major*. This differentiation of CD4 cells i.e. Th cells to Th1 or Th2 type of effector cells is based on priming during differentiation (Seder and Paul, 1994). $CD4^+$ Th1 which produce IFN-ϒ/IL-2 are associated with resistance to parasite survival whereas IL-4/IL-10 produced by CD4 Th2 cells is not host protective and make susceptible to the infection (Heinzel et al., 1989).

In most intracellular parasite infections, macrophages produces IL-12 along with other cytokines (IL-6, IL-18, IFN-ϒ and TNF-α) for Th1 type immune response. IL-18 is another proinflammatory cytokine that helps in evoking Th1 immune response particularly in collaboration with IL-12 (Tsuji-Takayama et al., 1999). IL-10, the Th2 cytokine, plays role in promoting disease probably by conditioning host macrophages for enhanced survival and growth of the parasite (Nylen and Gautam, 2010). IL-10 can render macrophages unresponsive to activation signals an inhibit killing of amastigotes by down-regulating the production of TNF-α and NO (Nylen and Gautam, 2010).

Chapter III

Materials and Methods

3.1 Selection of study site

Two different sectors (field and hospital) were chosen for the collection of blood samples. In the field sector, Khanar Village Development Committee (VDC) of Sunsari district was chosen, from where 53 randomly selected research participants were enrolled for the collection of blood. Similarly 22 and 25 blood samples were taken from the Bharatpur Hospital (Bharatpur, Chitwan) and District Health Office (Jaleshwor, Mahottari) respectively. The selected sites for sample collection are shown in the map of the respective districts (Fig. 3.1).

a b c

Fig. 3.1 Selected districts for sample collection (a) Bharapur Hospital, Chitwan (b) District Health office, Jaleshwor, Mohattari and (c) Khanar VDC, Sunsari. The red bubble labeled with alphabet A denotes the site for collection of sample. (Source: Google map 2013). The selected districts i.e. Sunsari and Mohattari were defined as endemic districts based on Pun *et al*., 2011. While Chitwan as buffer endemic district because this district lies at the juncture of endemic and non-endemic districts of Nepal for Kala-azar.

3.2 Collection and Transportation of Blood sample

For blood collection, the research participants satisfying the inclusion criteria (healthy donors, relatives or neighbors of VL patients living in sampling site) and exclusion criteria (having HIV, tuberculosis and other diseases, patients under long-term corticosteroid and immununo-suppressive therapy, below 10 years of age and pregnant woman) were clearly defined. After consent of hospital and a signed consent of research participant (RP), 3ml of

blood was withdrawn from the RP satisfying the inclusion criteria with the help of expert clinician using sterile syringe and was transferred to BD Vacutainer^{TM} (UK) followed by gentle shaking to mix with anticoagulant. Collected blood was then transported (in cold chain) to the laboratory of Central Department of Biotechnology and stored at 4^{o}C until down streaming processes.

3.3 Epidemiological study

Distributions of asymptomatic VL cases were studied in different analytical grounds.

1. Sampling site based epidemiology

In this analytical ground, research participants were divided into two different categories i.e. Field based category and Hospital based category.

2. Endemic region based epidemiology

The research participants were distributed into three different categories i.e. Non-endemic, Buffer-endemic and Endemic backgrounds based on the endemicity of the respective districts.

3. Gender based epidemiology

In gender based epidemiology, research participants were divided into male and female categories.

4. Age based epidemiology

Regarding age based epidemiology, research participants were divided into five different age groups i.e. Adolescent (11-20 yrs.), Youth (21-30 yrs.), Adult (30-40 yrs.), Elderly adult (41-50 yrs.) and Pre old (51-60 yrs.).

3.4 Processing of blood samples

As soon as possible, the collected blood samples were centrifuged at 3000 rpm (Bench-Top Centrifuge, Niive, NF400) for 10 min at room temperature to separate blood cells and serum. With the help of micropipette serum fraction was taken out into labeled microcentrifuge tube and stored at -20^{o}C for serological analysis. Likewise, approx. 200µl of white WBC ring was removed gently and headed to DNA extraction immediately to avoid fragmentation of DNA.

3.5 Extraction of DNA using Invitrogen PureLink™ Genomic DNA Mini kit

All the DNA extractions were carried out based on the methodology described on PureLink DNA Mini kit (Invitrogen, 2007).

3.5.1 Preparation of WBC lysate

For lysate preparation, 200µl of buffy coat i.e. white WBC ring was added to a sterile microcentrifuge tube followed by the addition of 20µl of Proteinase K and RNAse A respectively. Then the tube was briefly vortexed to ensure proper mixing and incubated at room temperature for 2 minutes. After incubation, 200µl of PureLink™ Genomic Lysis/Binding buffer was added to the tube and mixed well by vortexing. Then the tube was incubated at 55°C for 10 minutes to promote protein digestion. Finally, 200µl of 96-100% ethanol was added to the lysate, mixed well (vortexed for 5 seconds) and proceeded immediately to DNA binding.

3.5.2 Purification using spin column

Binding DNA

Approximately 640µl of the prepared lysate was transferred to PureLink™ spin column and centrifuged (Eppendorf Centrifuge 5418, Germany) at 10,000×g for 1 min at room temperature. The collection tube was discarded and spin column was placed into a clean PureLink™ Collection tube.

Washing DNA

Washing of DNA was carried out by adding 500µl of Wash Buffer 1 (prepared with ethanol) to the column and centrifuged at 10, 000×g for 1 min at RT. Then the collection tube was discarded and spin column was placed into fresh clean PureLink™ collection tube. Again 500µl of Wash Buffer 2 (prepared with ethanol) was added to the column and centrifuged at maximum speed for 3 minutes at RT. The collection tube was discarded and proceeded to eluting DNA.

Eluting DNA

For DNA elution, the spin column was placed in a sterile 1.5ml microcentrifuge tube and 150µl (recovers more than 90% DNA) of PureLink™ Genomic Elution Buffer was added (recovers more than 90% DNA). Then the column was incubated at 13,000 rpm for 1 min at RT to get purified genomic DNA.

Storing DNA

The purified DNA was stored at -20^{o}C into aliquots (to avoid repeated thawing and freezing) until PCR.

3.6 Quantification of DNA

The concentration of DNA of the given sample was determined because subsequent PCR requires particular amount of DNA for optimal performance. There are different methods like spectrophotometric quantification, UV fluorescence in the presence of DNA dye, etc. to determine the concentration of DNA. In this research study, spectrophotometric method (based on maximum absorption of UV by heterocyclic ring at 260nm) using quartz cuvette was employed to quantify DNA. First the spectrophotometer (Thermo Fisher Scientific, Genesys 10-S) was blanked using 490µl of distilled water plus 10µl of Elution Buffer. Then 10µl sample DNA was diluted in 490µl of distilled water and ODs at 260nm and 280nm were taken. Likewise, all the samples were measured and quantified using the relation:

$$\text{Concentration of DNA (µg/ml)} = 50 \times A_{260} \times \text{dilution factor}$$

Theoretically, the optical density (OD) of 1 corresponds to approx. 50µg/ml DNA. In addition, purity of DNA was determined by taking the ratio of absorbance *viz.* A_{260}/A_{280}. Absorbance value 1.8, <1.8 or >1.8 implies pure, protein contaminated and RNA contaminated DNA respectively (Sambrook and Russell, 2001).

3.7 Diagnostic Nested-PCR

Standardized reaction parameters and thermal cycling profile, described by Noyes *et al*., 1998, were used for diagnostic Nested-PCR (Noyes et al., 1998).

First Round PCR (PCR1)

In order to carry out the PCR1, the workstation was wiped with alcohol and exposed to UV for 15 minutes. All the PCR reagents were placed on ice. Then PCR1 mix was planned in a sterile microcentrifuge tube with total volume not more than 600µl (corresponding to 20 microcentrifuge tubes) to avoid problem of homogenization. Appropriate amount of each reagent as mentioned in table 3.1 (DNA grade water, Buffer 10×Roche, 10mM dNTPs, forward primer CSB2XF (C/GA/GTA/GCAGAAAC/TCCCGTTCA), reverse primer CSB1XR (ATTTTTCG/CGA/TTTT/CGCAGAACG) and 5U/µl Taq polymerase (Roche) were added sequentially. Thus prepared mix was vortexed for 1 min and 28µl of this mix was dispensed in each PCR tubes. A tube labeled with negative control (NC) was closed, while 2µl of samples DNA (5 to 50ng) was added to respective labeled PCR tubes in a different

workstation. Similarly, 2µl of *L. donovani* DNA was added in next PCR tube labeled with positive control (PC). To conclude PCR1, all the tubes were spun for seconds and loaded into a thermocycler (Cleaver Science Ltd, GTC, USA) programmed as shown in table 3.2.

Table 3.1 Preparation of master mix for PCR1

PCR1 components	Per reaction	mix for 20 tubes
DNA grade water	22.2 µl	444 µl
Buffer 10× Roche	3 µl	60 µl
dNTP 10mM	0.5 µl	10 µl
Primer CSB2XF	1 µl	20 µl
Primer CSB1XR	1 µl	20 µl
Taq Roche 5U/µl	0.3 µl	6 µl
Template DNA	2 µl	-

Table 3.2 Thermal cycling profile for PCR1

Steps	Cycles	Temp.	Time
Initial denaturation	1	94°C	2 min
Denaturation	40	94°C	0.5 min
Annealing		54°C	1 min
Extension		74°C	1.5 min
Final extension	1	72°C	10 min
Hold		4°C	Until PCR2

Second Round PCR (PCR2)

Similar to PCR1, another defined workstation was cleaned and exposed to UV. Then PCR2 mix was prepared by adding appropriate amount of reagents [DNA grade water, Buffer 10×Roche, 10mM dNTPs, primer LIR (TCGCAGAACGCCCCT), primer 13Z (ACTGGGGGTTGGTGTAAAATAG) and 5u/µl Taq polymerase Roche] in sequence as shown in table 3.3. Then 27µl prepared mix was dispensed in each tube and 3µl of template DNA was added to the respective tubes in defined workstation. Then these tubes were also short spun for few seconds and loaded into a thermocycler (Cleaver Science Ltd, GTC, USA) programmed as shown 3.4.

Table 3.3 Preparation of master mix for PCR2

PCR2 components	Per reaction	mix for 20 tubes
DNA grade water	21.2 µl	424 µl
Buffer 10× Roche	3 µl	60 µl
dNTP 10mM	0.5 µl	10 µl
Primer LIR	1 µl	20 µl
Primer 13Z	1 µl	20 µl
Taq Roche	0.3 µl	6 µl
PCR1 product	3 µl	

Table 3.4 Thermal cycling profile of PCR

Steps	Cycles	Temp.	Time
Initial denaturation	1	94°C	2 min
Denaturation	40	94°C	0.5 min
Annealing		56°C	1 min
Extension		74°C	40 sec
Final extension	1	72°C	10 min
Hold		4°C	Indefinitely

All the PCR products were stored at 8°C until, agarose gel-electrophoresis.

3.8 Agarose gel-electrophoresis

Agarose gel-electrophoresis was used to confirm the amplification. For this, 1.5% of DNA grade agarose was prepared in 0.5% TAE buffer and casted on gel electrophoresis tank (BIORAD). Ten microliters of PCR2 products stained with EZ-Vision^{TM} THREE (AMRESCO) dye were loaded along with suitable molecular weight ladder (100bp, Euromedex). Then the gel was run at 50V for one & half hours till the dye reached to the end of the gel. Finally, the gel was observed under UV transilluminator (Major Science, Taiwan) to confirm the amplified fragments and photographed (Sony) to keep the record.

3.9 Parasite culture

Nepalese strain *Leishmania donovani* (JKP01/2011) parasite, isolated from Janakpur Zonal Hospital, (Aganja, 2011) was maintained *in vitro* in culture flask containing 5ml Complete Rosewell Park Memorial Institute (cRPMI-1640, Himedia) media supplemented with 15% FBS (Gibco, Germany), and antibiotics (gentamycin 20 µg/ml, streptomycin 100 µg/ml and penicillin 100u/ml) throughout the study. Regular scrutiny of the shape, size, structure and color of the media were done so as to maintain the culture in uniform condition.

Promastigotes: Promastigote culture was maintained as described elsewhere (Manandhar, 2008) in a BOD incubator at 26^{o}C in the same cRPMI media (p^{H} 7.2-7.4). Presence of flagella at anterior end, spindle shaped with tapering at the tail, high motility and centrally located nucleus with transversely placed kinetoplast toward anterior end are peculiar to promastigotes (Fig. 4.3 c) morphology.

Promastigote subculture: Parasites cultures were regularly done for regular maintenance and availability of parasites. Briefly, 100µl of a stationary phase parasite culture with concentration greater than 1.5×10^{7} parasites per ml was transferred aseptically to a new culture flask with 5ml fresh cRPMI-1640. The culture flask was incubated at 26^{o}C in BOD incubator for 7 days to harvest stationary phase promastigotes.

Amastigotes: For the amastigote culture, stationary phase promastigotes was added in cRPMI media acidified to p^{H} 5.5. Then the culture was incubated at 37^{o}C in a humidified CO_2 incubator (SHEL LAB) with 5% CO_2. After two days, axenic amastigotes characterized by round and oval shape, aflagellated and very feeble motility were observed under the microscope. The giemsa staining of amastigotes is shown in Fig 4.4 b.

Cryopreservation: For cryopreservation, equal volume of stationary phage promastigotes with count $1\text{-}2\times10^{7}$ promastigotes/ml was mixed well with 3% DMSO

(Merck) prepared in heat inactivated FBS. The resulting solution was divided into aliquots of 1ml in cryovials & the vials were immediately transferred to Mc. Frosty Can containing chilled isopropanol. Then the vials were kept at -20^{o}C for overnight and finally, stored at -40^{o}C until reviving.

Thawing: For defrosting, the cryopreserved parasites were thawed in lukewarm water (37^{o}C) till a piece of ice was left. The parasite suspension was poured into a sterile 15ml polypropylene tube containing 10ml cRPMI and mixed quickly by inverting and reverting for 3/4 times. The tube was then centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and parasites were re-suspended in 5 ml cRPMI. The content was transferred to culture flask and incubated at 26^{o}C in BOD incubator for 7 days to harvest stationary phase promastigote.

Counting of parasite: Counting of parasite was done under light microscope using Neubauer's chamber or hemocytometer (MARIENFELD, REF 0610010). Neubauer's chamber is a thick glass microscope slide with a rectanglular indentation engraved with a laser-etched grid of perpendicular lines consisting 9 large squares (further divided in 16 smaller squares) each measuring 1mm$\times$1mm in area and 0.1mm in depth equating to a volume of 1mm^3. To determine parasite count 10µl of homogenous culture was loaded in the counting chamber, covered with cover slip and observed under light microscope. The relation to determine the count is:

$$\text{Parasite number per ml} = \frac{\text{Total no. of parasite counted in 4 corner squares}}{4} \text{ X dilution factor X } 10^4$$

3.10 Preparation of aqueous DAT antigen

Aqueous DAT antigen was prepared according to procedure described by Jacquet *et al*., 2006. Late log phase promastigote was harvested at a concentration of 1-2$\times$10^7 parasites per ml by centrifuging at 4000 rpm for 20 minutes in cold condition. With Phosphate-glucoose-saccharose (PGS) buffer solution ($Na_2HPO_4.2H_2O$, $NaH_2PO_4.H_2O$, glucose, saccharose and TDW) (p^H 8 to 8.1), the pellet was washed twice followed by digestion of surface protein with 0.4% trypsin for 1 hour at 37^{o}C with gentle orbital shaking (100 rpm). Again the content was washed with PGS. A 1% solution (v/v) was prepared in cold PGS and fixed with 4% formaldehyde solution for 20 hours at 4^{o}C. Then the excess fixative was removed through repeated washing with PBS (p^H 7.2) and stained the organism with 0.025% coomassie brilliant blue (CBB) in PBS containing 0.034 M sodium citrate for 20 hours at 4^{o}C. Finally, 5$\times$10^7 parasites/ml suspension was prepared in formaldehyde-citrate saline and stored at 4^{o}C until DAT assay was carried out.

3.11 Direct Agglutination Test (DAT)

Direct agglutination test was carried out based on the methodology already described by Manandhar 2008 with brief modifications. Primarily labeling of DAT microtitre plate was done as A1: antigen control, row A2-A12: positive control, row B2-B12 for negative control, row C to G: samples and row H: other disease (for cross-reactivity). Then 50µl DAT diluent (0.1% sodium azide, 0.2% gelatin in 0.9% NaCl) with 2-mercaptoethanol (240 µl 2-mercaptoethanol in 30 ml DAT diluent) was loaded in all wells except column 2. In column 2, 100 µl of DAT diluent and 1µl serum of positive, negative, samples and other disease were added respectively to their respective well. Dilution was carried out using a multi-channel pipette by transferring 50 µl of content from column 2 to column 3, mixed three times, again 50 µl was transferred to column 4 and continued until column 12. From the last column, 50 µl extra content was discarded. To all the well of microtitre plate 50 µl of prepared liquid DAT antigen was added and covered with lid or parafilm. The microtitre plate was then incubated at ambient temperature overnight. At the end, the result was read according to the formation of mat.

3.12 Analysis of agglutination test

The agglutinations were observed in V-shaped microtitre plate, after incubation of aqueous antigen with sera. Agglutination shown by samples was scored deeming the agglutination shown by positive Kala-azar serum. The sample detected negative by nested-PCR was used as healthy control, while samples shown positive by the same nested-PCR were used for diagnostic confirmation. In addition, positive malarial serum sample was used to check if cross-reactivity persists. Recording of the result was done by photography with the help of camera (Sony).

The sensitivity and specificity of liq. DAT antigen prepared in Central Department of Biotechnology was calculated based on given relation Kumar et al., 2006.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100$$

3.13 Preparation of Cocktail Crude Soluble Antigen (CSA)

Two different forms of *L. donovani* cultures, promastigote at late-logarithmic phase of growth and amastigote culture were mixed in equal proportion and collected in falcon tube

(50ml). The cocktail culture was centrifuged at 4,000 rpm for 20 minutes in cold condition to harvest parasites. Then the pellet of parasite was washed four times in PBS and stored at -40^{0}C until use or was employed simultaneously to prepare cocktail CSA. The pellet was dissolved in approximately equal volume (0.7ml) of lysis buffer prepared from Complete Protease Inhibitor Cocktail (Roche). Four alternative cycles of frosting (-20^{0}C) and defrosting (room temperature) followed by sonication (Sonicator-LOBA life) for 10 cycles for 30 seconds each in cold chain were carried to lyse the pellet. Ultimately, the resultant suspension was centrifuged at 3,000 rpm at 4^{o}C for 20 minutes to collect the supernatant called Cocktail CSA.

3.14 Quantification of Cocktail Crude Soluble Antigen (CSA)

To determine the concentration of protein in Cocktail CSA, Bradford Assay (Bradford, 1976) was done. Bradford assay is a colorimetric method based on binding of a dye, Coomassie Brilliant Blue G-250. This binding shifts the absorption maximum of dye to blue. The absorbance of the solution was measured spectrophotometrically at 595nm and was proportional to protein concentration when compared to standard curve. In this method Bovine Serum Albumin (BSA) was used to draw a standard curve. For quantification, test tubes were labeled and 1mg/ml stock solution of BSA was prepared. The standard curve was generated by preparing different dilutions of BSA, adding 1ml of Bradford reagent in each test tube and measuring the absorbance at 595nm after 20 minutes of incubation (as shown in table 3.5). Similarly, 5µl of Cocktail CSA in 95µl TDW along with 1ml of Bradford reagent was used to measure its absorbance to calculate the protein concentration of Cocktail CSA.

Table 3.5 Plan chart for BSA concentration standard curve determination

Test tubes	BSA Stock solution (1µg/µl)	TDW (µl)	Bradford Reagent (ml)	Final volume (ml)
A	1	99	1	1.1
B	5	95	1	1.1
C	10	90	1	1.1
D	15	85	1	1.1
E	20	80	1	1.1
F	25	75	1	1.1
G	30	70	1	1.1
H	35	65	1	1.1
I	40	60	1	1.1
Blank	-	100	1	1.1

3.15 Western Blotting (WB)

Based on Laemmli, 1970, SDS-PAGE was carried out with minor modifications. Briefly for Western Blotting (WB), the Cocktail CSA (45µg/well) along with prestained protein molecular weight marker (Fermentas) were electrophoresed at constant current of 25mA on SDS-PAGE (12% resolving gel & 5% stacking gel) using running buffer (39mM Tris, 48mM Glycine, 0.1% SDS) for about 4 hours. Then the gel was transferred to chilled Transfer Buffer (Tris base 25mM, glycine 192mM, methanol 5%) for 10 minutes. Eight equal sized blotting papers and exactly the same sized nitrocellulose membrane (0.45 µm, amersham pharmacia biotech, England) were cut and dipped into the same buffer. The sandwich for transblotting was prepared by placing four blotting papers, gel, nitrocellulose membrane and again four blotting papers in sequence such that nitrocellulose membrane faced towards anode (+) while gel towards cathode(-). Then protein transfer was carried out in transblotting unit (Cleaver Scientific Ltd.) containing Transfer buffer in cold chain at 60V for two and half hours. The pre-stained molecular weight marker was used for protein transfer confirmation. The nitrocellulose membrane was incubated in blocking buffer (1% BSA, 0.1% Tween-20 in PBS) overnight. Next day, lane containing molecular weight marker was cut off and remaining membrane was cut into equal strips of about 5mm. Each strip was then incubated with sera (1:100 in PBS) of different samples for 2 hours at RT and washed thrice with washing solution (0.1% BSA, 0.1% Tween-20 in PBS) with an interval of 15 minutes each. After washing, each strip was allowed to interact with goat antihuman IgG (1:1200) conjugated with alkaline phosphatase for 2 hours at 100 rpm. Washing was repeated thrice: twice with Washing solution and once with PBS, each for 15 minutes. Color was developed using BCIP-NBT (Santa cruz biotechnology Inc.) as substrate until blue bands were seen and the reaction was stopped with PBS. Finally, the strips were allowed to dry, photographed after labeling and stored at RT. All the above described processes of immunoblotting were based on Towbin *et al*., 1999.

Chapter IV

Results

4.1 Surveillance

Blood samples were collected from three major foci of Nepal and were found to be belonged into three endemic regions as represented in the table 4.1. Chitwan district has been categorized into buffer endemic region as this district lies at the juncture of endemic and non-endemic region towards the western region of Nepal. Besides, very few cases of leishmanaiasis have been reported from this district.

Table 4.1. Total number of samples collected from different districts.

S.N.	Collection Site	District	No. of samples	Endemicity	Collection month
1	Khanar VDC	Sunsari	53	Endemic	June
2	Bharatpur Hospital	Chitwan	22	Buffer-endemic	October
3	District Health Office	Mahottari	25	Endemic	July
Total			100		

VDC : Village Development Committee.

4.2 Parasite culture

The cultures of Nepalese strain parasite (JKP01/2011) in BOD incubator observed under inverted phase contrast microscope represented well grown culture of promastigote of *L. donovani*. The passaged 1×10^7 parasites/ml in fresh culture media at each passaging was found proliferated to 1×10^8 parasites/ml in 7 days which was in a late-logarithmic growth or stationary phase. The uniform growth of promastigote was found in all culture flasks as exhibited by color change from pink to yellowish (Fig. 4.1). Similarly, the promastigote culture incubated in 5% CO_2 for 2 days transformed into round, aflagellate and oval shaped with very feeble motile axenic amastigotes. The parasites were observed in temporary and permanent slides [Fig. 4.1 (c) and 4.2 (b)]. The later was prepared by giemsa stain both for promastigote and amastigote stage of *L. donovani* as mentioned in material and methods. The slides are preserved in Central Department of Biotechnology, Tribhuvan University.

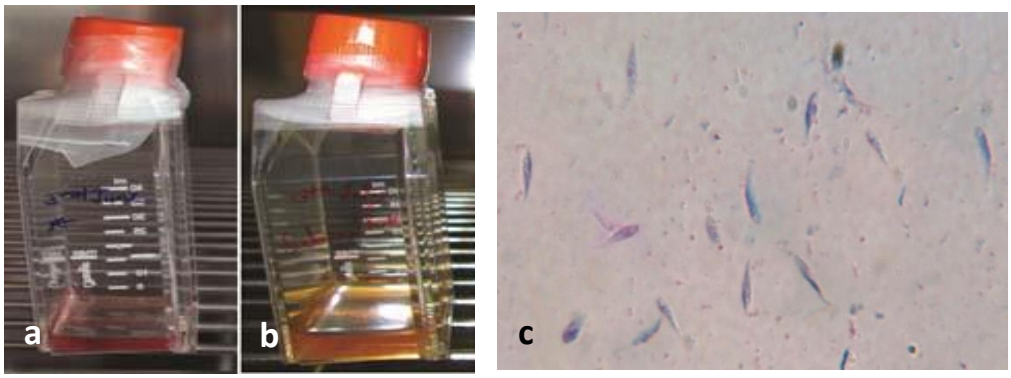


Fig. 4.1 (a) Lag phase promastigote culture (b) Stationary phase promastigote culture and (c) Giemsa stained slide of promastigotes (magnification 100X)

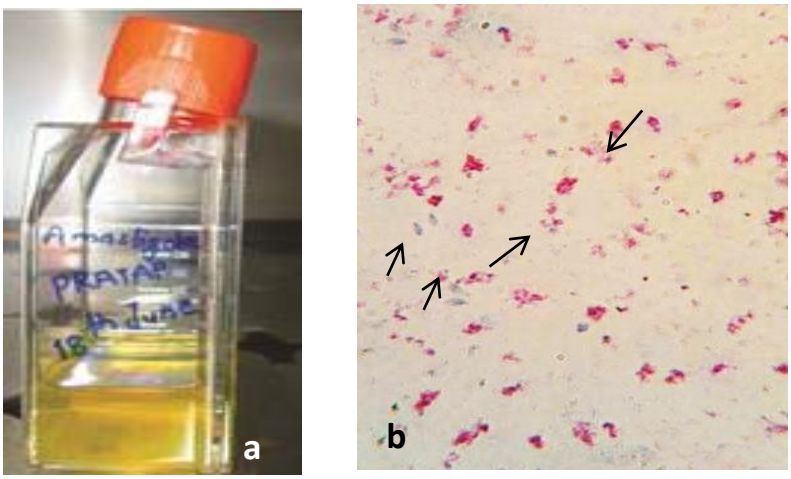


Fig. 4.2 (a) Stationary phase amastigote culture (b) Giemsa stained slide of amastigotes (magnification 100X)

4.3 Agarose gel electrophoresis

Distinct bands were observed in each extracted DNA samples after agarose gel electrophoresis on 1.5% DNA grade agarose prepared in 0.5X TAE. Very low or no contaminated DNA was extracted as shown by the absence of RNA bands [Fig. 4.3 (a)].

4.4 Quantification and Purity of DNA

The DNA extracted from blood samples using Genomic DNA Mini Kit (PureLink) was quantified and found between 10µg/ml to 190µg/ml, which were satisfactory yield. Those DNA samples having concentration higher than 50ng were diluted to the range of 5 to 50ng using elution buffer for PCR. According to Sambrook and Russel, DNA is pure enough when

$OD_{260/280}$ =1.8 (Sambrook and Russell, 2001) and the observed ratio of OD in the present investigation of 260nm and 280nm was close to 1.8 for majority of extracted DNA. The calculated concentration and purity of the DNA samples are tabulated below:

Table 4.2. Calculated concentration and purity of DNA samples from Khanar VDC, Sunsari.

SN	DNA sample code	Absorbance		Concentration (µg/ml)	Purity
		260nm	280nm		
1.	JB103	0.015	0.008	37.5	1.87
2.	U9110	0.023	0.012	57.5	1.91
3.	HK5	0.037	0.03	92.5	1.23
4.	Li102	0.029	0.016	72.5	1.81
5.	BiMa C-7	0.019	0.011	47.5	1.72
6.	MaRAC-3	0.028	0.02	70	1.4
7.	GoBd	0.011	0.006	27.5	1.83
8.	Pr C-2	0.027	0.015	67.5	1.80
9.	KhPr C-6	0.011	0.01	27.5	1.1
10.	BiBu C-5	0.014	0.013	35	1.07
11.	DbT C-2	0.036	0.02	90	1.8
12.	Du 202	0.02	0.017	50	1.17
13.	Ba 47	0.008	0.005	20	1.6
14.	Su 26	0.033	0.028	82.5	1.17
15.	Bibi C-4	0.017	0.009	42.5	1.88
16.	Ar 46	0.016	0.015	40	1.06
17.	Ar 48	0.031	0.037	77.5	0.83
18.	SbK C-5	0.008	0.007	20	1.14
19.	Sa Sh	0.01	0.006	25	1.66
20.	Ba 114	0.012	0.01	30	1.2
21.	NTM C-4	0.024	0.013	60	1.84
22.	11H5	0.01	0.009	25	1.11
23.	Si 127	0.017	0.01	42.5	1.7
24.	Ur 125	0.006	0.006	15	1.0
25.	68 Na	0.014	0.011	35	1.27
26.	Su Ra	0.007	0.008	17.5	0.87
27.	100An	0.01	0.006	25	1.66
28.	SaKa C-7	0.014	0.008	35	1.75
29.	PoBi C-7	0.004	0.003	10	1.33
30.	Am 7	0.019	0.017	47.5	1.11
31.	RoK C-6	0.043	0.04	107.5	1.07
32.	KH67	0.034	0.018	85	1.88
33.	13S4	0.042	0.025	105	1.68
34.	Rad 7	0.02	0.019	50	1.05
35.	Ni2	0.027	0.014	67.5	1.9
36.	Pr 9	0.03	0.016	75	1.87
37.	Ce 128	0.024	0.023	60	1.04
38.	Pu 199	0.021	0.02	52.5	1.05

39.	Ra 45	0.028	0.015	70	1.86
40.	Ri 28	0.011	0.006	27.5	1.83
41.	Kr 105	0.016	0.013	40	1.23
42.	Si15	0.021	0.019	52.5	1.10
43.	Ja32	0.006	0.004	15	1.5
44.	Di115	0.025	0.019	62.5	1.31
45.	TkBdr96	0.006	0.005	15	1.2
46.	Mu 95	0.016	0.012	40	1.33
47.	RuKaC-4	0.017	0.009	42.5	1.88
48.	Ra 78	0.004	0.003	10	1.3
49.	Ra 70	0.032	0.017	80	1.88
50.	Ma 73	0.027	0.024	67.5	1.12
51.	KA 77	0.029	0.019	72.5	1.52
52.	JA5	0.026	0.014	65	1.85
53.	TaMa	0.018	0.015	45	1.2

The rows with shedding were found as positive sample after n-PCR.

Table 4.3. Calculated concentration and purity of DNA samples from Bharatpur Hospital, Bharatpur, Chitwan.

SN	**DNA sample code**	**Absorbance**		**Concentration (µg/ml)**	**Purity**
		260nm	**280nm**		
54.	1SM	0.024	0.017	60	1.4
55.	2JM	0.036	0.024	90	1.5
56.	3MP	0.044	0.025	110	1.76
57.	4BB	0.030	0.016	75	1.87
58.	5SS	0.016	0.013	40	1.23
59.	6HT	0.015	0.008	37.5	1.87
60.	7JD	0.023	0.013	57.5	1.7
61.	8DB	0.012	0.006	30	2
62.	9SMM	0.014	0.012	35	1.16
63.	10CM	0.013	0.012	32.5	1.08
64.	11DS	0.016	0.009	40	1.77
65.	12SA	0.016	0.014	40	1.14
66.	13KT	0.015	0.012	37.5	1.25
67.	14TB	0.017	0.011	42.5	1.5
68.	15CK	0.015	0.008	37.5	1.87
69.	16DM	0.017	0.014	42.5	1.2
70.	17RL	0.011	0.006	27.5	1.83
71.	18US	0.026	0.014	65	1.85
72.	19AK	0.067	0.041	167.5	1.6
73.	20SLM	0.013	0.006	32.5	2.16
74.	21UD	0.014	0.01	35	1.4
75.	22UT	0.030	0.016	75	1.87

The rows with shedding were found as positive sample after n-PCR.

Table 4.4. Calculated concentration and purity of DNA samples from District Health Office, Jaleshwor, Mahottari.

SN	DNA sample code	Absorbance		Concentration (µg/ml)	Purity
		260nm	280nm		
76.	RA-1	0.023	0.013	57.5	1.76
77.	MK-2	0.01	0.008	25	1.25
78.	KS-3	0.076	0.062	190	1.22
79.	LD-4	0.022	0.012	55	1.83
80.	J23-5	0.025	0.019	62.5	1.31
81.	TW(12)-6	0.018	0.012	45	1.5
82.	RD-7	0.03	0.021	75	1.42
83.	PD-8	0.028	0.015	70	1.86
84.	RM-9	0.028	0.015	70	1.86
85.	MD-10	0.023	0.013	57.5	1.76
86.	SD-11	0.017	0.010	42.5	1.7
87.	J22-12	0.027	0.016	67.5	1.68
88.	UN-13	0.043	0.023	107.5	1.86
89.	AD-14	0.019	0.011	47.5	1.72
90.	FD-15	0.013	0.008	32.5	1.62
91.	MD-16	0.027	0.014	67.5	1.92
92.	GD-17	0.007	0.004	17.5	1.75
93.	J1-18	0.022	0.012	55	1.83
94.	J2-19	0.017	0.009	42.5	1.88
95.	J3-20	0.034	0.018	85	1.88
96.	UR-21	0.019	0.01	47.5	1.9
97.	BKR22	0.004	0.003	10	1.3
98.	J21-23 (A)	0.037	0.024	92.5	1.54
99.	F0D-24	0.032	0.017	80	1.88
100.	SuD-25	0.023	0.015	57.5	1.53

The rows with shedding were found as positive sample after n-PCR.

4.5 Diagnosis

Two types of very sensitive diagnostic techniques were used in this present investigation namely molecular and immunological as mentioned below.

4.5.1 Molecular diagnosis by Nested PCR

The collected samples from field and hospital were subjected to Nested-PCR along with positive (*Leishmania donovani* DNA) and negative control (no template). After nested-PCR, a sharp band sized about 680 bp were seen which represented positive to VL while absence of any bands represented negative as observed in negative control [Fig 4.3(b,c,d,e &f)]. In

Nested-PCR response, all the research participant's (n=100) DNA were subjected for nested-PCR. After agarose gel electrophoresis and observation under transilluminator 9% research participants (RP) were positive for Kala-azar and rest 91 % were negative to the disease [Fig 4.3 (b,c,d,e &f)].

The total numbers of research participants were studied for epidemiological analysis into five different analytical grounds i.e. sampling site based epidemiology, endemicity based epidemiology, gender based epidemiology and age based epidemiology.

4.5.1.1 Sampling site based epidemiology

Among the total 100 samples, the DNA samples with code U9110, Du202, Su26 and Ar48 were found positive as mentioned in table 4.3 and fig. 4.3. b & d. Hence, 7.55% (n=4 out of 53) healthy RP living in their native places without any complaint of VL were found VL positive. Similarly, 10.64% (n=5 out of 47) RP who came for treatment with different health complaints to the hospital were detected as VL positive. The DNA sample code of those RP found positive in the hospital setting were 16DM (Table 4.3 and Fig. 4.3 e) and PD-8, KS-3, LD-4 and RA-1 as shown in Table 4.4 & Fig. 4.3 f).

Table 4.5. Distribution of research participants into site based epidemiology.

SN	Site category	Research Participants				
		No. of Participants	No. of Positives	Positive %	No. of Negatives	Negative %
1	Field	53	4	7.55	49	92.45
2	Hospital	47	5	10.64	42	89.36

4.5.1.2 Endemic region based epidemiology

Regarding endemicity, the research participants (RP) were divided into three different endemic grounds i.e. non-endemic, buffer-endemic and endemic. Non-endemic regions included in this research were Nawalparasi and Darchula from where 2 and 1 research participants had participated in respective districts. None of the RP was found to be positive i.e. 100% were negative as showed by PCR. In the buffer-endemic region, the included district was Chitwan (categorized as buffer endemic because this district lies at the juncture of endemic and non-endemic region at the western region of leishmania endemic region of Nepal and very few cases of leishmanaiasis have been reported from this district) with 18 RP. None of them were noted as positive for Kala-azar i.e. 100% negative. Similarly, in endemic background the district included were Sunsari, Mahottari, Siraha and Parsa with 53, 24, 1 and 1 number of RP respectively.

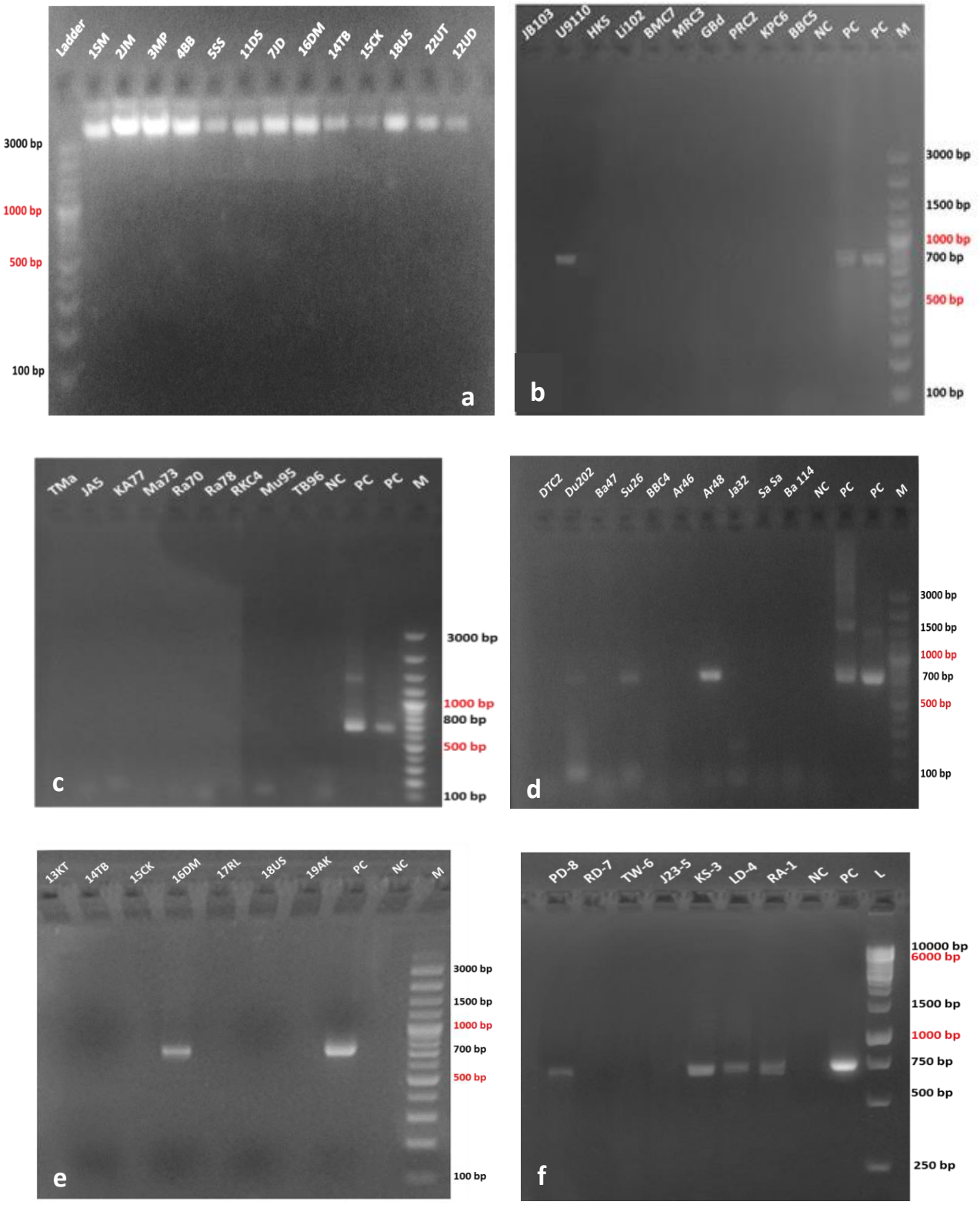


Fig. 4.3 (a) Agarose gel electrophoresis of extracted genomic DNA. Marker: 100 bp. Gel electrophoresis after Nested-PCR (b), (c) & (d) represents samples from Sunsari district, (e) represents samples from Chitwan and (f) represents samples from Mahottari district. NC; Negative control, PC; Positive control, M; 100 bp marker and L; 1Kb marker.

In total endemic samples (n=79), 9 (11.39%) were positive (Sample code U9110, Du202, Su26, Ar48, 16DM, PD-8, KS-3, LD-4 and RA-1) and 70 (86.61% of endemic) were negative for the disease (Table 4.6).

Table 4.6. Distribution of research participants in endemicity based epidemiology.

SN	Endemicity	Research Participants				
		No. of Participants	No. of Positives	Positive %	No. of Negatives	Negative %
1	Non-endemic	3	0	0.00	3	100
2	Buffer-endemic	18	0	0.00	18	100
3	Endemic	79	9	11.39	70	88.61

4.5.1.3 Gender based epidemiology

There was no statistical discrimination in the selection of research participants regarding gender issue. Male RP accounted for 47% while female RP accounted for 53% i.e. more females had participated in the research. As per the result shown by PCR, 8.51% (n=4 out of 47) of male RP were positive to Kala-azar and 9.45% (n=5 out of 53) of female RP were positive to the disease (Table 4.7.). These figures explain more females being exposed to the causative agent of the disease.

Table 4.7. Gender wise distribution of research participant

SN	Gender	Research Participants				
		No. of Participants	No. of Positives	Positive %	No. of Negatives	Negative %
1	Male	47	4	8.51	43	91.49
2	Female	53	5	9.43	48	90.57

4.5.1.4 Age based epidemiology

The RP were divided in five different age groups i.e. Adolescent (11-20 yrs.), Youth (21-30 yrs.), Adult (30-40 yrs.), Elderly adult (41-50 yrs.) and Pre old (51-60 yrs.), for evaluation. The data showed that highest (36) number of the RP fell into age group Youth (21-30) with 2.78 % were found VL positive and lowest (12) number of RP fell into age group Elderly adult (41-50) with 25% were found VL positive. Similarly, 8.33% (2 out of 24) of Adolescent, 13.33 % (2 out of 15) of Adult and 7.69% (1 out of 13) of Pre old age groups were detected to be positive for VL by nested PCR (Table 4.8.).

Table 4.8. Distribution of research participants into different age groups

SN	Age group	Research Participants				
		No. of Participants	No. of Positives	Positive %	No. of Negatives	Negative %
1	Adolescent (11-20)	24	2	8.33	22	91.67
2	Youth (21-30)	36	1	2.78	35	97.22
3	Adult (30-40)	15	2	13.33	13	86.67
4	Elderly adult (41-50)	12	3	25.00	9	75.00
5	Pre Old (51-60)	13	1	7.69	12	92.31

4.5.1.5 Summary Table

Table 4.9. Epidemiology of VL based on different analytical grounds

SN	Analytical ground		Research Participants				
			No. of Participants	No. of Positives	Positive %	No. of Negatives	Negative %
1	Sampling site	Field	53	4	7.55	49	92.45
		Hospital	47	5	10.64	42	89.36
2	Endemicity	Non-endemic	3	0	0.00	3	100
		Buffer-endemic	18	0	0.00	18	100
		Endemic	79	9	11.39	70	88.61
3	Gender	Male	47	4	8.51	43	91.49
		Female	53	5	9.43	48	90.57
4	Age	Adolescent (11-20)	24	2	8.33	22	91.67
		Youth (21-30)	36	1	2.78	35	97.22
		Adult (30-40)	15	2	13.33	13	86.67
		Elderly adult (41-50)	12	3	25.00	9	75.00
		Pre Old (51-60)	13	1	7.69	12	92.31

4.5.1.6 Follow up response:

None of those research participants (found positive in n-PCR) developed VL after follow up (phone interviewed).

4.5.2 Immunological Diagnosis

The antigen-antibody interactive immunodiagnostic techniques applied in this research work were DAT and WB. These tools are common in research aspects rather than clinical diagnosis.

4.5.2.1 Direct Agglutination Test (DAT)

About 50ml of *Leishmania donovani* culture (1×10^8 parasites per ml) was used to prepare the aqueous DAT antigen as described in materials and methods. Formaldehyde-citrate saline suspended parasite (maintained to 5×10^7 parasites/ml) used as aqueous DAT antigen showed the formation of mat formed due to the antigen-antibody interaction in confirmed patient serum. There was no agglutination developed as expressed by the formation of sharp end button on the bottom of v-bottom titer plate in VL negative cases. Deeming the agglutination reaction shown by Kala-azar serum (positive control), 4 samples i.e. RA1, PD-8, LD-4 and CS-3 showed agglutination (diffuse button formation at bottom) i.e. 44.44% of sera employed showed agglutination (Fig. 4.5 and 4.5), while other were negative as concluded by sharp end button formation as shown in Fig. 4.4. Further, sharp end button formation was found in the well containing sera of malaria patient interprets the non-cross reactivity of the produced aqueous antigen. The titre value from 1:200 was taken as positive cases. Referring to the cut off value 1:200, RA-1, PD-8, KS-3 and LD-4 samples were found VL positive with KS-3 crossed the titre value of positive control i.e. 1:800 serum dilutions. (Fig 4.4 and 4.6)

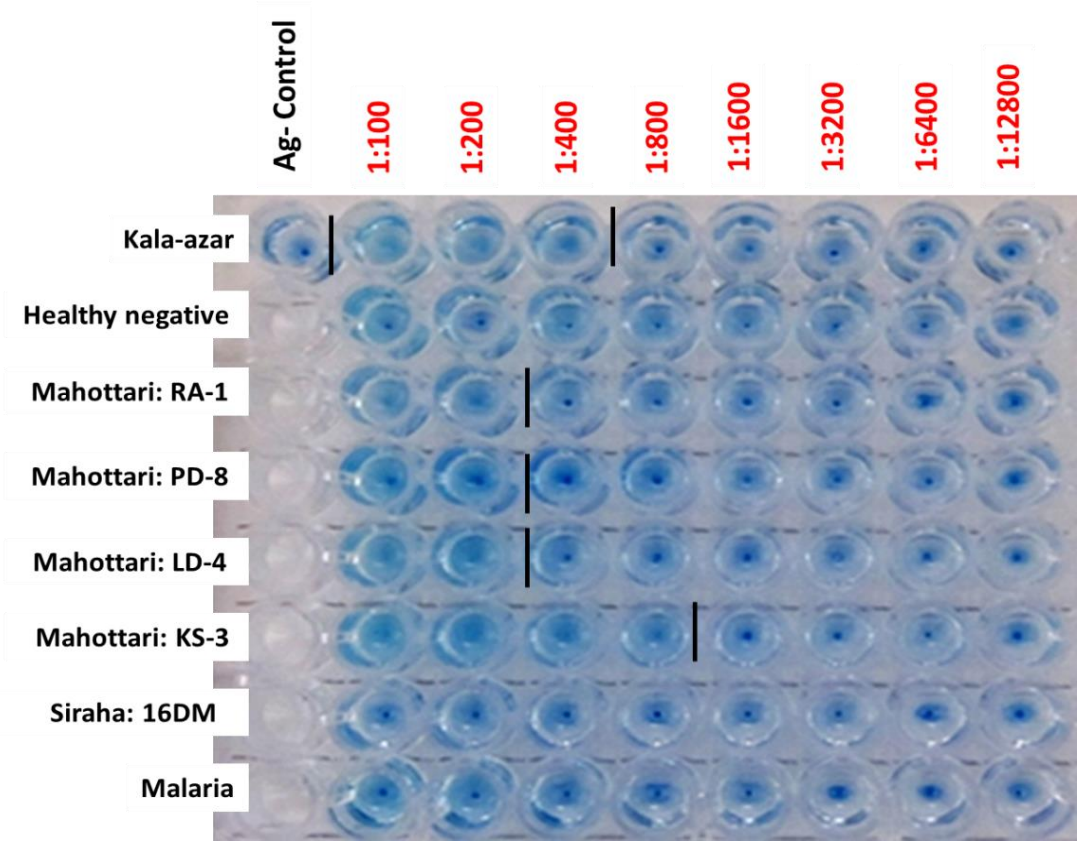


Fig. 4.4 DAT using aqueous DAT antigen and asymptomatic sera of nested PCR positive RP

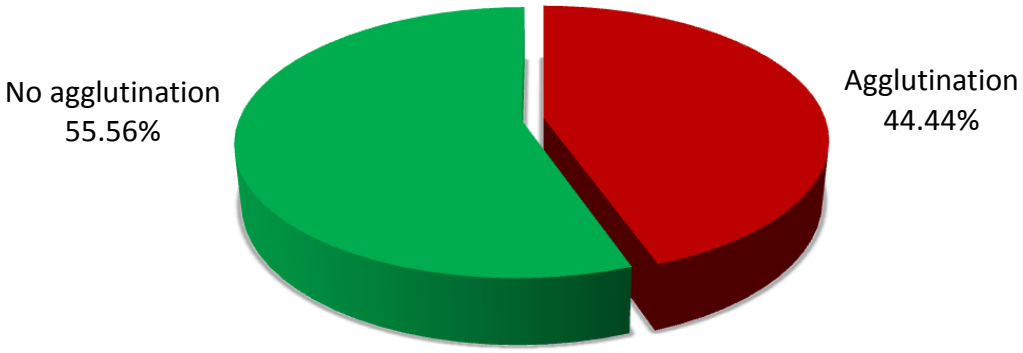


Fig. 4.5 Pie diagram illustrating the percentage of RP's sera showing agglutination, referring 1:200 as cut off value.

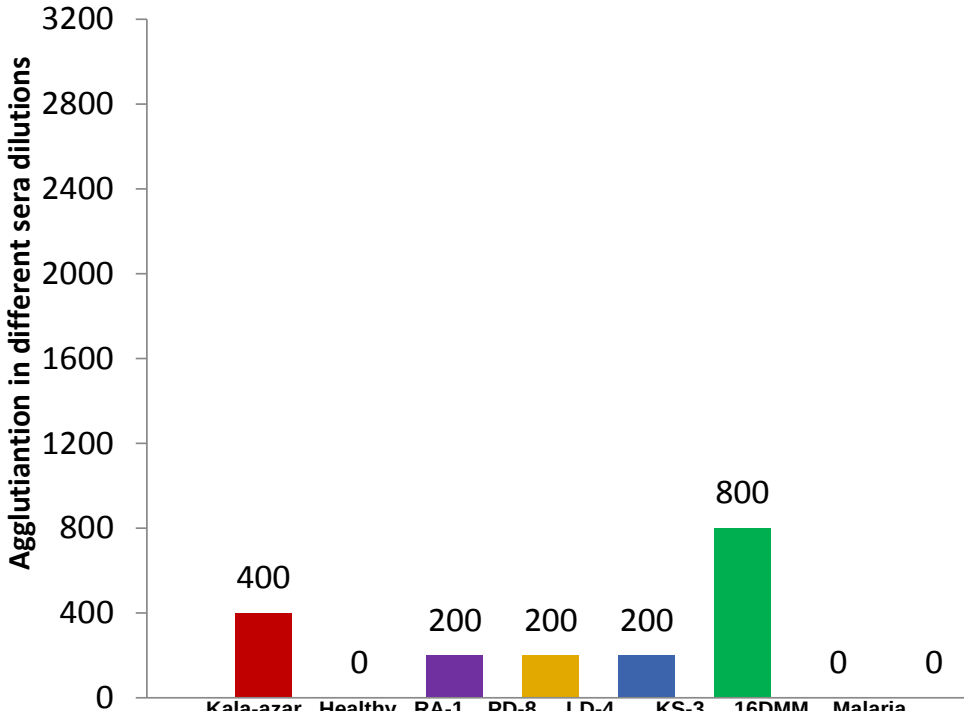


Fig. 4.6 Graphical representation of agglutination by different sera with their titre value.

4.5.2.2 Sensitivity and Specificity of DAT antigen

The sensitivity and specificity of aqueous DAT antigen was calculated using the relation given in materials and methods. Based on the agglutination of aqueous DAT by the sera of positive samples, the sensitivity of the prepared aqueous DAT antigen was found to be 44.44% when interpreted with cutoff titre 1:200. Similarly, the DAT antigen showed 100% specificity while using healthy negative serum.

Table 4.10. Sensitivity and Specificity of Direct agglutination test (DAT)

Serology	Direct Agglutination Test (DAT)	
	VL patient (n=9)	Healthy (n=1)
Positive	4	0
Negative	5	1
Sensitivity (%)	44.44	-
Specificity (%)	-	100

4.5.2.3 Collection and Quantification of cocktail CSA

Total 80ml stationary phase parasite cultures (40ml promastigote & 40ml amastigote) were collected during the research work and pelleted. The cocktail CSA collected from pellet was 1ml in total. The standard linear graph (Fig. 4.7) was obtained from Microsoft Excel 2010 (equation, y = 55.544x - 4.9084) using BSA as the standard solution. The 0.603 absorbance of prepared CSA at 595nm gave the concentration of protein as 28.54µg/10µl i.e. 2.854µg/1µl of CSA.

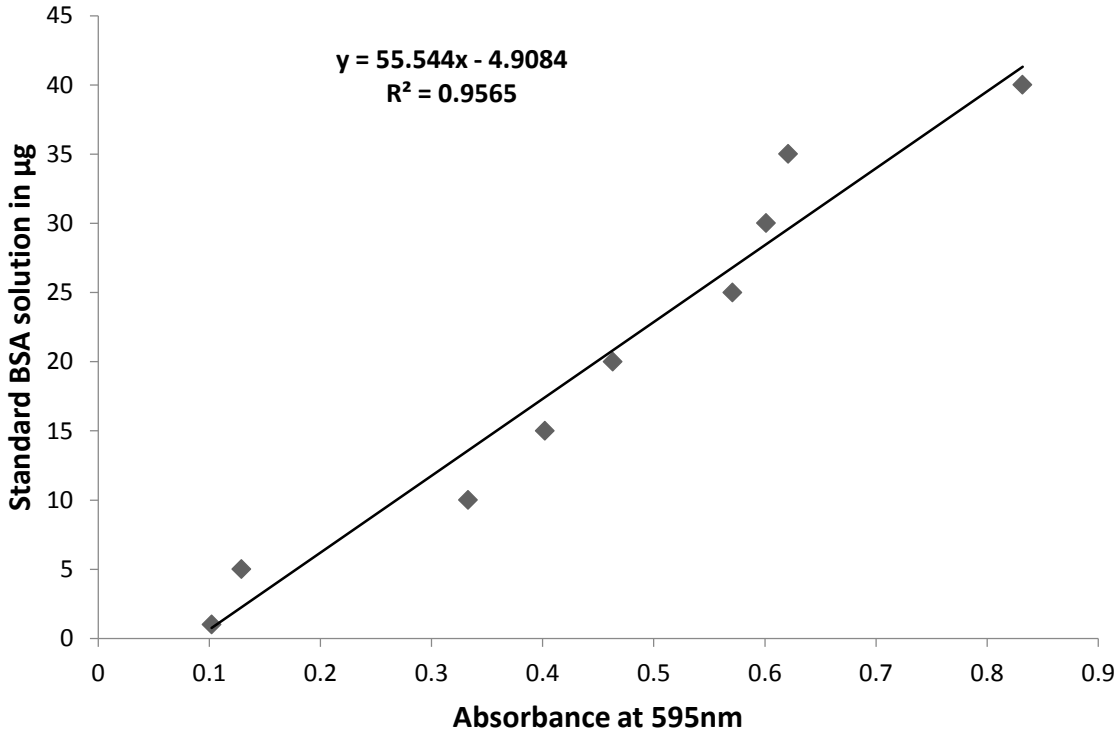


Fig. 4.7 Standard curve generated using different concentrations of BSA

4.5.2.4 Western blotting

The sera of RP which was positive to nested-PCR, when allowed to react with cocktail CSA, it produced bands after antigen-antibody interactions. Bands observed after immunoblotting were shown in the Fig. 4.8. Single band was observed in the sample RA-1 with molecular weight 16.26 kDa. Similarly, 5 bands sized 16.26, 18.27, 36, 64.61 and 71.26 kDa in sample KS-3 and two bands sized 21.36 and 25.3 kDa were observed as calculated based on the Rf value of marker and samples using Alpha Innotech (Version: 1.0.1.14) software (Table 4.10). However, in case of healthy negative and other samples (though shown positive by nested-PCR), no single band was observed.

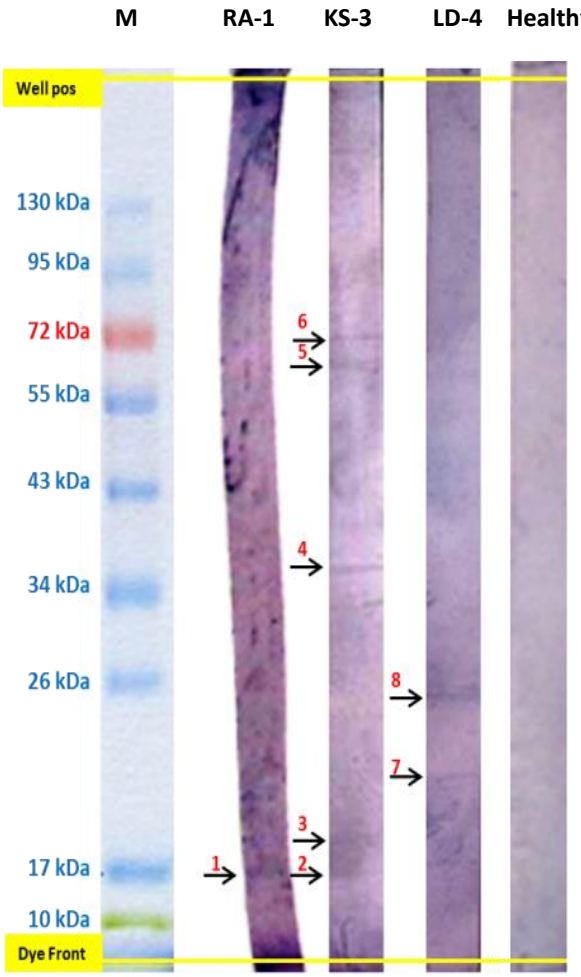


Fig. 4.8 WB profile for diagnosis of VL. Bands were shown by arrows. M: Protein marker. RA-1, KS-3, LD-4: Samples. The respective size of bands is given in table 4.11.

Table 4.11. Calculated Band position, Molecular weight and Retardation factor (Rf) of (a) Protein Marker (b) Samples using alpha innotech software.

(a)

Band	Position	Mol. Wt.	Rf
1	188	130	0.151
2	276	95	0.222
3	368	72	0.296
4	460	55	0.37
5	580	43	0.466
6	724	34	0.582
7	844	26	0.678
8	1100	17	0.884
9	1176	10	0.945

(b)

Samples	Band	Position	Mol. Wt.	Rf
RA-1	1	1108	16.26	0.891
	2	1108	16.26	0.891
	3	1064	18.27	0.855
KS-3	4	692	36	0.556
	5	408	64.61	0.328
	6	372	71.26	0.299
LD-4	7	976	21.36	0.785
	8	864	25.3	0.695

Molecular weight of bands were calculated by using Alpha Innotech software (Version: 1.0.1.14).

Chapter V
Discussion

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis accounting for an estimated 500,000 cases each year worldwide (Desjeux, 2001). After being first recorded in 1980 from Dhanusha district, VL has become endemic in 13 districts of terai lowlands with 8 million people at risk (Rijal et al., 2010). Asymptomatic forms to evident disease with subclinical forms are the sequel of VL infection. Nevertheless, symptomatic cases have prime role in transmission of disease, asymptomatic forms shouldn't be devalued since these acts as potential reservoir in transmission of VL (Sharma et al., 2000) and a proportion of asymptomatic infections may subsequently progress to clinical disease (Badaro et al., 1986). Asymptomatic forms ranging from 1:2.4 to 18:1 are depicted in various foci of the world (Zijlstra et al., 1994). Since the estimation of asymptomatic cases is difficult to access, a highly sensitive and specific test is mandatory. Invasive and risky technique involving demonstration of the parasite in stained preparations of bone marrow and splenic aspirate is the gold standard technique but is inappropriate for asymptomatic forms. Serological test have issues related to significant proportion of asymptomatic forms being positive and are unable to detect diseased or cured patient (Srivastava et al., 2011). Molecular techniques have been successfully used for diagnosis of symptomatic as well as asymptomatic VL. Besides, asymptomatic infections have been associated with high PCR positivity (Bhattarai et al., 2009).

With the aim to explore *Leishmania* infection among asymptomatic individuals in the context of anthroponotic VL, the present investigation has been carried out. Sampling was carried out from three selected districts i.e. Sunsari (Khanar), Chitwan (Bharatpur Hospital) and Mahottari (Distict Health Office) during higher sandfly density months (June to September). In Nepal vector density was observed to start rising from April, peaking in May and then decline from September to a negligible level in December (Das, 1998). All the representative districts of this study are endemic districts according to Pun *et al*., 2011. The reported incidence of the disease were 1.6 per 10,000 from Mahottari (Barnett and Rai, 2009) and 1.5 per 10,000 from Sunsari (Rijal et al., 2010) while one case and one death of patient was reported in 2003 from Chitwan (Joshi et al., 2006).

For molecular diagnosis, buffy coat was choice source for DNA extraction because *Leishmania* are obligate intracellular parasites of macrophage blood cells and therefore, are supposedly more concentrated in buffy coat. Extracted DNA after agarose gel electrophoresis followed by UV transillumination, elucidated sharp DNA bands in majority of extractions [Fig. 4.3(a)]. An absence of RNA bands depicted fairly uncontaminated DNA (from RNA) in the gel. In order to generate reproducible PCR result, quality and quantity of

template DNA have been considered as one of the major factor. Therefore, quantification of DNA were carried out using spectrophotometer, based on absorption of UV light by heterocyclic ring maximally at 260nm which showed adequate quantity of the extracted DNA for nested PCR (as n-PCR requires 5 to 50ng of DNA concentration). The ratio of absorbance at 260nm and 280nm, used to assess the purity of DNA and RNA, were close to 1.8 for majority of extracted DNA pure enough (Sambrook and Russell, 2001).

Molecular diagnosis by Nested-PCR

PCR based assay targeting several multicopy genes, eg. rRNA and miniexon genes, kDNA minicircles and repetitive nuclear DNA sequences form the mainstay of molecular diagnosis (Santos-Gomes et al., 2000; Attar et al., 2001; El Tai et al., 2001). In the present investigation, the optimized sensitive reaction parameters *viz*. template DNA concentration, primer concentration (lower or higher concentration of primer leads to amplification failure and primer dimer formation respectively, $MgCl_2$ concentration (Mg^{++} affect primer annealing and template denaturation), dNTPs concentrations, *Taq* polymerase (intensity of band increases with *Taq*) concentration and n-PCR cycling condition were used for standard n-PCR protocol (Noyes et al., 1998). The two sets of primers, targeting abundantly found kinetoplast minicirlce DNA, gave 680 bp sized fragment after n-PCR followed by agarose gel electrophoresis as observed by Noyes *et al*., 1998. In this study, 9% (n=100) of the research participants were found to be positive for asymptomatic VL. Nepal reports the VL incidence of 5 per 10,000 annually (Rijal et al., 2010) which is much lower (18 folds) than the incidence of asymptomatic VL as detected in this research. Similar findings like 9% of subjects found to be infected and asymptomatic by the Kalanet survey (WHO, 2010), 17.6% of PCR positivity among healthy Nepalese subjects living in areas endemic for Kala-azar (Bhattarai et al., 2009), 7.9% (n=28) of PCR positivity in asymptomatic individuals in Bihar (Topno et al., 2010), etc. are supportive to the findings this research. Within the follow up period (by phone interviewed), of six months for samples collected form Sunsari & Chitwan and three months for samples collected from Mahottari, none of those asymptomatic positive cases were converted into the disease. Other studies have shown 18.42% (Topno et al., 2010), to 25% (Sharma et al., 2000) of asymptomatic infections showed clinical symptoms such as enlargement of spleen, liver or both organs, and fever development peculiar to the visceral leishmaniasis within 3-6 months.

Analyzing VL epidemiology among the research participants for asymptomatic cases based on sample collection site analytical ground, higher percentage (10.64%) of the subjects from hospital based sample were positive to asymptomatic VL as compared to field based sample (7.55%) (Table 4.5). As far as the available information no specific studies have been

done on asymptomatic VL in patients with complains other than VL. This result indicates that people from endemic region with complain of health deterioration might have chance of 10.64% Kala-azar. But it has also been reported that people coming to hospital with possibility of Kala-azar represents at least 80% confirmation in the community (Adhikari et al., 2010).

Regarding endemic region based epidemiology, 11.39% of subjects belonging to endemic region were positive to asymptomatic Kala-azar using n-PCR. This finding is also in line with the other findings i.e. 9% (WHO, 2010). However, less frequent asymptomatic VL was observed in this study while compared to 17.6% (Bhattarai et al., 2009) among healthy Nepalese subjects living in areas endemic for Kala-azar. This difference could be due to technical factors (analytical sensitivity of PCR) or possible sampling bias (size and site). Though there are reports of VL in non-endemic regions of the country (Pandey et al., 2011), this present investigation with two districts as non-endemic candidate didn't show as positive subjects. This observation could be explained by sampling bias i.e. sampling district and sample size.

In the gender based epidemiology, this study portrayed contradictory finding. Previous studies reported the diseases as more frequent in males than in females (Ostyn et al., 2011; Pun et al., 2011). In this investigation more females (9.43%) and less number of males (8.15%) showed positivity by n-PCR affecting the gender-specific distribution of VL. Kala-azar has been known as disease of poor and poorest (Mondal et al., 2009). In this decade, Nepalese people are seeking for overseas employment lured by lucrative salaries. This outflow of Nepalese people for 2008/2009 was 219, 965 out of which 211,371 were males (96%) and 8,594 were females (4%) (IOM, 2013). This huge male population outflowing for overseas employment could change the behavior risk factors or greater mobility of females to high-risk areas and ultimately the higher incidence of the disease in females.

Considering age-specific epidemiology, more adults were found to be the carrier of asymptomatic VL. The finding of this study is in line with one of the previous observations, which reported asymptomatic persons were adults (15-44 years) and children (5-14 years) (Topno et al., 2010). Furthermore, host factor that can directly or indirectly influence the frequency of asymptomatic cases supports the results of this study. For example, the proportion of asymptomatic carrier increased with the age reflecting cumulative transmission over multiple years (Marty et al., 1992; Morillas et al., 1996; Biglino et al., 2010). A large number of mobile population, mostly adults, travelling between Nepal and India have been increasing in recent years (Bhattrai, 2007) further to strengthen our findings.

Direct agglutination test (DAT)

DAT is a well validated test for the diagnosis of Kala-azar and has been widely used for more than 25 years. In this research, comassie brilliant blue stained promastigote (prepared in the laboratory of Central Department of Biotechnology using Nepalese strain parasite JKPO1/2011) used as an aqueous antigen were agglutinated by the collected sera samples. CBB dye binds most readily to arginyl and lysyl residues (Compton and Jones, 1985) of membrane proteins of parasite that gave the aqueous antigen a bluish color. The use of citrate to antigen suspension ensured antigen stability by maintaining the intracellular repulsion forces that are responsible for overcoming spontaneous clumping (el Harith et al., 1988). Similarly, the use of 2-mercaptoethanol in DAT removed the non-specific agglutination reactions while augmenting or at least maintaining specific ones (el Harith et al., 1988). Using aqueous antigen prepared in our lab, the parasitologically confirmed Kala-azar serum showed agglutination at 1:400 times of serum dilution. Keeping the titre value as cut-off point, the sera of four samples (shown positive by nested-PCR) were positive to Kala-azar showing maximum agglutination at 1:800. Additionally, the non-agglutinating property as shown by malaria positive serum in this DAT explains the non-cross reactivity of the formulated aqueous antigen. Leishmanial antigen agglutinated by serum from malaria patient shows cross reactivity (Sinha and Sehgal, 1994) which was not observed in this research, validating the antigen as potent tool for DAT based diagnosis. The calculated sensitivity and specificity of the prepared aqueous DAT antigen was 44.44% and 100% respectively while taking 1:200 as cut-off titre value (as per the parasitologically confirmed positive). DAT accuracy is critically dependent on the choice of DAT cut-off titer for positivity. In several reports on VL, a DAT titre of 1:3200 is used as diagnostic limit, since it give highest specificity and sensitivity. Several studies have reported high sensitivity for DAT, but each used a different definition of a positive test, with threshold titers ranging from 1:800 to 1:6,400 (Pal et al., 1991; Singla et al., 1993; Zijlstra et al., 1998). A false positive diagnosis of VL has the implication of subjecting someone to an expensive although relatively safe treatment regimen. A false negative is also harmful in the sense that it will cause a delay of treatment, assuming patient will come back to the health service if they don't feel better. In view of these considerations, the decision on DAT cut-off titer has to be made in the context of the country, laboratories and health service concerned. Boelaert *et al* strongly warned against the use of a single, universal DAT cut-off titer for all purposes (Boelaert et al., 1999). DAT result obtained at CDBT laboratory, which do not function under highly controlled and sophisticated conditions (on contrary to ITMA, Institute of Tropical Medicine in Antwerp laboratory) might have also reduced the quality of prepared aqueous antigen.

Immunoblotting

Parasites in culture were very active metacyclic (days 3-5) to stationary phase (day 7). The counts, 1×10^8, in 7-day culture before harvest were as according to previous reports (Manandhar, 2008).

The cocktail CSA prepared from the parasite lysate was a heterogeneous mixture containing cytoplasmic and membrane (organelles) bound proteins components. CSA was found to contain total protein concentration of 2.8mg/ml. A similar study done by Kumar *et al* and Aganja *et al* have obtained 4.4mg/ml and 9.4mg/ml respectively, but Kumar *et al* used Lowry method (Kumar et al., 2002). Using the cocktail CSA i.e. proteins form amastigotes and promastigotes, it is likely to improve the detection as higher the number of proteins higher the chances of interaction by primary antibody and so the detection. Proteins are polypeptides present in CSA got denatured in the presence of detergent like SDS and reduced in the presence of beta-mercaptoethanol. The denatured protein complexes with SDS in the ratio of 1.4gm SDS per gram of protein giving overall negative charge to the complex (Voet and Voet, 2004). On applying the electric field, the SDS protein complex moved toward the anode separating the proteins based on difference in charge they possess and their molecular weight. The separated polypeptides by PAGE were transblotted to the nitrocellulose membrane by Western blotting technique. The different polypeptides bands observed after incubation with primary antibody followed by alkaline phosphatase enzyme conjugated secondary antibody were analyzed by Alpha innotech software. Analysis resulted 16.26 (~16 kDa), 18.27 (~18 kDa), 36 kDa, 64.61 (~65 kDa) 71.26 (~72 kDa), 21.36 (~22 kDa) and 25.26 (~26kDa) polypeptide bands (Fig 4.8) in the blotting profile obtained due to interaction between CSA and primary antibody of nested-PCR positive sera samples. The healthy control did not produce any bands. Different antigen polypeptides like 26 kDa and 22 kDa (Aganja, 2011), 65 kDa (Kumar et al., 2002), 74 kDa (Manandhar, 2008), 120 kDa, 91 kDa, 72 kDa, 63 kDa, 51 kDa, 34 kDa and 31 kDa (Ravindran et al., 2004) has been reported as better diagnostic marker for Vl in Indian sub-continents. In addition, detection of reported size of polypeptides like 22 kDa, 26 kDa, 65 kDa and 72 kDa also strengthen the statement that validates the positivity to VL. Parasite antigen potentially useful for specific immunodiagnosis of VL includes proteins such as gp63 (Okong'o-Odera et al., 1993), p32 of *L. donovani*, *L. infantum* promastigotes (Tebourski et al., 1994). Purified proteins of 70 and 72 kDa from *L. donovani* promastigote (Jaffe and Zalis, 1988; Jaffe and Zalis, 1988), recombinant proteins such as rK39, a 39-amino acid repeat part of a 230 kDa protein predominant in *L. chagasi* amastigote (Burns et al., 1993) and recombinant gp63 antigens form *L. chagasi* and *L. donovani* (Shreffler et al., 1993) are reported as useful for VL diagnosis.

The antibodies against 14, 16 kDa and / or 18kDa antigens are important for the diagnosis of asymptomatic infections (Mary et al., 1992; Sakru et al., 2007) and detection of 16kDa and 18 kDa polypeptide bands in the sera of the RP of this research confirmed positive to asymptomatic VL. By this research, 16kDa can be validated as marker for asymptomatic VL of Nepal.

Chapter VI

Summary

Visceral leishmaniasis is one of the major public health disease caused by protozoan parasite *Leishmania donovani* and transmitted by sandfly, *Phlebotomus argentipes*. It especially occurs among the socially marginalized and the poorest communities living in the remote rural regions with no or little access to health amenities. It accounts for more than 90% of the global VL incidence in just five countries i.e. Brazil, India, Sudan Bangladesh and Nepal. In the three countries *viz.* Nepal, India and Bangladesh of the SEAR, about 189 million people in 109 districts are at risk. Although 13 districts in the eastern and central terai regions of Nepal are endemic, it is being reported form non-endemic regions making theoretical outbreaks probable at any time. Anthroponotic disease and lack of anti-leishmanial vaccine are major cause for increasing endemicity. Considering the intensity and socio-economic impact of this disease, WHO/TDR has recognized Leishmaniasis as a public health concern. Similarly, national governments of Nepal, India and Bangladesh have expressed their commitment to eliminate VL in these countries by 2015. The outcome of VL manifests asymptomatic forms to obvious disease. The symptomatic case of the disease is characterized by fever for more than two weeks with splenomegaly, anemia, progressive weight loss and sometimes darkening of skin. Symptomatic infections plays a critical role in transmission of the disease while asymptomatic forms cannot be devalued because these acts as a potential reservoir in transmission dynamic of VL and a proportions of asymptomatic infections may subsequently progress to clinical disease. Asymptomatic cases ranged from 1:2.4 to 18:1 in various foci of the world. Moreover, Kala-azar and HIV coinfections have emerged as a health problem in recent years. Till date great numbers of epidemiological surveys are being done for the estimation of symptomatic VL using immunological and molecular tools but none or very few have done on asymptomatics. To carry out the above aim arrays of diagnostic tools are available but complete satiating tools are not on hand. The most popular diagnostic tool is rK39 but cannot discriminate asymptomatic, diseased and cured individuals. It gives positive result to PKDL cases causing dilemma in drug administration. In light of this devastation, the use of new diagnostic approaches to study asymptomatic population is mandatory.

PCR is the most common molecular technique successfully used for diagnosis and differentiation of species. Asymptomatic infections have been associated with high PCR positivity. This study was designed so far to rationalize the detection of *L. donovani* kDNA using n-PCR. In this section of research, three different districts were seriously scrutinized as site for blood sample collection. With the help of expert clinician, blood samples were collected and separated into buffy coat and serum. From the buffy coat, DNA was extracted

and quantified between 10 µg/ml to 190 µg/ml. The calculated purity was close to 1.8 for majority of extracted DNA samples. The DNA samples were subjected to n-PCR, using two sets of primers [external primers (targets CSB1 & CSB2) CSB2XF (C/GA/GTA/GCAGAAAC/TCCCGTTCA) and CSB1XR (ATTTTTCG/CGA/TTTT/CGCAGAACG) and internal primers (targets CSB3 & CSB1) LiR (TCGCAGAACGCCCCT) and 13Z (ACTGGGGGTTGGTGTAAAATAG)] that targets kDNA minicircle, gave 680bp sized fragment in positive case. The n-PCR can detect 1/10 of the *Leishmania* DNA with maximum theoretically possible sensitivity as reported. After completion of n-PCR the research participants were analyzed into different analytical grounds. On reviewing all the samples in n-PCR ground 9% (n=100) of RP were detected positive i.e. 9% incidence rate of asymptomatic case in different settings included in this study. In the sampling site based epidemiology, 7.55% (n=53) of healthy RP living in their native places without any complaint of VL and 10.64% (n=47) of RP who came for treatment with different health complaints other than that of VL were found to be Kala-azar positive. Concerning endemic region based epidemiology, only RP from endemic region were positive i.e. 11.39% (n=79) while all RP belonging to non-endemic and buffered endemic were negative to the disease. Gender wise division of RP disclosed, male RP accounted for 47% while female RP accounted for 53% and more females (9.43%, n=53) being affected than males (8.5% in 47). Age wise distribution of data exhibited the incidence of VL highest (25%) in elderly adults and lowest, (2.78%) youngs. Similarly, adults, adolescent and pre old age group of research participants were 13.33%, 8.33% and 7.69% positive to Kala-azar respectively. On follow up (phone interviewed) response none of those RP (found positive to VL in n-PCR) developed the disease.

The entry of the *Leishmania* parasite inside the human body activates the immune system to combat against the parasite. The activation of immune system involves (i) the production of specific antibodies by B cells to interact with the epitopes of the leishmanial protein for clearance (ii) the cell mediated immunity to control the disease. Under the basis of production of antibody, immunological techniques (DAT and Immunoblot) were carried out for diagnosis.

DAT assay was carried out by agglutinating the aqueous DAT antigen, prepared by harvesting Nepalese strain promastigote (JKP01/2011) at stationary phase, by the sera of the RP that were shown positive by n-PCR. Thus prepared aqueous DAT antigen (first time in Nepal) showed agglutination in 44.44% (n=9) of RP on account of agglutination shown by confirmed positive sample (taking cut off value 1:200). And the sensitivity and specificity of the aqueous DAT antigen was found to be 44.44% and 100% respectively.

WB was carried out using cocktail CSA prepared from equal volumes of stationary phase promastigote and amastigote culture. The CSA obtained from parasite lysate was quantified

to contain 2.854 µg/µl proteins based on Bradford assay. The cocktail CSA after SDS-PAGE and immunoblotting disclosed 16kDa, 18kDa, 22kDa, 36kDa, 65kDa and 72kDa distinct polypeptides bands in different sera (shown positive by n-PCR) and have been reported as better diagnostic marker.

Research work being conducted on such neglected disease in the country like ours is a matter of honor. Despite the hurdles, the findings like higher incidence of asymptomatic VL alerts government in achieving the Kala-azar elimination target. Further encouragement, support and dedication from national and international bodies can help in the improvement of DAT and Immunoblot in regional level.

Chapter VII

Conclusion

Visceral leishmaniasis is a neglected disease since it is mostly confined to poor class of society, however huge number of world population are in threat. Diagnostic test capable of detecting asymptomatic infections is of utmost importance for screening of asymptomatic infections and verification of elimination and epidemiological studies. Furthermore, the diagnostic test should be simple and affordable which is seldom defended by molecular techniques. DNA based molecular techniques add up a new dimension in the identification, diagnostic, and genetic diversity of *Leishmania* parasite. In the pool of various molecular techniques, n-PCR has major attention because of its high sensitivity and specificity. This epidemiological and diagnostic study, carried out using molecular tool endorsed with serology, disclosed the high incidence (9%) of asymptomatic carrier of VL in the endemic regions of Nepal. In sampling site based epidemiology, 10.64% and 7.55% of research participants from hospital and field were positive to asymptomatic VL. Regarding endemicity, 11.39% of RP belonging to endemic were positive to the disease. Gender based epidemiology depicted 9.43% of females and 8.15% of males positive to VL. Further, more elderly adults of age 41-50 years (25%) were found to be carrier of asymptomatic VL. Serological tests, DAT and Immunoblot were also able to detect asymptomatic VL employing Nepalese strain parasite culture. DAT detected 4 samples (out of 9) as positive while immunoblot analysis revealed 16kDa, 18kDa, 36kDa, 65kDa, 72kDa, 22kDa and 26kDa polypeptide bands. The presence of 16kDa and 18kDa fraction have validated the marker for asymptomatic cases The ongoing governmental control program for more than decades, look futile in the present perspective unless remedial steps are taken. This work also emphasized the need for strengthening the existing health system.

Scope and Recommendation:

- ✓ As many asymptomatic cases could be detected with PCR, Government should be vigilant to improve Surveillance in endemic and other risk areas.
- ✓ Schizodeme analysis and sequencing of n-PCR positive samples may be carried out to find the genetic diversity.
- ✓ Preparation of FD antigen from Nepalese strain parasite could be appealing in the diagnosis of Kala-azar in the context of Nepal.
- ✓ Needs further investigation of 14kDa, 16kDa and 18kDa peptides that may have diagnostic value.

References

Adams ER, Schoone GJ, Ageed AF, Safi SE and Schallig HD (2010) Development of a reverse transcriptase loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of Leishmania parasites in clinical samples. *Am J Trop Med Hyg* 82(4): 591-6

Addy M and Nandy A (1992) Ten years of kala-azar in west Bengal, Part I. Did post-kala-azar dermal leishmaniasis initiate the outbreak in 24-Parganas? *Bull World Health Organ* 70(3): 341-6

Adhikari S, Supakankunti S and Khan M (2010) Kala azar in Nepal: Estimating the effects of socioeconomic factors on disease incidence. *Kathmandu University Medical Journal* 8(1): 73-79

Adhya S, Chatterjee M, Hassan MQ, Mukherjee S and Sen S (1995) Detection of Leishmania in the blood of early kala-azar patients with the aid of the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 89(6): 622-4

Aga E, Katschinski DM, van Zandbergen G, Laufs H, Hansen B, Muller K, Solbach W and Laskay T (2002) Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite Leishmania major. *J Immunol* 169(2): 898-905

Aganja RP (2011) *Potential diagnostic antigenic leishmanial protein detection for Kala-azar*. Master Thesis submitted in Tribhuvan University, Kirtipur, Kathmandu, Nepal: 1-110

Ahuja SS, Reddick RL, Sato N, Montalbo E, Kostecki V, Zhao W, Dolan MJ, Melby PC and Ahuja SK (1999) Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection. *J Immunol* 163(7): 3890-7

Alvar J, Aparicio P, Aseffa A, Boer DM, Canavate C, Dedet JP, Gradoni L, Horst RT, Lopez-Velez R and Moreno J (2008) <The Relationship between Leishmaniasis and hiv, 10 years experience.pdf>. *Clinical Microbiology Reviews* 21(2): 334-359

Alvar J, Canavate C, Gutierrez-Solar B, Jimenez M, Laguna F, Lopez-Velez R, Molina R and Moreno J (1997) Leishmania and human immunodeficiency virus coinfection: the first 10 years. *Clin Microbiol Rev* 10(2): 298-319

Angelici MC, Gramiccia M and Gradoni L (1989) Study on genetic polymorphism of Leishmania infantum through the analysis of restriction enzyme digestion patterns of kinetoplast DNA. *Parasitology* 99 Pt 3: 301-9

Anjili CO, Mbati PA, Mwangi RW, Githure JI and Koech DK (1996) A simple method for maintaining, detecting and recovering virulent Leishmania donovani in hamsters. *Acta Trop* 60(4): 263-7

Antinori S, Calattini S, Longhi E, Bestetti G, Piolini R, Magni C, Orlando G, Gramiccia M, Acquaviva V, Foschi A, Corvasce S, Colomba C, Titone L, Parravicini C, Cascio A and

Corbellino M (2007) Clinical use of polymerase chain reaction performed on peripheral blood and bone marrow samples for the diagnosis and monitoring of visceral leishmaniasis in HIV-infected and HIV-uninfected patients: a single-center, 8-year experience in Italy and review of the literature. *Clin Infect Dis* 44(12): 1602-10

Arana BA, Roca M, Rizzo NR, Mendoza CE and Kroeger A (1999) Evaluation of a standardized leishmanin skin test in Guatemala. *Trans R Soc Trop Med Hyg* 93(4): 394-6

Aseffa A, Gumy A, Launois P, MacDonald HR, Louis JA and Tacchini-Cottier F (2002) The early IL-4 response to Leishmania major and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory CD4+CD25+ T cells. *J Immunol* 169(6): 3232-41

Ashford DA, Bozza M, Freire M, Miranda JC, Sherlock I, Eulalio C, Lopes U, Fernandes O, Degrave W, Barker RH, Jr. and et al. (1995) Comparison of the polymerase chain reaction and serology for the detection of canine visceral leishmaniasis. *Am J Trop Med Hyg* 53(3): 251-5

Ashford RW (1997) The leishmaniases as model zoonoses. *Ann Trop Med Parasitol* 91(7): 693-701

Ashford RW and Bates PA (1998) Leishmaniasis in Old World, The Wellcome Trust illustrated history of tropical diseases. In: Cox FEG, Kreier JP and Wakelin D (Eds). *Parasitology, Topley & Wilson's microbiology and microbial infection*(Oxford University Press: New York, USA): 215-240

Assreuy J, Cunha FQ, Epperlein M, Noronha-Dutra A, O'Donnell CA, Liew FY and Moncada S (1994) Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major. *Eur J Immunol* 24(3): 672-6

Attar ZJ, Chance ML, el-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C and Hommel M (2001) Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. *Acta Trop* 78(1): 11-6

Awasthi A, Mathur RK and Saha B (2004) Immune response to Leishmania infection. *Indian J Med Res* 119(6): 238-58

Badaro R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R and Johnson WD, Jr. (1986) New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis* 154(6): 1003-11

Balana-Fouce R, Reguera RM, Cubria JC and Ordonez D (1998) The pharmacology of leishmaniasis. *Gen Pharmacol* 30(4): 435-43

Barker DC (1987) DNA diagnosis of human leishmaniasis. *Parasitol Today* 3(6): 177-84

Barnett C and Rai AM (2009) Elimination of Kala-azar in Mahottari District, Nepal *International Rescue Committee*: 1-10

Basu R, Bhaumik S, Basu JM, Naskar K, De T and Roy S (2005) Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-

sensitive and -resistant strains of Leishmania donovani that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. *J Immunol* 174(11): 7160-71

Bates PA (1994) Complete developmental cycle of Leishmania mexicana in axenic culture. *Parasitology* 108 (Pt 1): 1-9

Bates PA (2007) Transmission of Leishmania metacyclic promastigotes by phlebotomine sand flies. *Int J Parasitol* 37(10): 1097-106

Bates PA and Rogers ME (2004) New insights into the developmental biology and transmission mechanisms of Leishmania. *Curr Mol Med* 4(6): 601-9

Ben-Ismail R, Smith DF, Ready PD, Ayadi A, Gramiccia M, Ben-Osman A and Ben-Rachid MS (1992) Sporadic cutaneous leishmaniasis in north Tunisia: identification of the causative agent as Leishmania infantum by the use of a diagnostic deoxyribonucleic acid probe. *Trans R Soc Trop Med Hyg* 86(5): 508-10

Berkovic D, Fleer EA, Eibl H and Unger C (1992) Effects of hexadecylphosphocholine on cellular function. *Prog Exp Tumor Res* 34: 59-68

Berman JD, Gallalee JV and Best JM (1987) Sodium stibogluconate (Pentostam) inhibition of glucose catabolism via the glycolytic pathway, and fatty acid beta-oxidation in Leishmania mexicana amastigotes. *Biochem Pharmacol* 36(2): 197-201

Bern C, Hightower AW, Chowdhury R, Ali M, Amann J, Wagatsuma Y, Haque R, Kurkjian K, Vaz LE, Begum M, Akter T, Cetre-Sossah CB, Ahluwalia IB, Dotson E, Secor WE, Breiman RF and Maguire JH (2005) Risk factors for kala-azar in Bangladesh. *Emerg Infect Dis* 11(5): 655-62

Bhatia A, Daifalla NS, Jen S, Badaro R, Reed SG and Skeiky YA (1999) Cloning, characterization and serological evaluation of K9 and K26: two related hydrophilic antigens of Leishmania chagasi. *Mol Biochem Parasitol* 102(2): 249-61

Bhattarai NR, Van der Auwera G, Khanal B, De Doncker S, Rijal S, Das ML, Uranw S, Ostyn B, Praet N, Speybroeck N, Picado A, Davies C, Boelaert M and Dujardin JC (2009) PCR and direct agglutination as Leishmania infection markers among healthy Nepalese subjects living in areas endemic for Kala-Azar. *Trop Med Int Health* 14(4): 404-11

Bhattrai R (2007) Open borders, closed citizenship: Nepali labors migrants in Delhi. *South Asian Study Centre*

Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A and Ferroglio E (2010) Asymptomatic Leishmania infantum infection in an area of northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. *J Clin Microbiol* 48(1): 131-6

Bista MB (1998) National overview of kala-azar in Nepal. In: *Kala-azar in Nepal: Principals, Practice and Public Health Perspectives* (ed Bastola S, Karki P, Rijal S and Gautam A) EDCD/BPKIHSWHO, Kathmandu. 1-5

Bista MB (1998) National overview of kala-azar in Nepal. In: *Kala-azar in Nepal: Principals, Practice and Public Health Perspectives* (ed Bastola S, Karki P, Rijal S and Gautam A) EDCD/BPKIHSWHO, Kathmandu. 1-5

Boelaert M, Criel B, Leeuwenburg J, Van Damme W, Le Ray D and Van der Stuyft P (2000) Visceral leishmaniasis control: a public health perspective. *Trans R Soc Trop Med Hyg* 94(5): 465-71

Boelaert M, El Safi S, Jacquet D, de Muynck A, van der Stuyft P and Le Ray D (1999) Operational validation of the direct agglutination test for diagnosis of visceral leishmaniasis. *Am J Trop Med Hyg* 60(1): 129-34

Boelaert M, El-Safi S, Hailu A, Mukhtar M, Rijal S, Sundar S, Wasunna M, Aseffa A, Mbui J, Menten J, Desjeux P and Peeling RW (2008) Diagnostic tests for kala-azar: a multi-centre study of the freeze-dried DAT, rK39 strip test and KAtex in East Africa and the Indian subcontinent. *Trans R Soc Trop Med Hyg* 102(1): 32-40

Boelaert M, Rijal S, Regmi S, Singh R, Karki B, Jacquet D, Chappuis F, Campino L, Desjeux P, Le Ray D, Koirala S and Van der Stuyft P (2004) A comparative study of the effectiveness of diagnostic tests for visceral leishmaniasis. *Am J Trop Med Hyg* 70(1): 72-7

Bossolasco S, Gaiera G, Olchini D, Gulletta M, Martello L, Bestetti A, Bossi L, Germagnoli L, Lazzarin A, Uberti-Foppa C and Cinque P (2003) Real-time PCR assay for clinical management of human immunodeficiency virus-infected patients with visceral leishmaniasis. *J Clin Microbiol* 41(11): 5080-4

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-54

Brajtburg J and Bolard J (1996) Carrier effects on biological activity of amphotericin B. *Clin Microbiol Rev* 9(4): 512-31

Brajtburg J, Elberg S, Schwartz DR, Vertut-Croquin A, Schlessinger D, Kobayashi GS and Medoff G (1985) Involvement of oxidative damage in erythrocyte lysis induced by amphotericin B. *Antimicrob Agents Chemother* 27(2): 172-6

Bray RS (1985) Immunodiagnosis of leishmaniasis. *Leishmaniasis*: 177-81

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112(2): 195-203

Burns JM, Jr., Shreffler WG, Benson DR, Ghalib HW, Badaro R and Reed SG (1993) Molecular characterization of a kinesin-related antigen of Leishmania chagasi that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci U S A* 90(2): 775-9

Chappuis F, Mueller Y, Nguimfack A, Rwakimari JB, Couffignal S, Boelaert M, Cavailler P, Loutan L and Piola P (2005) Diagnostic accuracy of two rK39 antigen-based dipsticks and the

formol gel test for rapid diagnosis of visceral leishmaniasis in northeastern Uganda. *J Clin Microbiol* 43(12): 5973-7

Chappuis F, Rijal S, Jha UK, Desjeux P, Karki BM, Koirala S, Loutan L and Boelaert M (2006) Field validity, reproducibility and feasibility of diagnostic tests for visceral leishmaniasis in rural Nepal. *Trop Med Int Health* 11(1): 31-40

Chappuis F, Rijal S, Soto A, Menten J and Boelaert M (2006) A meta-analysis of the diagnostic performance of the direct agglutination test and rK39 dipstick for visceral leishmaniasis. *BMJ* 333(7571): 723

Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S, Peeling RW, Alvar J and Boelaert M (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 5(11): 873-82

Chen J, Englund PT and Cozzarelli NR (1995) Changes in network topology during the replication of kinetoplast DNA. *EMBO J* 14(24): 6339-47

Chen J, Rauch CA, White JH, Englund PT and Cozzarelli NR (1995) The topology of the kinetoplast DNA network. *Cell* 80(1): 61-9

Chulay JD and Bryceson AD (1983) Quantitation of amastigotes of Leishmania donovani in smears of splenic aspirates from patients with visceral leishmaniasis. *Am J Trop Med Hyg* 32(3): 475-9

Claborn DM (2010) The Biology and Control of Leishmaniasis Vectors. *J Glob Infect Dis* 2(2): 127-134

Clark RA, Chong B, Mirchandani N, Brinster NK, Yamanaka K, Dowgiert RK and Kupper TS (2006) The vast majority of CLA+ T cells are resident in normal skin. *J Immunol* 176(7): 4431-9

Clerici M and Shearer GM (1994) The TH1-TH2 hypothesis of HIV infection: new insights. *Immunol Today* 15(12): 575-581

Coler RN, Goto Y, Bogatzki L, Raman V and Reed SG (2007) Leish-111f, a recombinant polyprotein vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. *Infect Immun* 75(9): 4648-54

Compton SJ and Jones CG (1985) Mechanism of dye response and interference in the Bradford protein assay. *Analyt. Biochem.* 151: 369–374.

Costa CH, Stewart JM, Gomes RB, Garcez LM, Ramos PK, Bozza M, Satoskar A, Dissanayake S, Santos RS, Silva MR, Shaw JJ, David JR and Maguire JH (2002) Asymptomatic human carriers of Leishmania chagasi. *Am J Trop Med Hyg* 66(4): 334-7

Cox FE (2002) History of human parasitology. *Clin Microbiol Rev* 15(4): 595-612

Croft SL, Snowdon D and Yardley V (1996) The activities of four anticancer alkyllysophospholipids against Leishmania donovani, Trypanosoma cruzi and Trypanosoma brucei. *J Antimicrob Chemother* 38(6): 1041-7

Cruz I, Morales MA, Noguer I, Rodriguez A and Alvar J (2002) Leishmania in discarded syringes from intravenous drug users. *Lancet* 359(9312): 1124-5

Cunningham ML, Titus RG, Turco SJ and Beverley SM (2001) Regulation of differentiation to the infective stage of the protozoan parasite Leishmania major by tetrahydrobiopterin. *Science* 292(5515): 285-7

da Silva ES, Gontijo CM, Pacheco Rda S and Brazil RP (2004) Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. *Genet Mol Res* 3(2): 251-7

Daneshvar H, Coombs GH, Hagan P and Phillips RS (2003) Leishmania mexicana and Leishmania major: attenuation of wild-type parasites and vaccination with the attenuated lines. *J Infect Dis* 187(10): 1662-8

Das A and Ali N (2012) Vaccine Development Against Leishmania donovani. *Front Immunol* 3: 99

Das ML. (1998) "Sandfly the vectors of kala-azar," in Kala-azar in Nepal: Principles, Practice and Public Health Perspective. EDCD/BPKIHS/WHO, Kathmandu, Nepal. pp. 47-52

Davies CR, Kaye P, Croft SL and Sundar S (2003) Leishmaniasis: new approaches to disease control. *Bmj* 326(7385): 377-82

De Colmenares M, Portus M, Riera C, Gallego M, Aisa MJ, Torras S and Munoz C (1995) Short report: detection of 72-75-kD and 123-kD fractions of Leishmania antigen in urine of patients with visceral leishmaniasis. *Am J Trop Med Hyg* 52(5): 427-8

Deborggraeve S, Laurent T, Espinosa D, Van der Auwera G, Mbuchi M, Wasunna M, El-Safi S, Al-Basheer AA, Arevalo J, Miranda-Verastegui C, Leclipteux T, Mertens P, Dujardin JC, Herdewijn P and Buscher P (2008) A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. *J Infect Dis* 198(10): 1565-72

Degu GE (2006) *An investigation into some aspects of the epidemiology of cutaneous leishmaniasis in Saris, Addis Ababa*. PhD Thesis submitted in Addis Ababa University, Addis Ababa, Ethopia: 1-69

Desjeux P (1996) Leishmaniasis. Public health aspects and control. *Clin Dermatol* 14(5): 417-23

Desjeux P (2001) The increase in risk factors for leishmaniasis worldwide. *Trans R Soc Trop Med Hyg* 95(3): 239-43

Desjeux P (2004) Leishmaniasis: current situation and new perspectives. *Comp Immunol Microbiol Infect Dis* 27(5): 305-18

Desjeux P and Alvar J (2003) Leishmania/HIV co-infections: epidemiology in Europe. *Ann Trop Med Parasitol* 97 Suppl 1: 3-15

EDCD. (2010) Report of the Internal Assessment of Malaria Control and Kala-azar Elimination Activities 2007, 2008 and 2009. Goverment of Nepal MoHaP, Department of Health Services, Epidemiology and Disease Control Division, Teku, Kathmandu, Nepal. pp. 51-68

el Harith A, Kolk AH, Leeuwenburg J, Muigai R, Huigen E, Jelsma T and Kager PA (1988) Improvement of a direct agglutination test for field studies of visceral leishmaniasis. *J Clin Microbiol* 26(7): 1321-5

El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi SH, Presber WH and Schonian G (2001) Leishmania donovani: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. *Exp Parasitol* 97(1): 35-44

el Tai NO, Osman OF, el Fari M, Presber W and Schonian G (2000) Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. *Trans R Soc Trop Med Hyg* 94(5): 575-9

ElHassan AM, Gaafar A and Theander TG (1995) Antigen-presenting cells in human cutaneous leishmaniasis due to Leishmania major. *Clin Exp Immunol* 99(3): 445-53

Elliott J. (1863) Report on epidemic and remittent fever occuring in parts of Burdwan and Neddea divisions. Office BS, Calcutta, India. pp. 1-23

Elnaiem DA, Aboud MA, El Mubarek SG, Hassan HK and Ward RD (1999) Impact of pyrethroid-impregnated curtains on Phlebotomus papatasi sandflies indoors at Khartoum, Sudan. *Med Vet Entomol* 13(2): 191-7

El-Safi SH, Abdel-Haleem A, Hammad A, El-Basha I, Omer A, Kareem HG, Boelaert M, Chance M and Hommel M (2003) Field evaluation of latex agglutination test for detecting urinary antigens in visceral leishmaniasis in Sudan. *East Mediterr Health J* 9(4): 844-55

el-Safi SH, Peters W and Evans DA (1991) Studies on the leishmaniases in the Sudan. 3. Clinical and parasitological studies on visceral and mucosal leishmaniasis. *Trans R Soc Trop Med Hyg* 85(4): 465-70

Evans KJ and Kedzierski L (2012) Development of Vaccines against Visceral Leishmaniasis. *J Trop Med* 2012: 892817

Fakhar M, Motazedian MH, Hatam GR, Asgari Q, Kalantari M and Mohebali M (2008) Asymptomatic human carriers of Leishmania infantum: possible reservoirs for Mediterranean visceral leishmaniasis in southern Iran. *Ann Trop Med Parasitol* 102(7): 577-83

Franco MA, Barbosa AC, Rath S and Dorea JG (1995) Antimony oxidation states in antileishmanial drugs. *Am J Trop Med Hyg* 52(5): 435-7

Gangneux JP, Menotti J, Lorenzo F, Sarfati C, Blanche H, Bui H, Pratlong F, Garin YJ and Derouin F (2003) Prospective value of PCR amplification and sequencing for diagnosis and typing of old world Leishmania infections in an area of nonendemicity. *J Clin Microbiol* 41(4): 1419-22

Gasim S, Elhassan AM, Khalil EA, Ismail A, Kadaru AM, Kharazmi A and Theander TG (1998) High levels of plasma IL-10 and expression of IL-10 by keratinocytes during visceral leishmaniasis predict subsequent development of post-kala-azar dermal leishmaniasis. *Clin Exp Immunol* 111(1): 64-9

Geneva, Switzerland: 1-78

Gibson ME (1983) The identification of kala-azar and the discovery of Leishmania donovani *Med Hist* 27(2): 203-13

Gold W, Stout A, Pagona JF and Donovivk R (1956) Amphotericin A and B: antifungal antibiotics produed by a streptomyces. *Antibiot. Annu.*: 579-586

Gradoni L (2001) An update on antileishmanial vaccine candidates and prospects for a canine Leishmania vaccine. *Vet Parasitol* 100(1-2): 87-103

Gurubacharya RL, Gurubacharya SM, Gurubacharya DL, J Q and Gurubacharya VL (2006) Prevalence of visceral leishmania & HIV co-infection in Nepal. *Indian Journal of Medical Research* 123(3): 473-475

Haldar JP, Ghose S, Saha KC and Ghose AC (1983) Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. *Infect Immun* 42(2): 702-7

Handman E (2001) Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev* 14(2): 229-43

Harhay MO, Olliaro PL, Vaillant M, Chappuis F, Lima MA, Ritmeijer K, Costa CH, Costa DL, Rijal S, Sundar S and Balasegaram M (2011) Who is a typical patient with visceral leishmaniasis? Characterizing the demographic and nutritional profile of patients in Brazil, East Africa, and South Asia. *Am J Trop Med Hyg.* 84(4): 543-550

Harith AE, Kolk AH, Kager PA, Leeuwenburg J, Faber FJ, Muigai R, Kiugu S and Laarman JJ (1987) Evaluation of a newly developed direct agglutination test (DAT) for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis: comparison with IFAT and ELISA. *Trans R Soc Trop Med Hyg* 81(4): 603-6

Harith AE, Kolk AH, Kager PA, Leeuwenburg J, Muigai R, Kiugu S and Laarman JJ (1986) A simple and economical direct agglutination test for serodiagnosis and sero-epidemiological studies of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 80(4): 583-36

Heinzel FP, Sadick MD, Holaday BJ, Coffman RL and Locksley RM (1989) Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med* 169(1): 59-72

Herwaldt BL (1999) Leishmaniasis. *Lancet* 354(9185): 1191-9

Hirve S, Singh SP, Kumar N, Banjara MR, Das P, Sundar S, Rijal S, Joshi A, Kroeger A, Varghese B, Thakur CP, Huda MM and Mondal D (2010) Effectiveness and feasibility of active and passive case detection in the visceral leishmaniasis elimination initiative in India, Bangladesh, and Nepal. *Am J Trop Med Hyg* 83(3): 507-11

Ho EA, Soong T-H and Li Y (1948) Comparative merits of sternum, spleen and liver punctures in the study of human visceral leishmaniasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 41(5): 629-636

Hoare CA (1938) Early discoveries regarding the parsites of oriental sore. *Trans R Soc Trop Med Hyg* 32: 67-92

Hoerauf A, Andrade PP, Andrade CR, Solbach W and Rollinghoff M (1992) Immunoblotting as a valuable tool to differentiate human visceral leishmaniasis from lymphoproliferative disorders and other clinically similar diseases. *Res Immunol* 143(4): 375-83

Holaday BJ, Pompeu MM, Jeronimo S, Texeira MJ, Sousa Ade A, Vasconcelos AW, Pearson RD, Abrams JS and Locksley RM (1993) Potential role for interleukin-10 in the immunosuppression associated with kala azar. *J Clin Invest* 92(6): 2626-32

Hommel M (1999) Visceral leishmaniasis: biology of the parasite. *J Infect* 39(2): 101-11

Hori S, Haury M, Lafaille JJ, Demengeot J and Coutinho A (2002) Peripheral expansion of thymus-derived regulatory cells in anti-myelin basic protein T cell receptor transgenic mice. *Eur J Immunol* 32(12): 3729-35

Houghton RL, Petrescu M, Benson DR, Skeiky YA, Scalone A, Badaro R, Reed SG and Gradoni L (1998) A cloned antigen (recombinant K39) of Leishmania chagasi diagnostic for visceral leishmaniasis in human immunodeficiency virus type 1 patients and a prognostic indicator for monitoring patients undergoing drug therapy. *J Infect Dis* 177(5): 1339-44

Hu XS, Yang WT, Lu HG, Yan HP, Cheng JP, Ma Y, Jin BQ and Zhang T (2000) Sequencing a specific kinetoplast DNA fragment of Leishmania donovani for polymerase chain reaction amplification in diagnosis of leishmaniasis in bone marrow and blood samples. *J Parasitol* 86(4): 822-6

Ibrahim ME, Lambson B, Yousif AO, Deifalla NS, Alnaiem DA, Ismail A, Yousif H, Ghalib HW, Khalil EA, Kadaro A, Barker DC and El Hassan AM (1999) Kala-azar in a high transmission focus: an ethnic and geographic dimension. *Am J Trop Med Hyg* 61(6): 941-4

IOM (2013) Country Information - Nepal.

Jaffe CL and McMahon-Pratt D (1983) Monoclonal antibodies specific for Leishmania tropica. I. Characterization of antigens associated with stage- and species-specific determinants. *J Immunol* 131(4): 1987-93

Jaffe CL and Zalis M (1988) Purification of two Leishmania donovani membrane proteins recognized by sera from patients with visceral leishmaniasis. *Mol Biochem Parasitol* 27(1): 53-62

Jaffe CL and Zalis M (1988) Use of purified parasite proteins from Leishmania donovani for the rapid serodiagnosis of visceral leishmaniasis. *J Infect Dis* 157(6): 1212-20

Jha TK, Sundar S, Thakur CP, Felton JM, Sabin AJ and Horton J (2005) A phase II dose-ranging study of sitamaquine for the treatment of visceral leishmaniasis in India. *Am J Trop Med Hyg* 73(6): 1005-11

Joshi DD, Sharma M and Bhandari S (2006) Visceral leishmaniasis in Nepal during 1980-2006. *J Commun Dis* 38(2): 139-48

Joshi S, Bajracharya BL and Baral MR (2006) Kala-azar (visceral Leishmaniasis) from Khotang. *Kathmandu Univ Med J (KUMJ)* 4(2): 232-4

Kager PA, Rees PH, Manguyu FM, Bhatt KM and Bhatt SM (1983) Splenic aspiration; experience in Kenya. *Trop Geogr Med* 35(2): 125-31

Kamhawi S (2006) Phlebotomine sandflies and Leishmania parasite: friends or foes? *Trends Parasitol* 22(2): 439-445

Kar K (1995) Serodiagnosis of leishmaniasis. *Crit Rev Microbiol* 21(2): 123-52

Katakura K, Kawazu S, Naya T, Nagakura K, Ito M, Aikawa M, Qu JQ, Guan LR, Zuo XP, Chai JJ, Chang KP and Matsumoto Y (1998) Diagnosis of kala-azar by nested PCR based on amplification of the Leishmania mini-exon gene. *J Clin Microbiol* 36(8): 2173-7

Kean BH, Mott KE and Russell AJE (1978). Tropical medicine and parasitology: classic investigation, Cornell University Press, Ithaca, N. Y

Kedzierski L, Sakthianandeswaren A, Curtis JM, Andrews PC, Junk PC and Kedzierska K (2009) Leishmaniasis: current treatment and prospects for new drugs and vaccines. *Curr Med Chem* 16(5): 599-614

Killick-Kendrick R, Leaney AJ, Peters W, Rioux JA and Bray RS (1985) Zoonotic cutaneous leishmaniasis in Saudi Arabia: the incrimination of Phlebotomus papatasi as the vector in the Al-Hassa oasis. *Trans R Soc Trop Med Hyg* 79(2): 252-5

Koirala S, Karki P, Das ML, Parija SC and Karki BM (2004) Epidemiological study of kala-azar by direct agglutination test in two rural communities of eastern Nepal. *Trop Med Int Health* 9(4): 533-7

Kumar P, Pai K, Tripathi K, Pandey HP and Sundar S (2002) Immunoblot analysis of the humoral immune response to Leishmania donovani polypeptides in cases of human visceral leishmaniasis: its usefulness in prognosis. *Clin Diagn Lab Immunol* 9(5): 1119-23

Kumar R, Pai K, Kumar P, Pandey HP and Sundar S (2006) Sero-epidemiological study of kala-azar in a village of Varanasi district, India. *Trop Med Int Health* 11(1): 41-8

Kumar R, Pai K, Pathak K and Sundar S (2001) Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. *Clin Diagn Lab Immunol* 8(6): 1220-4

Lainson R (1996) New World Leishmaniasis. *In F. E. G. Cox(ed) The wellcome Trust illustrated history of tropical diseases.*: 218-29

Lainson R and Shaw JJ. (1987) Evolution, classification and geographical distribution. In: Peters W and Killick-Kendrick R (Eds), The leishmaniasis in biology in biology and medicine. Press A, London. pp. 1-120

Lane RP. (1993) Sandflies (Phlebotominae). Medical Insects and Arachnids. Chapman and Hall,London. pp. 78-119

Lang T, Warburg A, Sacks DL, Croft SL, Lane RP and Blackwell JM (1991) Transmission and scanning EM-immunogold labeling of Leishmania major lipophosphoglycan in the sandfly Phlebotomus papatasi. *Eur J Cell Biol* 55(2): 362-72

le Fichoux Y, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D and Kubar J (1999) Occurrence of Leishmania infantum parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. *J Clin Microbiol* 37(6): 1953-7

Lira R, Contreras LM, Rita RM and Urbina JA (2001) Mechanism of action of anti-proliferative lysophospholipid analogues against the protozoan parasite Trypanosoma cruzi: potentiation of in vitro activity by the sterol biosynthesis inhibitor ketoconazole. *J Antimicrob Chemother* 47(5): 537-46

Lux H, Hart DT, Parker PJ and Klenner T (1996) Ether lipid metabolism, GPI anchor biosynthesis, and signal transduction are putative targets for anti-leishmanial alkyl phospholipid analogues. *Adv Exp Med Biol* 416: 201-11

Lux H, Heise N, Klenner T, Hart D and Opperdoes FR (2000) Ether--lipid (alkyl-phospholipid) metabolism and the mechanism of action of ether--lipid analogues in Leishmania. *Mol Biochem Parasitol* 111(1): 1-14

Maalej IA, Chenik M, Louzir H, Ben Salah A, Bahloul C, Amri F and Dellagi K (2003) Comparative evaluation of ELISAs based on ten recombinant or purified Leishmania antigens for the serodiagnosis of Mediterranean visceral leishmaniasis. *Am J Trop Med Hyg* 68(3): 312-20

Malla N and Mahajan RC (2006) Pathophysiology of visceral leishmaniasis - some recent concepts. *Indian J Med Res* 123(3): 267-74

Malla N, Sengupta C, Dubey ML, Sud A, Ansari NA and Salotra P (2003) Antigenaemia and antibody response to Leishmania donovani stage-specific antigens and rk39 antigen in human immunodeficiency virus-infected patients. *Br J Biomed Sci* 60(4): 210-6

Manandhar KD (2008) *A study on immunological and diagnostic aspects of Visceral Leishmaniasis*. PhD Thesis submitted in Banarus Hindu University, Varanasi-221005, India: 1-50

Manandhar KD, Yadav TP, Prajapati VK, Kumar S, Rai M, Dube A, Srivastava ON and Sundar S (2008) Antileishmanial activity of nano-amphotericin B deoxycholate. *J Antimicrob Chemother* 62(2): 376-80

Manandhar KD. (2011) Study of interindividual phenotypic differentiation as a permissive or non permissive macrophage host cells for *Leishmania donovani*, Study of Biomarkers associated with human infection with *Leishmania* in the risk population of Nepal. (IRD) IdRplD, Montpllier, France. pp. 1-3

Manson-Bahr PE (1961) The leishmanin test and immunity in kala azar. *East Afr Med J* 38: 165-7

Manson-Bahr PEC (1986) Old World Leishmaniasis. *In F.E.G. Cox(ed) The Wellcome Trust illustrated history of tropical diseases*. (The Wellcome Trust: London, United Kingdom).

Martin-Sanchez J, Pineda JA, Morillas-Marquez F, Garcia-Garcia JA, Acedo C and Macias J (2004) Detection of Leishmania infantum kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between polymerase chain reaction results and other Leishmania infection markers. *Am J Trop Med Hyg* 70(5): 545-8

Marty P, Le Fichoux Y, Giordana D and Brugnetti A (1992) Leishmanin reaction in the human population of a highly endemic focus of canine leishmaniasis in Alpes-Maritimes, France. *Trans R Soc Trop Med Hyg* 86(3): 249-50

Mary C, Lamouroux D, Dunan S and Quilici M (1992) Western blot analysis of antibodies to Leishmania infantum antigens: potential of the 14-kD and 16-kD antigens for diagnosis and epidemiologic purposes. *Am J Trop Med Hyg* 47(6): 764-71

Mathis A and Deplazes P (1995) PCR and in vitro cultivation for detection of Leishmania spp. in diagnostic samples from humans and dogs. *J Clin Microbiol* 33(5): 1145-9

Matlashewski G, Arana B, Kroeger A, Battacharya S, Sundar S, Das P, Sinha PK, Rijal S, Mondal D, Zilberstein D and Alvar J (2011) Visceral leishmaniasis: elimination with existing interventions. *Lancet Infect Dis* 11(4): 322-5

Maurya R, Singh RK, Kumar B, Salotra P, Rai M and Sundar S (2005) Evaluation of PCR for diagnosis of Indian kala-azar and assessment of cure. *J Clin Microbiol* 43(7): 3038-41

Mbongo N, Loiseau PM, Billion MA and Robert-Gero M (1998) Mechanism of amphotericin B resistance in Leishmania donovani promastigotes. *Antimicrob Agents Chemother* 42(2): 352-7

Meredith SE, Kroon NC, Sondorp E, Seaman J, Goris MG, van Ingen CW, Oosting H, Schoone GJ, Terpstra WJ and Oskam L (1995) Leish-KIT, a stable direct agglutination test based on freeze-dried antigen for serodiagnosis of visceral leishmaniasis. *J Clin Microbiol* 33(7): 1742-5

Mishra BB, Singh RK, Srivastava A, Tripathi VJ and Tiwari VK (2009) Fighting against Leishmaniasis: search of alkaloids as future true potential anti-Leishmanial agents. *Mini Rev Med Chem* 9(1): 107-23

Mishra J, Saxena A and Singh S (2007) Chemotherapy of leishmaniasis: past, present and future. *Curr Med Chem* 14(10): 1153-69

Mohapatra TM, Singh DP, Sen MR, Bharti K and Sundar S (2010) Compararative evaluation of rK9, rK26 and rK39 antigens in the serodiagnosis of Indian visceral leishmaniasis. *J Infect Dev Ctries* 4(2): 114-7

Molynex DH and Killick Kendrick R (1987) Morphology, ultrastructure and life cycles. In: Peters, W., Killick-Kendrick, R. (Eds.). *The leishmaniasis in Biology and Medicine* 1: 121-176

Momeni AZ, Jalayer T, Emamjomeh M, Khamesipour A, Zicker F, Ghassemi RL, Dowlati Y, Sharifi I, Aminjavaheri M, Shafiei A, Alimohammadian MH, Hashemi-Fesharki R, Nasseri K, Godal T, Smith PG and Modabber F (1999) A randomised, double-blind, controlled trial

of a killed L. major vaccine plus BCG against zoonotic cutaneous leishmaniasis in Iran. *Vaccine* 17(5): 466-72

Mondal D and Khan MG (2011) Recent advances in post-kala-azar dermal leishmaniasis. *Curr Opin Infect Dis* 24(5): 418-22

Mondal D, Singh SP, Kumar N, Joshi A, Sundar S, Das P, Siddhivinayak H, Kroeger A and Boelaert M (2009) Visceral leishmaniasis elimination programme in India, Bangladesh, and Nepal: reshaping the case finding/case management strategy. *PLoS Negl Trop Dis* 3(1): e355

Monroy-Ostria A and Sanchez-Tejeda G (2002) Molecular probes and the polymerase chain reaction for detection and typing of Leishmania species in Mexico. *Trans R Soc Trop Med Hyg* 96 Suppl 1: S101-4

Montenegro J (1926) Cutaneous reaction in leishmaniasis. *Arch Derm Syph* 13: 187-94

Morillas F, Sanchez Rabasco F, Ocana J, Martin-Sanchez J, Ocana-Wihelmi J, Acedo C and Sanchiz-Marin MC (1996) Leishmaniosis in the focus of the Axarquia region, Malaga province, southern Spain: a survey of the human, dog, and vector. *Parasitol Res* 82(6): 569-70

Murray HW, Berman JD, Davies CR and Saravia NG (2005) Advances in leishmaniasis. *Lancet* 366(9496): 1561-77

Murray HW, Brooks EB, DeVecchio JL and Heinzel FP (2003) Immunoenhancement combined with amphotericin B as treatment for experimental visceral leishmaniasis. *Antimicrob Agents Chemother* 47(8): 2513-7

Nagill R, Mahajan R, Sharma M and Kaur S (2009) Induction of cellular and humoral responses by autoclaved and heat-killed antigen of Leishmania donovani in experimental visceral leishmaniasis. *Parasitol Int* 58(4): 359-66

Napier LE (1922) A new serum test for kala-azar. *Indian Journal of medical research* 9: 830-846

Napier LE (1926) An epidemiological consideration of the transmission of kala-azar in India. *Indian Medical Research Memoirs* 4: 219-265

Nigro L, Rizzo ML, Vancheri C, La Rosa R, Mastruzzo C, Tomaselli V, Ragusa A, Manuele R and Cacopardo B (2007) CCR5 and CCR3 expression on T CD3+ lymphocytes from HIV/Leishmania co-infected subjects. *Med Microbiol Immunol* 196(4): 253-5

Noyes HA, Reyburn H, Bailey JW and Smith D (1998) A nested-PCR-based schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. *J Clin Microbiol* 36(10): 2877-81

Nuzum E, White F, 3rd, Thakur C, Dietze R, Wages J, Grogl M and Berman J (1995) Diagnosis of symptomatic visceral leishmaniasis by use of the polymerase chain reaction on patient blood. *J Infect Dis* 171(3): 751-4

Nylen S and Gautam S (2010) Immunological perspectives of leishmaniasis. *J Glob Infect Dis* 2(2): 135-46

Okong'o-Odera EA, Kurtzhals JA, Hey AS and Kharazmi A (1993) Measurement of serum antibodies against native Leishmania gp63 distinguishes between ongoing and previous L. donovani infection. *Apmis* 101(8): 642-6

Opperdoes FR (1990) The glycosome of trypanosomes and Leishmania. *Biochem Soc Trans* 18(5): 729-31

Oskam L, Nieuwenhuijs JL and Hailu A (1999) Evaluation of the direct agglutination test (DAT) using freeze-dried antigen for the detection of anti-Leishmania antibodies in stored sera from various patient groups in Ethiopia. *Trans R Soc Trop Med Hyg* 93(3): 275-7

Osman OF, Oskam L, Zijlstra EE, Kroon NC, Schoone GJ, Khalil ET, El-Hassan AM and Kager PA (1997) Evaluation of PCR for diagnosis of visceral leishmaniasis. *J Clin Microbiol* 35(10): 2454-7

Ostyn B, Gidwani K, Khanal B, Picado A, Chappuis F, Singh SP, Rijal S, Sundar S and Boelaert M (2011) Incidence of symptomatic and asymptomatic Leishmania donovani infections in high-endemic foci in India and Nepal: a prospective study. *PLoS Negl Trop Dis* 5(10): e1284

Otero AC, da Silva VO, Luz KG, Palatnik M, Pirmez C, Fernandes O and Palatnik de Sousa CB (2000) Short report: occurrence of Leishmania donovani DNA in donated blood from seroreactive Brazilian blood donors. *Am J Trop Med Hyg* 62(1): 128-31

Pagliano P, Carannante N, Rossi M, Gramiccia M, Gradoni L, Faella FS and Gaeta GB (2005) Visceral leishmaniasis in pregnancy: a case series and a systematic review of the literature. *J Antimicrob Chemother* 55(2): 229-33

Pal A, Mukerji K, Basu D, Naskar K, Mallick KK and Ghosh DK (1991) Evaluation of direct agglutination test (DAT) and ELISA for serodiagnosis of visceral leishmaniasis in India. *J Clin Lab Anal* 5(5): 303-6

Palatnik-de-Sousa CB, Gomes EM, Paraguai-de-Souza E, Palatnik M, Luz K and Borojevic R (1995) Leishmania donovani: titration of antibodies to the fucose-mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 89(4): 390-3

Pandey BD, Babu E, Thapa S and Thapa LB (2006) First case of cutanous leishmaniasis in Nepalese patient. *Nepal Med Coll J* 8(3): 213-4

Pandey BD, Pandey K, Kaneko O, Yanagi T and Hirayama K (2009) Relapse of visceral leishmaniasis after miltefosine treatment in a Nepalese patient. *Am J Trop Med Hyg* 80(4): 580-2

Pandey BD, Pun SB, Kaneko O, Pandey K and Hirayama K (2011) Case report: Expansion of visceral leishmaniasis to the western hilly part of Nepal. *Am J Trop Med Hyg* 84(1): 107-8

Parida M, Posadas G, Inoue S, Hasebe F and Morita K (2004) Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of West Nile virus. *J Clin Microbiol* 42(1): 257-63

Paris C, Loiseau PM, Bories C and Breard J (2004) Miltefosine induces apoptosis-like death in Leishmania donovani promastigotes. *Antimicrob Agents Chemother* 48(3): 852-9

Parra LE, Borja-Cabrera GP, Santos FN, Souza LO, Palatnik-de-Sousa CB and Menz I (2007) Safety trial using the Leishmune vaccine against canine visceral leishmaniasis in Brazil. *Vaccine* 25(12): 2180-6

Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, Colombara D, Abdoon AO, Mukhtar MM, Mondal D, Esfandiari J, Kumar S, Chun P, Reed SG and Bhatia A (2010) Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. *PLoS Negl Trop Dis* 4(9)

Pintado V and Lopez-Velez R (2001) HIV-associated visceral leishmaniasis. *Clin Microbiol Infect* 7(6): 291-300

Pintado V, Martin-Rabadan P, Rivera ML, Moreno S and Bouza E (2001) Visceral leishmaniasis in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. A comparative study. *Medicine (Baltimore)* 80(1): 54-73

Prajapati VK, Awasthi K, Yadav TP, Rai M, Srivastava ON and Sundar S (2011) An oral formulation of amphotericin B attached to functionalized carbon nanotubes is an effective treatment for experimental visceral leishmaniasis. *J Infect Dis* 205(2): 333-6

Puentes SM, Da Silva RP, Sacks DL, Hammer CH and Joiner KA (1990) Serum resistance of metacyclic stage Leishmania major promastigotes is due to release of C5b-9. *J Immunol* 145(12): 4311-6

Pun SB, Sato T, Pandey K and Pandey BD (2011) Changing trends in visceral leishmaniasis: 10 years' experience at a referral hospital in Nepal. *Trans R Soc Trop Med Hyg* 105(10): 550-4

Rai RN and Sundar S (1996) Epidemiology of Kala-azar in India. *In: Indian Kala-azar* 1-9

Raj VS, Ghosh A, Dole VS, Madhubala R, Myler PJ and Stuart KD (1999) Serodiagnosis of leishmaniasis with recombinant ORFF antigen. *Am J Trop Med Hyg* 61(3): 482-7

Ramesh V and Mukherjee A (1995) Post-kala-azar dermal leishmaniasis. *Int J Dermatol* 34(2): 85-91

Ravindran R, Anam K, Bairagi BC, Saha B, Pramanik N, Guha SK, Goswami RP, Banerjee D and Ali N (2004) Characterization of immunoglobulin G and its subclass response to Indian kala-azar infection before and after chemotherapy. *Infect Immun* 72(2): 863-70

Ray DS (1989) Conserved sequence blocks in kinetoplast minicircles from diverse species of trypanosomes. *Mol Cell Biol* 9(3): 1365-7

Reed SG, Badaro R and Lloyd RM (1987) Identification of specific and cross-reactive antigens of Leishmania donovani chagasi by human infection sera. *J Immunol* 138(5): 1596-601

Reiner SL and Locksley RM (1993) Cytokines in the differentiation of Th1/Th2 CD4+ subsets in leishmaniasis. *J Cell Biochem* 53(4): 323-8

Remer KA, Apetrei C, Schwarz T, Linden C and Moll H (2007) Vaccination with plasmacytoid dendritic cells induces protection against infection with Leishmania major in mice. *Eur J Immunol* 37(9): 2463-73

Renart J, Reiser J and Stark GR (1979) Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: a method for studying antibody specificity and antigen structure. *Proc Natl Acad Sci U S A* 76(7): 3116-20

Reynes J, Portales P, Segondy M, Baillat V, Andre P, Avinens O, Picot MC, Clot J, Eliaou JF and Corbeau P (2001) CD4 T cell surface CCR5 density as a host factor in HIV-1 disease progression. *Aids* 15(13): 1627-34

Riera C, Fisa R, Udina M, Gallego M and Portus M (2004) Detection of Leishmania infantum cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. *Trans R Soc Trop Med Hyg* 98(2): 102-10

Rijal S, Boelaert M, Regmi S, Karki BM, Jacquet D, Singh R, Chance ML, Chappuis F, Hommel M, Desjeux P, Van der Stuyft P, Le Ray D and Koirala S (2004) Evaluation of a urinary antigen-based latex agglutination test in the diagnosis of kala-azar in eastern Nepal. *Trop Med Int Health* 9(6): 724-9

Rijal S, Uranw S, Chappuis F, Picado A, Khanal B, Paudel IS, Andersen EW, Meheus F, Ostyn B, Das ML, Davies C and Boelaert M (2010) Epidemiology of Leishmania donovani infection in high-transmission foci in Nepal. *Trop Med Int Health* 15 Suppl 2: 21-8

Rijal S. (2006) Kala-azar in Nepal: from clinical evidence to control. Publishers APS, Antwerpen. pp. 1-142

Ritmeijer K, Melaku Y, Mueller M, Kipngetich S, O'Keeffe C and Davidson RN (2006) Evaluation of a new recombinant K39 rapid diagnostic test for Sudanese visceral leishmaniasis. *Am J Trop Med Hyg* 74(1): 76-80

Rosenthal E, Marty P, Poizot-Martin I, Reynes J, Pratlong F, Lafeuillade A, Jaubert D, Boulat O, Dereure J, Gambarelli F and et al. (1995) Visceral leishmaniasis and HIV-1 co-infection in southern France. *Trans R Soc Trop Med Hyg* 89(2): 159-62

Ross R. (1899) Report on the nature of kala azar. Printing OotSoG, Culcutta, India.

Russell DG and Talamas-Rohana P (1989) Leishmania and the macrophage: a marriage of inconvenience. *Immunol Today* 10(10): 328-33

Ryan JR, Smithyman AM, Rajasekariah GH, Hochberg L, Stiteler JM and Martin SK (2002) Enzyme-linked immunosorbent assay based on soluble promastigote antigen detects immunoglobulin M (IgM) and IgG antibodies in sera from cases of visceral and cutaneous leishmaniasis. *J Clin Microbiol* 40(3): 1037-43

Sakru N, Korkmaz M, Ozbel Y, Ertabaklar H, Sengul M and Toz SO (2007) Investigation of asymptomatic visceral leishmaniasis cases using western blot in an endemic area in Turkey. *New Microbiol* 30(1): 13-8

Salam MA, Khan MG and Mondal D (2011) Urine antigen detection by latex agglutination test for diagnosis and assessment of initial cure of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 105(5): 269-72

Salotra P, Raina A and Ramesh V (1999) Western blot analysis of humoral immune response to Leishmania donovani antigens in patients with post-kala-azar dermal leishmaniasis. *Trans R Soc Trop Med Hyg* 93(1): 98-101

Salotra P, Sreenivas G, Pogue GP, Lee N, Nakhasi HL, Ramesh V and Negi NS (2001) Development of a species-specific PCR assay for detection of Leishmania donovani in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. *J Clin Microbiol* 39(3): 849-54

Sambrook J and Russell DW (2001). Molecular Cloning A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press. 1

Santa-Rita RM, Santos Barbosa H, Meirelles MN and de Castro SL (2000) Effect of the alkyl-lysophospholipids on the proliferation and differentiation of Trypanosoma cruzi. *Acta Trop* 75(2): 219-28

Santos WR, de Lima VM, de Souza EP, Bernardo RR, Palatnik M and Palatnik de Sousa CB (2002) Saponins, IL12 and BCG adjuvant in the FML-vaccine formulation against murine visceral leishmaniasis. *Vaccine* 21(1-2): 30-43

Santos-Gomes G, Gomes-Pereira S, Campino L, Araujo MD and Abranches P (2000) Performance of immunoblotting in diagnosis of visceral Leishmaniasis in human immunodeficiency virus-Leishmania sp.-coinfected patients. *J Clin Microbiol* 38(1): 175-8

Sarkari B, Chance M and Hommel M (2002) Antigenuria in visceral leishmaniasis: detection and partial characterisation of a carbohydrate antigen. *Acta Trop* 82(3): 339-48

Sassi A, Louzir H, Ben Salah A, Mokni M, Ben Osman A and Dellagi K (1999) Leishmanin skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic Leishmania major infection in Tunisia. *Clin Exp Immunol* 116(1): 127-32

Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F and Cassatella MA (2000) The neutrophil as a cellular source of chemokines. *Immunol Rev* 177: 195-203

Schaefer KU, Schoone GJ, Gachihi GS, Muller AS, Kager PA and Meredith SE (1995) Visceral leishmaniasis: use of the polymerase chain reaction in an epidemiological study in Baringo District, Kenya. *Trans R Soc Trop Med Hyg* 89(5): 492-5

Schallig HD and Oskam L (2002) Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. *Trop Med Int Health* 7(8): 641-51

Schenkel K, Rijal S, Koirala S, Vanlerberghe V, Van der Stuyft P, Gramiccia M and Boelaert M (2006) Visceral leishmaniasis in southeastern Nepal: a cross-sectional survey on Leishmania donovani infection and its risk factors. *Trop Med Int Health* 11(12): 1792-9

Schulz A, Mellenthin K, Schonian G, Fleischer B and Drosten C (2003) Detection, differentiation, and quantitation of pathogenic leishmania organisms by a fluorescence resonance energy transfer-based real-time PCR assay. *J Clin Microbiol* 41(4): 1529-35

Seder RA and Paul WE (1994) Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol* 12: 635-73

Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D and Nakhasi HL (2009) Intracellular replication-deficient Leishmania donovani induces long lasting protective immunity against visceral leishmaniasis. *J Immunol* 183(3): 1813-20

Sergent E, Sergent E, Parrott L, Donatien A and Beguet M (1921) Transmission du clou de Biskra par le phelobotome (Phlebotomus papatasi Scop.). *C r Seances Soc Biol* 73: 1030-2

Sharifi I, FeKri AR, Aflatonian MR, Khamesipour A, Nadim A, Mousavi MR, Momeni AZ, Dowlati Y, Godal T, Zicker F, Smith PG and Modabber F (1998) Randomised vaccine trial of single dose of killed Leishmania major plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. *Lancet* 351(9115): 1540-3

Sharma MC, Gupta AK, Das VN, Verma N, Kumar N, Saran R and Kar SK (2000) Leishmania donovani in blood smears of asymptomatic persons. *Acta Trop* 76(2): 195-6

Sharma U and Singh S (2008) Insect vectors of Leishmania: distribution, physiology and their control. *J Vector Borne Dis* 45(4): 255-72

Shatry AM, Oster CN, Mebrahtu YB, Perkins PV and Hendricks LD (1988) Mouse foot-pad inoculation as an aid to the isolation of Leishmania spp. from patients. *Trans R Soc Trop Med Hyg* 82(5): 701-3

Shaw JJ (1994) Taxonomy of the genus Leishmania: present and future trends and their implications. *Mem Inst Oswaldo Cruz* 89(3): 471-8

Sheline C and Ray DS (1989) Specific discontinuities in Leishmania tarentolae minicircles map within universally conserved sequence blocks. *Mol Biochem Parasitol* 37(2): 151-7

Shlomai J (1994) The assembly of kinetoplast DNA. *Parasitol Today.* 10(9): 341-6

Shreffler WG, Burns JM, Jr., Badaro R, Ghalib HW, Button LL, McMaster WR and Reed SG (1993) Antibody responses of visceral leishmaniasis patients to gp63, a major surface glycoprotein of Leishmania species. *J Infect Dis* 167(2): 426-30

Shrestha SL and Pant SK (1994) Seasonal distribution of Phlebotomine sand flies-vector of Visceral Leishmaniasis. *Journal of Nepal Medical Association* 32(237-246)

Simpson L (1986) Kinetoplast DNA in trypanosomid flagellates. *Int Rev Cytol* 99: 119-79

Simpson L (1997) The genomic organization of guide RNA genes in kinetoplastid protozoa: several conundrums and their solutions. *Mol Biochem Parasitol* 86(2): 133-41

Singh N, Nagar S, Roy U and Sundar S (1995) Antigenic analysis of soluble fraction of Leishmania donovani by SDS-PAGE and immunoblotting. *Indian J Exp Biol* 33: 313-315

Singh R, Subba Raju BV, Jain RK and Salotra P (2005) Potential of direct agglutination test based on promastigote and amastigote antigens for serodiagnosis of post-kala-azar dermal leishmaniasis. *Clin Diagn Lab Immunol* 12(10): 1191-4

Singh S (2006) New developments in diagnosis of leishmaniasis. *Indian J Med Res* 123(3): 311-30

Singh S and Sivakumar R (2003) Recent advances in the diagnosis of leishmaniasis. *J Postgrad Med* 49(1): 55-60

Singh S, Dey A and Sivakumar R (2005) Applications of molecular methods for Leishmania control. *Expert Rev Mol Diagn* 5(2): 251-65

Singh S, Kumari V and Singh N (2002) Predicting kala-azar disease manifestations in asymptomatic patients with latent Leishmania donovani infection by detection of antibody against recombinant K39 antigen. *Clin Diagn Lab Immunol* 9(3): 568-72

Singla N, Singh GS, Sundar S and Vinayak VK (1993) Evaluation of the direct agglutination test as an immunodiagnostic tool for kala-azar in India. *Trans R Soc Trop Med Hyg* 87(3): 276-8

Sinha R and Sehgal S (1994) Comparative evaluation of serological tests in Indian kala-azar. *J Trop Med Hyg* 97(6): 333-40

Sivakumar R, Sharma P, Chang KP and Singh S (2006) Cloning, expression, and purification of a novel recombinant antigen from Leishmania donovani. *Protein Expr Purif* 46(1): 156-65

Sokal JE (1975) Editorial: Measurement of delayed skin-test responses. *N Engl J Med* 293(10): 501-2

Squier MK, Sehnert AJ and Cohen JJ (1995) Apoptosis in leukocytes. *J Leukoc Biol* 57(1): 2-10

Srivastava P, Dayama A, Mehrotra S and Sundar S (2010) Diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 105(1): 1-6

Srivastava P, Mehrotra S, Tiwary P, Chakravarty J and Sundar S (2011) Diagnosis of Indian visceral leishmaniasis by nucleic acid detection using PCR. *PLoS One* 6(4): e19304

Srividya G, Kulshrestha A, Singh R and Salotra P (2011) Diagnosis of visceral leishmaniasis: developments over the last decade. *Parasitol Res*

Stager S, Maroof A, Zubairi S, Sanos SL, Kopf M and Kaye PM (2006) Distinct roles for IL-6 and IL-12p40 in mediating protection against Leishmania donovani and the expansion of IL-10+ CD4+ T cells. *Eur J Immunol* 36(7): 1764-71

Stanford. (2012) History. [Online] <http://www.stanford.edu/class/humbio103/ParaSites2006/Leishmaniasis/history.htm >

Strauss-Ayali D, Jaffe CL, Burshtain O, Gonen L and Baneth G (2004) Polymerase chain reaction using noninvasively obtained samples, for the detection of Leishmania infantum DNA in dogs. *J Infect Dis* 189(9): 1729-33

Sundar S (2003) Diagnosis of kala-azar--an important stride. *J Assoc Physicians India* 51: 753-5

Sundar S and Benjamin B (2003) Diagnosis and treatment of Indian visceral leishmaniasis. *J Assoc Physicians India* 51: 195-201

Sundar S and Chakravarty J (2008) Paromomycin in the treatment of leishmaniasis. *Expert Opin Investig Drugs* 17(5): 787-94

Sundar S and Chatterjee M (2006) Visceral leishmaniasis - current therapeutic modalities. *Indian J Med Res* 123(3): 345-52

Sundar S and Rai M (2002) Laboratory diagnosis of visceral leishmaniasis. *Clin Diagn Lab Immunol* 9(5): 951-8

Sundar S, Kumar K, Singh VP and Mahopatra TM (1991) Diagnostic lag period in kala-azar: test for early diagnosis needed. *J Assoc Physicians India* 39(8): 651

Sundar S, Reed SG, Sharma S, Mehrotra A and Murray HW (1997) Circulating T helper 1 (Th1) cell- and Th2 cell-associated cytokines in Indian patients with visceral leishmaniasis. *Am J Trop Med Hyg* 56(5): 522-5

Sundar S, Reed SG, Singh VP, Kumar PC and Murray HW (1998) Rapid accurate field diagnosis of Indian visceral leishmaniasis. *Lancet* 351(9102): 563-5

Sundar S, Singh RK, Maurya R, Kumar B, Chhabra A, Singh V and Rai M (2006) Serological diagnosis of Indian visceral leishmaniasis: direct agglutination test versus rK39 strip test. *Trans R Soc Trop Med Hyg* 100(6): 533-7

Swaminath CS, Shortt HE and Anderson LAP (1942) Transmission Indian Kala-azar to man by the bites of Phlebotomus argentipes. *Indian J Med Res* 30(473-7)

Tanaka T, Hu-Li J, Seder RA, Fazekas de St Groth B and Paul WE (1993) Interleukin 4 suppresses interleukin 2 and interferon gamma production by naive T cells stimulated by accessory cell-dependent receptor engagement. *Proc Natl Acad Sci U S A* 90(13): 5914-8

Tavares CA, Fernandes AP and Melo MN (2003) Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn* 3(5): 657-67

Tebourski F, el Gaied A, Louzir H, Ben Ismail R, Kammoun R and Dellagi K (1994) Identification of an immunodominant 32-kilodalton membrane protein of Leishmania donovani infantum promastigotes suitable for specific diagnosis of Mediterranean visceral leishmaniasis. *J Clin Microbiol* 32(10): 2474-80

Tewary P, Sukumaran B, Saxena S and Madhubala R (2004) Immunostimulatory oligodeoxynucleotides are potent enhancers of protective immunity in mice immunized with recombinant ORFF leishmanial antigen. *Vaccine* 22(23-24): 3053-60

Thakur CP (1997) A comparison of intercostal and abdominal routes of splenic aspiration and bone marrow aspiration in the diagnosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 91(6): 668-70

Topno RK, Das VN, Ranjan A, Pandey K, Singh D, Kumar N, Siddiqui NA, Singh VP, Kesari S, Bimal S, Kumar AJ, Meena C, Kumar R and Das P (2010) Asymptomatic infection with visceral leishmaniasis in a disease-endemic area in bihar, India. *Am J Trop Med Hyg* 83(3): 502-6

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76(9): 4350-4

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76(9): 4350-4

Tsuji-Takayama K, Aizawa Y, Okamoto I, Kojima H, Koide K, Takeuchi M, Ikegami H, Ohta T and Kurimoto M (1999) Interleukin-18 induces interferon-gamma production through NF-kappaB and NFAT activation in murine T helper type 1 cells. *Cell Immunol* 196(1): 41-50

Turgay N, Balcioglu IC, Toz SO, Ozbel Y and Jones SL (2010) Quantiferon-Leishmania as an epidemiological tool for evaluating the exposure to Leishmania infection. *Am J Trop Med Hyg* 83(4): 822-4

Uranw S, Ostyn B, Rijal A, Devkota S, Khanal B, Menten J, Boelaert M and Rijal S (2011) Post-Kala-azar Dermal Leishmaniasis in Nepal: A Retrospective Cohort Study (2000-2010). *PLoS Negl Trop Dis* 5(12): e1433

Vianna G (1911) Sobre uma nova espe' cie de *Leishmania*. *Bras. Med.* 25: 411

Vinayak VK, Mahajan D, Sobti RC, Singla N and Sundar S (1994) Anti-66 kDa antileishmanial antibodies as specific immunodiagnostic probe for visceral leishmaniasis. *Indian J Med Res* 99: 109-14

Voet D and Voet JG (2004). Biochemistry. New Jershey, United States of Amaerica, John Wiley & Sons Inc.

Volpini AC, Passos VM, Oliveira GC and Romanha AJ (2004) PCR-RFLP to identify Leishmania (Viannia) braziliensis and L. (Leishmania) amazonensis causing American cutaneous leishmaniasis. *Acta Trop* 90(1): 31-7

Webster P and Russell DG (1993) The flagellar pocket of trypanosomatids. *Parasitol Today* 9(6): 201-6

Werneck GL (2008) Forum: geographcal spread and urbanization of visceral leishmaniasis in Brazil. Introduction. *Cad Saude Publica.* 24(2): 2937-2940

Werneck GL, Batista MS, Gomes JR, Costa DL and Costa CH (2003) Prognostic factors for death from visceral leishmaniasis in Teresina, Brazil. *Infection* 31(3): 174-7

Wheeler RJ. (2005) Diagram illustrating the process of nested PCR. [Online] <http://en.wikipedia.org/wiki/Nested_PCR>

WHO (1990) Control of the leishmaniases. Report of a WHO Expert Committee. *World Health Organ Tech Rep Ser* 793: 1-158

WHO (1996) Mannual on Visceral Leishmaniasis Control. *World Health Organization, Division of Control of Tropical Diseases*

WHO (2008). Kala-azar elimination in the South-East Asia Region: Training module. Indraprastha Estate, Mahatma Gandhi Marg,New Delhi 110 002, India, World Health Organization, Regional Office for South-East Asia: pp: 1-83

WHO (2011) Visceral Leishmaniasis Rapid Diagnostic Test Performance. *World Health Organization, Avenue Appia, 1211 Geneva, Switzerland*: 1-46

WHO (2013) Leishmaniasis.

WHO. (1998) Advances in the batle against leishmaniasis. *TDR New.* 57(2)

WHO. (2001) The World Health Report 2001. WHO Geneva.: pp.

WHO. (2010) Control of leishmaniases: Report of a meeting of the WHO Expeert Committee on the Control of Leishmaniases. 20 Avenue Appia, 1211 Geneva 27, Switzerland. pp. 1-202

Wilson R. Arthropod Vectors of Human Diseases. [Online] <http://www.raywilsonbirdphotography.co.uk/Galleries/Invertebrates/vectors/sand_fly.html>

Wolday D, Berhe N and Britton S (1999) Leishmania-HIV interaction: Immunopathogenic mechanisms. *Parasitol Today* 15(5): 182-187

Wortmann G, Sweeney C, Houng HS, Aronson N, Stiteler J, Jackson J and Ockenhouse C (2001) Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. *Am J Trop Med Hyg* 65(5): 583-7

Wyllie S, Cunningham ML and Fairlamb AH (2004) Dual action of antimonial drugs on thiol redox metabolism in the human pathogen Leishmania donovani. *J Biol Chem* 279(38): 39925-32

Zhao C, Papadopoulou B and Tremblay MJ (2004) Leishmania infantum enhances human immunodeficiency virus type-1 replication in primary human macrophages through a complex cytokine network. *Clin Immunol* 113(1): 81-8

Zhao C, Papadopoulou B and Tremblay MJ (2004) Leishmania infantum promotes replication of HIV type 1 in human lymphoid tissue cultured ex vivo by inducing secretion of the proinflammatory cytokines TNF-alpha and IL-1 alpha. *J Immunol* 172(5): 3086-93

Zijlstra EE, Ali MS, el-Hassan AM, el-Toum IA, Satti M, Ghalib HW and Kager PA (1992) Kala-azar: a comparative study of parasitological methods and the direct agglutination test in diagnosis. *Trans R Soc Trop Med Hyg* 86(5): 505-7

Zijlstra EE, Daifalla NS, Kager PA, Khalil EA, El-Hassan AM, Reed SG and Ghalib HW (1998) rK39 enzyme-linked immunosorbent assay for diagnosis of Leishmania donovani infection. *Clin Diagn Lab Immunol* 5(5): 717-20

Zijlstra EE, el-Hassan AM, Ismael A and Ghalib HW (1994) Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. *Am J Trop Med Hyg* 51(6): 826-36

Zijlstra EE, Khalil EA, Kager PA and El-Hassan AM (2000) Post-kala-azar dermal leishmaniasis in the Sudan: clinical presentation and differential diagnosis. *Br J Dermatol* 143(1): 136-43

Zijlstra EE, Musa AM, Khalil EA, el-Hassan IM and el-Hassan AM (2003) Post-kala-azar dermal leishmaniasis. *Lancet Infect Dis* 3(2): 87-98

Appendices

RPMI media

RPMI powder	10.4 gm
$NaHCO_3$	2 gm
HEPES	1.40 gm
L-Glutamine	2 mM
Gentamycin	20 µg/ml
Streptomycin	100 mg
Penicillin	100 U/ml
pH	7.2-7.4
TDW	1000ml

PBS (Phosphate Buffer Saline)

NaCl	8 gm
$Na_2HPO_4.2H_2O$	1.44 gm
KCl	0.2 gm
KH_2PO_4	0.2 gm
pH	7.3 to 7.4
TDW	1000ml

Preparation of Giemsa stain

Giemsa stock solution:

giemsa powder	1 gm
glycerol	60 ml
methanol	66 ml

Giemsa buffer:

Na_2HPO_4	9.5 gm/L
KH_2PO_4	9.07 gm/L

Giemsa stain:

10% giemsa stock in giemsa buffer

Bradford Reagent

Coomassie Brialliant Blue	10mg
Ethanol (95%)	10ml
Orthophosphoric acid (85%)	10ml
DW	to 100ml

SDS-PAGE

12% Resolving gel

TDW	3.3ml	4.9ml
30% Acrylamide	4ml	6ml
1.5% Tris (P^H 8.8)	2.5ml	3.8ml
10% SDS	0.1ml	0.15ml
10% $(NH_4)_2S_2O_8$	0.1ml	0.15ml
TEMED	0.004ml	0.006ml
Final volume	10ml	15ml

5% Stacking gel

TDW	2.1ml	2.7ml
30% Acrylamide	0.5ml	0.67ml
1.5% Tris (P^H 6.8)	0.38ml	0.5ml
10% SDS	0.03ml	0.04ml
10% $(NH_4)_2S_2O_8$	0.03ml	0.04ml
TEMED	0.003ml	0.004ml
Final volume	3ml	4ml

Phosphate Glucose Saccharose buffer (PGS)

$Na_2HPO_4.2H_2O$	0.67 gm
$NaH_2PO_4.H_2O$	0.27 gm
P^H	8.0

Just before use, add

Anhydrous glucose	15 gm
Anhydrous saccharose	34.98 gm
TDW	1000ml

DAT diluent

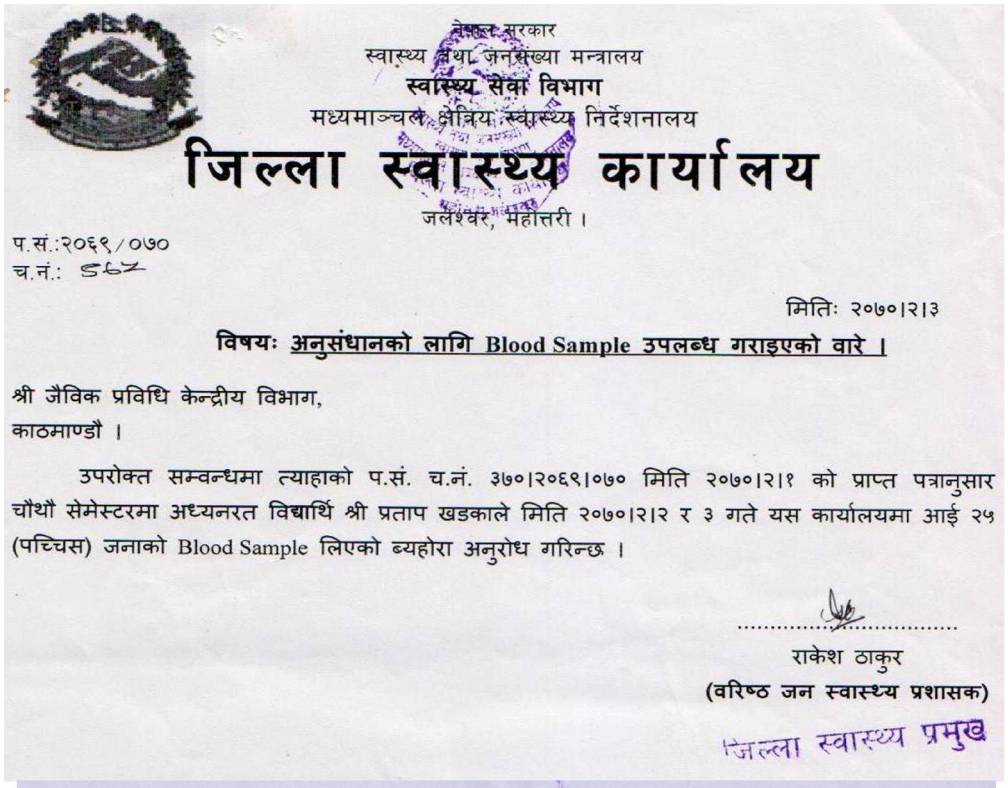
Sodium citrate	0.056M
Sodium chloride	0.15M
Gelatin	0.2%

Heated at 56^{o}C for 10 min. to dissolve gelatin and left to cool at room temperature

2-Mercaptoethanol	0.1M

TAE **50X**

Tris base	242 gm
Glacial acetic acid	57.1ml
0.5M EDTA	100ml
P^H	8.0



नेपाल सरकार
स्वास्थ्य तथा जनसंख्या मन्त्रालय
स्वास्थ्य सेवा विभाग
मध्यमाञ्चल क्षेत्रिय स्वास्थ्य निर्देशनालय

जिल्ला स्वास्थ्य कार्यालय

जलेश्वर, महोत्तरी ।

प.सं.:२०६९/०७०
च.नं.: ८६२

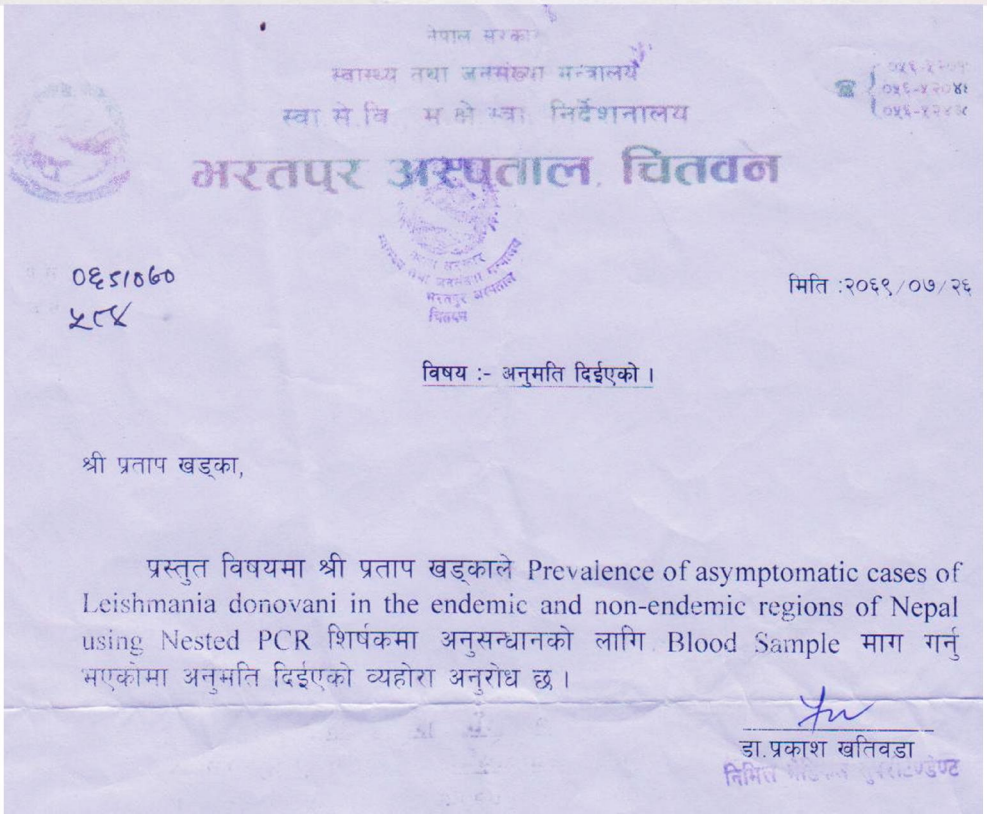
मितिः २०७०।२।३

विषयः <u>अनुसंधानको लागि Blood Sample उपलब्ध गराइएको वारे ।</u>

श्री जैविक प्रविधि केन्द्रीय विभाग,
काठमाण्डौ ।

उपरोक्त सम्वन्धमा त्याहाको प.सं. च.नं. ३७०।२०६९।०७० मिति २०७०।२।१ को प्राप्त पत्रानुसार चौथौ सेमेस्टरमा अध्यनरत विद्यार्थि श्री प्रताप खडकाले मिति २०७०।२।२ र ३ गते यस कार्यालयमा आई २५ (पच्चिस) जनाको Blood Sample लिएको ब्यहोरा अनुरोध गरिन्छ ।

.....
राकेश ठाकुर
(वरिष्ठ जन स्वास्थ्य प्रशासक)
जिल्ला स्वास्थ्य प्रमुख



नेपाल सरकार
स्वास्थ्य तथा जनसंख्या मन्त्रालय
स्वा से वि म को ग्रा निर्देशनालय

भरतपुर अस्पताल, चितवन

०६५/०७०
५८४

मिति :२०६९/०७/२६

<u>विषय :- अनुमति दिईएको ।</u>

श्री प्रताप खड्का,

प्रस्तुत विषयमा श्री प्रताप खड्काले Prevalence of asymptomatic cases of Leishmania donovani in the endemic and non-endemic regions of Nepal using Nested PCR शिर्षकमा अनुसन्धानको लागि Blood Sample माग गर्नु भएकोमा अनुमति दिईएको व्यहोरा अनुरोध छ ।

डा.प्रकाश खतिवडा

Fig. Permission granted for blood collection

सहमति पत्र

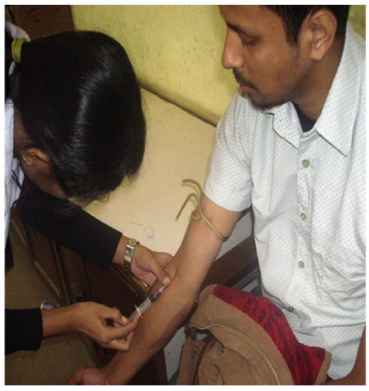
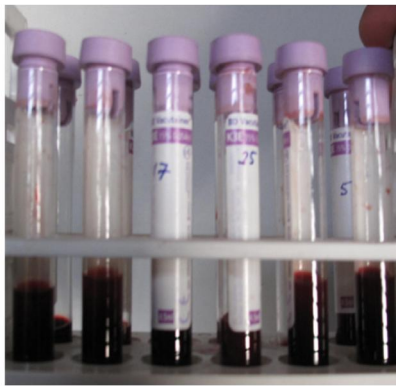
म.....जौरीगञ्ज चितवन.....मा स्थाई बसोवास गर्ने श्री/श्रीमती/सुश्री.....उज्जल श्रेष्ठ, २६ CM).....लाई जैविक प्रविधि केन्द्रिय विभाग त्रिभुवन विश्वविद्यालयले गर्न लागेको अनुसन्धान ***नेपालमा कालाज्वर रोगबाट पिडित हुन सक्ने जनसंख्याको अध्ययन***को उदेश्यहरुको बारेमा र सो अनुसन्धान गर्ने प्रविधि बारे स्पष्ट जानकारी गराउनु भयो । सो जानकारी बमोजिम यस अनुसन्धानबाट प्रकोप र अन्य क्षेत्रमा बसोवास गर्ने नेपालीलाई सहयोग पुग्ने कुरा बुझें । मेरो जिज्ञासाको सबै प्रश्नहरु सोध्ने मेरो अधिकार बमोजिम मैले सोधेका प्रश्नहरुको सन्तुष्ट जवाफ पाएँ । अतः उक्त अनुसन्धानको लागि म आफ्नो रगत दिन मन्जुरी जनाएकोछु । मलाई मेरो रगतको जाँचबाट निस्केको रिपोर्ट पछि सो अनुसन्धानको लागि सुरक्षित राखेमा मेरो कुनै आपत्ति रहने छैन । म आफ्नो उपचारमा बाधा नपर्ने गरी यस अनुसन्धानबाट बाहिरिन पनि सक्छु र मेरो नामलाई गोप्य राखिने कुरामा म विश्वस्त छु ।

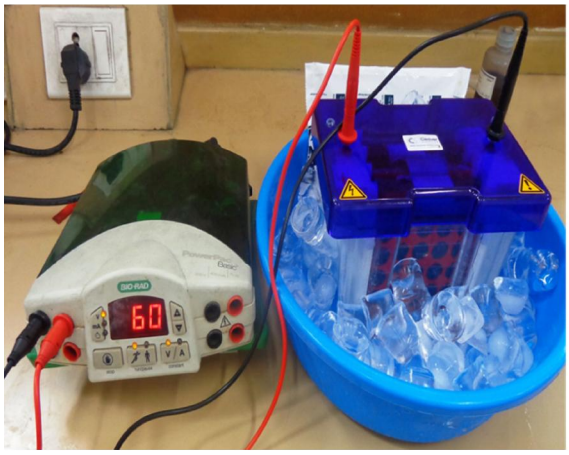
स्थान : भरतपुर चितवन

मिति : २०६५.०७.२७

दस्तखत :

साक्षी (निरक्षरको लागि):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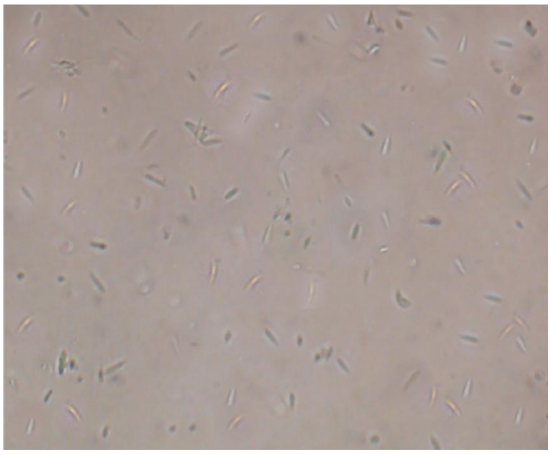


जलेश्वर अस्पताल