IMMUNO-MOLECULAR STUDY OF CUTANEOUS LEISHMNIASIS: AN ALARMING AND EMERGING DISEASE OF NEPAL

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Submitted to
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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/her original findings. I/we, hereby, recommend this thesis for final evaluation.

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Certificate of Evaluation

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Dedicated

To

My beloved Parents

And

Our respected teachers

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Glossary Acronyms

APC:	Allophycocyanin
BLAST:	Basic Local Alignment Search Tool
CD:	Cluster of Differentiation
CL:	Cutaneous Leishmaniasis
CMI:	Cell mediated immunity
cRPMI:	Complete Roswell Park Memorial Institute medium
CSB:	Conserved Sequence Blocks
DCL:	Diffuse Cutaneous Leishmaniasis
DCs:	Dendritic cells
DNA:	Deoxyribonucleic acid
DTH:	Delayed-type hypersensitivity
EDTA:	Ethylenediamine Tetraacetic Acid
ELISA:	Enzyme-linked immunosorbent assay
FACS:	Fluorescence Assisted Cell Sorting
FITC:	Fluorescence isothiocyanate
FRET:	Fluorescence Resonance Energy Transfer
HSPs:	Heat shock proteins
IL:	Interleukin
INF-γ:	Interferron-γ
iNOS:	isoform of nitric oxide synthase
LPG:	lipophosphoglycan
MCL:	Mucocutaneous Leishmaniasis
MEGA7:	Molecular Evolutionary Genetics Analysis Version 7
NCBI:	National Center for Biotechnology Information
NETs:	Neutrophil Extracellular Traps
NGMCTH:	Nepalgunj Medical College and Teaching hospital

NHRC:	Nepal Health Research Council
NK:	Natural Killer
NNN:	Novy-McNeal Nicolle medium
NOS:	Nitric oxide synthase
PBMC:	Peripheral Blood Mononuclear Cells
PBS:	Phosphate buffer saline
PCR:	Polymerase Chain Reaction
PE:	Phycoerythrin
PerCp:	Peridinin-Chlorophyll-protein
PKDL:	Post Kala-azar Dermal Leishmaniasis
RPMI:	Rosewell Park Memorial Institute medium
STIDH:	Sukraraj Tropical and Infectious Disease Hospital
TAE:	Tris base, acetic acid and EDTA
TB	Tuberculosis
Th1/Th2:	T helper1/ Thelper2
TNF:	Tumor Necrosis Factor
Treg:	regulatory T cells
VL:	Visceral Leishmaniasis
WHO:	World Health Organization

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Abstract

Immuno-molecular study of cutaneous leishmaniasis: an alarming and emerging disease of Nepal.

Cutaneous Leishmaniasis is a vector borne, parasitic disease caused by the bite of an infected sand fly. The disease is rare in Nepal with only few cases reported till date. The diagnosis of CL might be difficult, in particular in areas where the disease is uncommon and where other skin diseases with similar clinical symptoms occur. The main objective of the study is to improve the diagnosis of CL and the identification of causal Leishmania species and studying the immune response of the patients. A total of 17 patients presenting with cutaneous lesions suggestive of CL were sampled for parasitological diagnosis by direct examination (DE), kinetoplast DNA (kDNA) nested PCR (CSB1X/CSB2X and 13Z/LiR primers). Immunological status was analyzed by flow cytometer and compared with healthy controls. The data were statistically analyzed using graph pad prism ver.7. A total of 17 patients with age ranging from 8 years to 85 years were included in the study. Most patients were seen from January to September and had 1-3 lesions (duration = 3 to > 12 months) that involved mainly the face (64.70%). Mean age was 36 ± 22.05 years. Most patients were in the age group 21-40 years (35.29%) followed by ≤20 years (29.41%). Male: Female ratio was 1.4:1. LD bodies were observed in (11.76%) of the cases. Out of 17 cases only 5.88% had travel history outside Nepal in Leishmaniasis endemic country. PCR positive cases was 58.82%. The diagnostic criteria of CL in KDNA-PCR were based on observation of 720bp, 680bp and 560bp for *Leishmania donovani* complex, *Leishmania infantum* and *Leishmania major* respectively. Immunological study showed significant difference in $CD8^+$ T cells in CL patients with active lesions but not in $CD4^+$ T cells and in B cells between the CL patients and the healthy controls. Cutaneous leishmaniasis is not common in Nepal. So, it is often neglected and misdiagnosed. It is in an increasing trend. The PCR-based assays used increased the speed and sensitivity of the diagnosis of CL as well as in species identification compared to the conventional techniques.

Key words: Cutaneous leishmaniasis, kinetoplast DNA, flow cytometer, Leishmania, nested PCR.

Chapter I

INTRODUCTION

1.1 Background

Leishmaniasis remain one of the world's most devastating neglected tropical diseases (NTDs) (Mouttaki et al., 2014). The disease is caused by an obligate intracellular protozoan parasites of the genus *Leishmania*. This parasites typically lives in infected sand flies and are carried by 30 species of female sand fly that belongs to the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world (Desjeux, 2004; Singh et al., 2018;). A total of about 21 *Leishmania* spp. have been identified to be pathogenic to human (Aransay et al., 2000) and is transmitted by 30 species of sandfly (Herwaldt, 1999). These sand flies that carry the parasite typically reside in tropical and subtropical environments.

According to the World Health Organization (WHO), up to 350 million people are at risk in 98 countries around the world. It is considered that approximately 12 million people are currently infected, and 2 million new infections occur every year. In most countries, the incidence numbers are probably underestimated because cases are not recognized and reporting is not mandatory (Mcgwire & Satoskar, 2014, Mouttaki et al., 2014) of which an estimated 1.5 million cases are cutaneous (Alvar et al., 2012).

The clinical manifestations of leishmaniasis depend on complex interactions between the virulence characteristics of the infecting *Leishmania* species and the immune responses of its human host. The result is a spectrum of disease ranging from localized skin lesions to diffuse involvement of the reticuloendothelial system (Sharma & Singh, 2008). Leishmaniasis is categorized into different forms; different types of species and strains of Leishmania parasite are involved in such cases. The severity of leishmaniasis ranges from Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL) to Diffuse Cutaneous Leishmaniasis (DCL) and Visceral Leishmaniasis (VL) to Post Kala azar Dermal Leishmaniasis (PKDL) depending upon the parasite species.

1.2 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, which had been found graved in the 10th century (Manson-Bahr, 1986). CL and MCL (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).

Old World Leishmaniasis or Kala-azar was first noted in Jessore (now in Bangladesh) in India in 1825 (Elliott, 1863; Gibson, 1983; Cox, 2002). In the year following 1858 an epidemic of quinine-resistant fever was reported in the district Burdwan (Gibson, 1983). The cause remained unidentified (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 with Kala-azar(Hoare, 1938; Kean et al., 1978). 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 the context of Nepal, 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). 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 Kala-azar to be endemic in the southern Terai (Shrestha and Pant, 1994). In Nepal, VL was officially recorded in 1980 from Dhanush 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) (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). In Nepal, the cases of CL has not any long time history. The first imported case of CL was reported by Parija et al., (Parija et al., 1998) and the first reported case of CL in Nepal was in 2006. (Pandey et al., 2006).

1.3 Leishmania Parasite

1.3.1 Systematic position

Kingdom: Protista (Haeckel, 1866)
Sub-Kingdom: Protozoa (Goldfuss, 1817)
Phylum: Sarcomastigophora (Honigberg and Balamuth, 1963)
Sub-Phylum: Mastigophora (Deising, 1866)
Class: Zoomastigophorea (Calkins, 1909)
Order: Kinetoplastida (Honigber, 1963, *emend*. Vickerman, 1976)
Sub order: Trypanosomatina (Kent, 1880)
Family: Trypanosomatidae (Doflein, 1901, *emend.* Grobben, 1905)
Genus: *Leishmania* (Ross, 1903)

1.3.2 Morphology of the parasite

Leishmania are one of the several genera within the family Trypanosomatidae, and are characterized by the possession of a kinetoplast, a unique form of mitochondrial DNA. (2).

Leishmania parasites exhibit a variety of different cell morphologies and a number of cell types (developmental forms) that are adapted to either the host or the vector (Sunter & Gull, 2017) and throughout the life cycle. The parasite is able to exist in two different morphological forms, i.e. dimorphic parasite: intracellular amastigote when it is in the vertebrate host (human), and extracellular promastigote when it exists in the invertebrate host, vector (sandfly)(Mishra *et al.,* 2009). In these two hosts, the *Leishmania* parasite adapts in a varied and heterogeneous environments, e.g.:

(i) temperature from 37oC in mammalian host to ambient temperature in sandfly and *in vitro*;
(ii) pH—from neutral to highly acidic in sandfly stomach and the macrophage phagolysosome;
(iii) nutrients and oxygen contents; and
(iv) to immune attack—complement, antibodies and T-lymphocytes (Singh *et al.,*2005, Mishra *et al.,* 2009).

This rapid adaptation to the environment must have been due to the ability of *Leishmania* to modulate the gene expression, which probably occurs by the specific gene amplification or by having several tandem repeat (Singh *et al.,* 2005).

1.3.3 Leptomonad form

This form is also known as promastigote form. Promastigotes are slender and spindle shaped, and flagellated form found in alimentary tract of sandflies, measuring about 15–20 µm ×1·5–3·5 µm with 15–28 µm flagellum. It is an extracellular and motile form. A long flagellum (about the body length) is projected externally at the anterior end. In the promastigote form, the nucleus lies at the centre, and in front of it are the kinetoplast, The kinetoplast is the unique features of these parasites with the presence of DNA containing granule located within the single mitochondrion and associated with the flagellar base. The parasite divides by longitudinal binary fission at 27°C in the sandfly. Promastigotes can be grown in vitro at 25°C temperature on NNN medium, which has a solid phase of blood agar and a liquid phase containing a physiologic salt solution (8). The mode of nutrition of parasite is absorptive, multiply asexually by binary fission and are morphologically similar to those grown in vitro.

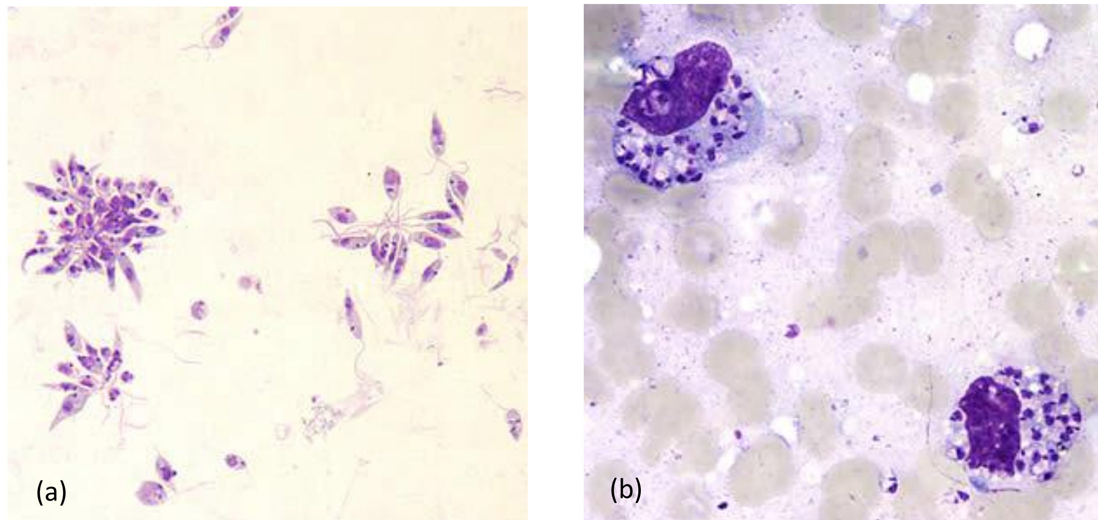


Fig 1.1: (a) Giemsa stained promastigotes (b) Giemsa stained Intracellular amastigotes
Source: CDC website

1.3.4 Leishmanial form

This form is also known as amastigote form. The amastigote, literally "without a flagellum," is the intracellular, non-motile form, resides the parasitophorous vacuole in the macrophages of the reticuloendothelial cells of vertebrate host, and divides by longitudinal binary fission at 37ºC. Intracellular amastigotes are 3-6 um in length and 1.5-3.0 um in width. The amastigote is also called the Leishman-Donovan (LD) body. The amastigote is not really devoid of a flagellum, it is simply that the flagellum does not protrude beyond the body surface and by light microscopy cannot be seen (8). The centrally located round/oval nucleus and transversely placed small rid shaped kinetoplast provides a distinctive feature. Kinetoplast is the dense mass of mitochondrial DNA and composed of several thousands of circular DNA molecules linked together in a catenated network (Shlomai, 1994).

1.3.5 Tranformation of forms

All leishmania parasite exist in two forms i.e. flagellated promastigote form in the gut of sandflies and non-flagellated amastigotes in the mammalian host. Promastigote-to-amastigote differentiation is a complex process that is accompanied by a number of morphological and biochemical changes. Parasites change shape from elongated to spherical and lose most of their flagellum. They undergo a major shift in metabolism, especially in the rate and pH optima for several processes, including DNA synthesis and nutrient uptake (Killick-Kendrick, 1990; Hommel 1999)

1.4 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 (Sharma and Singh, 2008). Out of 600 species of sandfly, only 30 species have been implicated in the transmission of leishmaniasis (Desjeux, 1996; Ashford, 1997). In 1921, it has been experimentally proved that sandflies of genus *Phlebotomus* are responsible for transmission of the disease (Sergent et al., 1921).

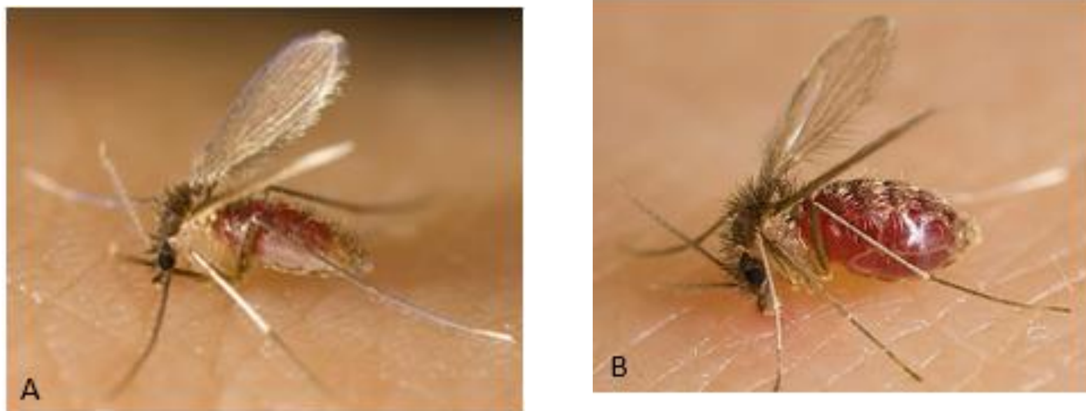


Fig1.2: Biting on human arm (A) *Phlebotomus argentipes* (B) *Lutzomyis longipalpis* Source: http://www.raywilsonbirdphotography.co.uk/Galleries/Invertebrates/vectors/sand_fly.html

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, and termitaria. Burrows of several reservoir hosts of leishmaniasis, damp portion of a cave and termite hills provide ideal habitats for the breeding of vector species. 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, HC, Dieldrin and Malthion, are effective in sandfly control.

1.5 Life cycle of Leishmania parasite

As part of their life cycle, Leishmania alternate between the alimentary tract of the sandfly vector (where they grow as extracellular flagellated promastigotes and differentiate into infective non-dividing metacyclic forms) and the phagolysosome of the vertebrate host macrophages (where parasites differentiate into aflagellated replicative amastigotes).

Important morphological and biochemical changes underlie the differentiations involved in the life cycle and are most likely the result of regulated changes in gene expression in response to environmental signals (e.g. temperature change and pH shift)(Adaui et al., 2011, Saxena et al., 2007, Singh *et al.,* 2005).When an invertebrate host (female sandfly) 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). The non-flagellated amastigotes convert to flagellated promastigote which keep on dividing by binary fission and transform into procyclic promastigotes in posterior midgut of sandfly. Procyclic promastigotes continue to divide and transform to nectomonad forms. After 3 days, these migratory forms that accumulate at the anterior end of peritrophic matrix, breakout of the blood meal facilitated by the action of parasite secretory cartilage (Schlein et al., 1991). Then they move forwards to the anterior midgut of the host (Killick-Kendrick, 1990;Lang and Kaye, 1991), until they reach the stomodeal valve (cardia) that guard the junction between foregut and midgut. These nectomonad promastigotes mediate the establishment phase of the infection that makes a true vector i.e. persistence beyond the blood meal and avoidance of expulsion during defecation. Once they reach the stomodeal valve the nectomonads transform to leptomonads and the leptomonads secret (Promastigotes Secreting Gel) PSG important in transmission. After 5 days in the anterior midgut, some of the nectomonads/leptomonads differentiate into haptomonads and attach to the stomodeal valve. From 5th days 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 probpscis. Within the human host, the promastigotes transforms to amastigote and continue to grow and divide by binary fission within the phagolysosomal compartment. The heavily populated phagolysosomes bound to burst releasing amastigotes in blood circulation. From the blood they reach to liver, spleen, bone marrow and lymph node. These parasites again will be taken by sandfly to be transmitted to new host and cycle thus repeats.

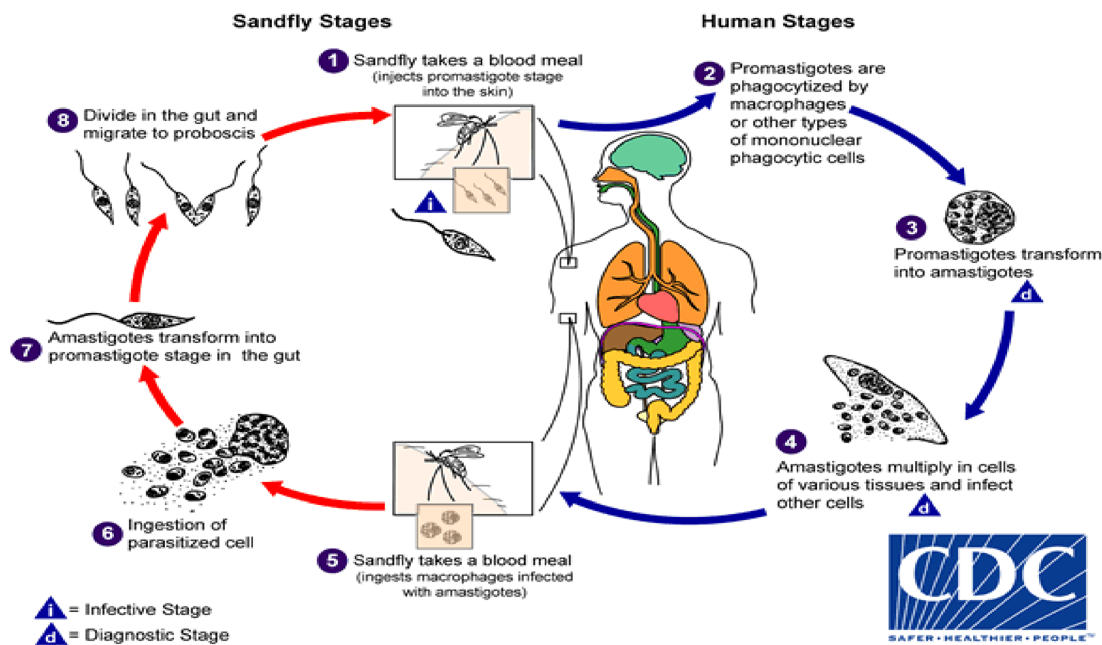


Fig1.3: Life cycle of Leishmania parasite (Source: CDC).

1.6 Clinical Spectrum of Leishmaniasis:

Different form of leishmanisis has been detected regarding different aspects like parasite species, disease distribution pattern according to geographical region vector type and immune response to the disease.

1.6.1 Visceral Leishmaniasis

Visceral leishmaniasis (VL) is the most severe form of the leishmaniasis caused by the obligate protozoan parasite of the genus *Leismania.* In the Old world, VL is caused by *Leishmania donovani* (in regions of India, Pakistan, China and Africa) and *Leishmania infantum* (in the Mediterranean region). In the New World, VL is also caused by *L. infantum* (also known as *Leishmania chagasi* or *L. infantum chagasi*). VL, also known as kala-azar or Dumdum fever, (referring to hyperpigmentation with fever during the disease) results from the infection of phagocytes within the reticuloendothelial system (liver, spleen) and bone marrow due to metastasis of parasites and parasite-infected macrophages from the initial site of cutaneous infection (Mcgwire & Satoskar, 2014).

The clinical spectra of VL ranges from sub clinical or asymptomatic (80-90%) to oligosymptomatic and fully characterized Kala-ajar. The symptoms of visceral leishamaniasis starts generally within 3 months with fever, weakness, night sweats, anorexia and weight loss that progress over weeks to months. The other prominent manifestations are splenomegaly, hepatomegaly, polyclonal-hypergammaglobulinaemia (IgG and IgM), leukopenia, thrombocytopenia, hypoalbulinemia, lymphadenopathy, severe cachexia, and pancytopenia (Chappuis et al., 2007). If untreated the disease proves

fatal within 2-3 years and the death is often because of secondary infection (Malla and Mahajan, 2006).

Post Kala-azar Dermal Leishmaniasis (PKDL)

Post Kala-azar dermal leishmaniasis (PKDL) also known as Post Kala-azar dermatosis, is a well-recognized complication of visceral leishmaniasis (VL) or kala-azar. It is the relapse case (recurrence) of VL even after the successful treatment, incomplete drug dose treatment or inadequate immune response to infection of may be genetic predisposition (Mondal and Khan, 2011). It is characterised by a macular, maculopapular, and nodular rash in a patient who has recovered from VL and who is otherwise well. The rash usually starts around the mouth from where it spreads to other parts of the body depending on severity. It is largely restricted to areas where *Leishmania donovani* is the causative parasite. A few cases from *L. infantum* or *L. chagasi* have been reported (Brahmachari, 1922). There is increasing evidence that the pathogenesis is largely immunologically mediated; high concentrations of interleukin 10 in the peripheral blood of VL patients predict the development of PKDL. During VL, interferon gamma is not produced by peripheral blood mononuclear cells (PBMC). Diagnosis is mainly clinical, but parasites can be seen by microscopy in smears with limited sensitivity. PCR and monoclonal antibodies may detect parasites in more than 80% of cases. Serological tests and the leishmanin skin test are of limited value (Zijlstra et al., 2003).

PKDL occurs in about 10-20% in Indian subcontinent and much higher (50%) in Sudan (Zijlstra et al., 2003). And a retrospective cohort study (2000-2010) showed 5.4% presented active skin lesions suspect of PKDL with 2.05% confirmed PKDL in south eastern region of Nepal (Uranw et al., 2011).

1.6.2 Cutaneous Leishmaniasis

Cutaneous Leishmaniasis (also known as oriental sore, tropical sore, chiclero ulcer, chiclero's ulcer or Aleppo boil)(Calvopina et al., 2013) is characterized by the single or multiple localized lesion on the exposed areas of skin caused by the number of Leishmania species from both the old world and new world. The hallmark of CL includes the self-healing or nonhealing skin lesions mostly developing into papules, nodules or nodules ulcers. The clinical features of cutaneous leishmaniasis tend to vary between and within regions due to different species of Leishmania. The patients with cutaneous leishmaniasis do not show visceral manifestation. A classical lesion starts as a papule or nodule at the site of inoculation, followed by formation of crusts (Adhikari & Shah, 2017).

Although cutaneous leishmaniasis (CL) is not life threatening, it can cause devastating effects on local communities. Indeed, the disfiguring lesions can lead to affected persons being stigmatized, with consequences such as ostracism, impaired education, and

economic loss. It can become disseminated and produce generalized debilitating disease in immune suppressed persons (e.g. HIV-affected patients) (https://www.dndi.org/ diseases-projects/leishmaniasis/disease-background-cl/).

Cutaneous leishmaniases are of following types:

Old world cutaneous leishmaniasis:

It is usually caused by *L. major, L. tropica,* and *L. aethiopica*. Cutaneous leishmaniasis caused by *L. major* is found in central Asia, Middle East, North Africa, North India and Pakistan. There are few reports from Nepal and authors have also seen cases of cutaneous leishmaniasis. Multiple inflamed lesions are seen on the nose, lips and limbs. *L. major* infection self-cures within 2-4 months. Cutaneous leishmaniasis caused by *L. tropica* produces painless dry ulcers of the skin on the face, feet, legs and arms and does not heal rapidly; difficult to treat and requires 6-15 months. (WHO, 2010). Children are usually affected and this disease is prevalent in Afghanistan, Greece, North India, Iran, Iraq, Israel, Kuwait, Lebanon, Morocco, Pakistan, Saudi Arabia, Syria, Tunisia and Turkey (Chatterjee et al., 2009).

Leishmaniasis recidivans (LR) also known as lupoid or tuberculoid leishmaniasis is also caused by *L. tropica* and characterized by slowly progressive skin lesion on the face. Cutaneous leishmaniasis caused by *L. aethiopica* gives rise to small cutaneous lesion on the face. Ulceration is absent. Rarely this type of leishmaniasis may distort the nostrils and lips. Diffuse cutaneous leishmaniasis results from specific deficiency of cell-mediated immunity to leishmania antigen. It is caused by *L. aethiopica* and reported from Ethiopia and Kenya. It starts with single lesion and spreads over the face, extremities and whole body.

In Nepal, cutaneous leishmaniasis is caused by *L. tropica* (Parija et al., 1998) and *L. major* (Kumar et al., 2008), is reported from Dharan (eastern part of Nepal). Nepalese cutaneous leishmaniasis presented with infiltrating erythematous plaque with ill-defined border and extensive crusting ulceration is noted in some cases. These lesions were localized in face & neck region (Neupane et al., 2008), however other sites like wrist, leg and arm are also involved.

New world cutaneous leishmaniasis:

New world Cutaneous Leishmaniasis is caused by *L. amazonensis* and *L. Mexicana, L. infantum* and *L. chagasi. L. braziliensis, L. panamensis*, *L. peruvinia* and *L. guyanensis* account for the more severe form of CL called mucocutaneous leishmaniasis, which is only prevalent in the New World and affects the mouth, nose, and occasionally the ear tissues (Zufferey & Whyte, 2017). Multiple *Leishmania* species cause wide range of clinical

features in South and Central America. A substantial proportion of infections are asymptomatic. The clinical forms are localized, disseminated, diffuse and atypical cutaneous and muco-cutaneous leishmaniasis. Localized cutaneous leishmaniasis is caused by multiple species of both the *Leishmania* and *Viannia* subgenera. Cutaneous lesions are characterized by macule at the site of inoculation, followed by papule that ulcerates. Lymphadenitis may be seen especially when it is caused by Vianna subgenus(Adhikari & Shah, 2017).

1.6.3 Mucocutaneous Leishmaniasis (MCL)

Mucocutaneous Leishmaniasis is the most feared form of cutaneous leishmaniasis because it produces destructive and disfiguring lesions. MCL is usually found in New World caused by *L. braziliensis* and *L. panamensis.* Most cases (nearly 90%) are reported from Bolivia, Brazil and Peru. There are two phases, a primary cutaneous lesion, sometimes followed by a secondary mucosal involvement. Nasal mucous membranes, pharynx, larynx and upper lip are involved. The infection is confined to mucus secreting organs such nose, throat, larynx and anus. After initial skin lesions which heal slowly but spontaneously, chronic ulcers appear after months or years with destruction of underlying tissues (nasal cartilage). Death might result from severe respiratory tract infection due to massive destruction of pharynx (Elhassan, 1994).

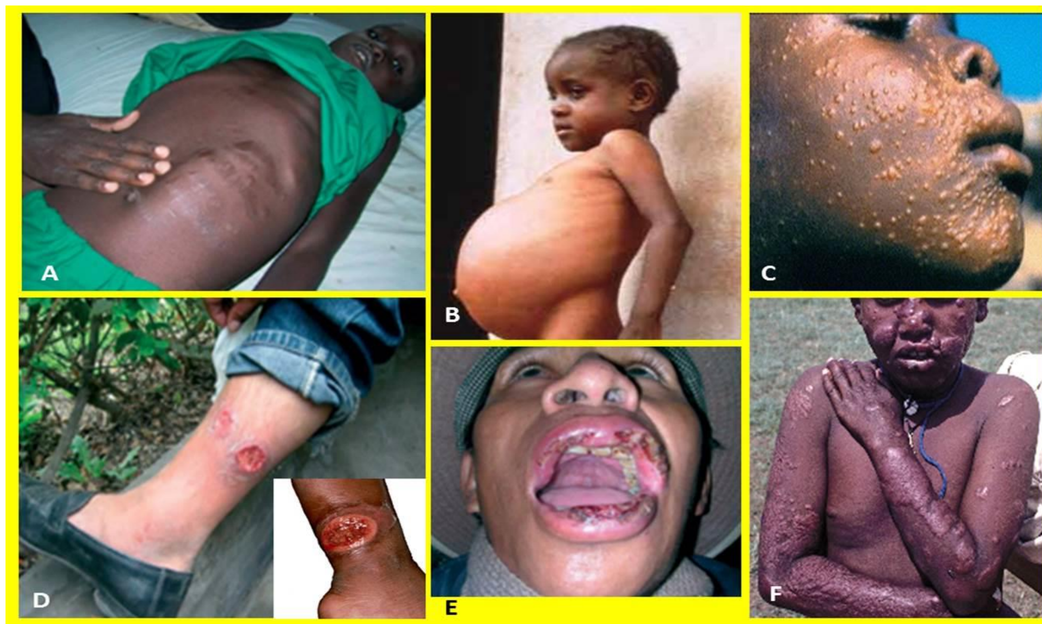


Fig 1.4: Clinical spectrum of Leishmaniasis.(A & B) Visceral leishmaniasis (C) Post kala-azar dermal leishmaniasis (PKDL) (D) Cutaneous leishmaniasis and (E) Mucocutaneous leishmaniasis. (F) Diffuse cutaneous leishmaniasis (Zijlstra et al., 2003; Chappuis et al., 2007; Manandhar, 2008)

1.7 Clinical Presentation of CL

CL is characterized by skin lesions (open or closed sores), which typically develop within several weeks or months after exposure. In some people, the sores first appear months or years later, in the context of trauma (such as skin wounds or surgery). The sores can change in size and appearance over time. They typically progress from small papules to nodular plaques, and often lead to open sores with a raised border and central crater (ulcer), which can be covered with scales or crust. The lesions usually are painless but can be painful, particularly if open sores become infected with bacteria. Satellite lesions, regional lymphadenopathy, and nodular lymphangitis can be noted. The sores usually heal eventually, even without treatment. However, they can last for months or years and typically result in scarring.

Some parasites might spread from the skin to the mucosal surfaces of the nose or mouth and cause sores there. This form of leishmaniasis, mucosal leishmaniasis (ML), might not be noticed until years after the original skin sores appear to have healed. Although ML is uncommon, it has occurred in travelers and expatriates whose cases of CL were not treated or were inadequately treated. The initial clinical manifestations typically involve the nose (chronic stuffiness, bleeding, and inflamed mucosa or sores) and less often the mouth; in advanced cases, ulcerative destruction of the nose, mouth, pharynx, and larynx can be noted such as perforation of the nasal septum.

1.8 Differential diagnosis of cutaneous leishmaniasis

Skin lesions of cutaneous leishmaniasis may clinically mimic variety of skin diseases, inflammatory like impetigo, eczema, or granulomatous like sarcoidosis, lupus vulgaris, to skin tumor like basal cell carcinoma & squamous cell carcinoma. Some authors reported a patient with an uncommon cutaneous leishmaniasis lesion that clinically resembled allergic contact dermatitis (Uzun et al., 1999). Unusual clinical forms of cutaneous leishmaniasis have been reported including the palmoplantar form, chancriform lesion at the glans penis and penile shaft, zosteri form lesions at the trunk, erysipeloid form at the upper lip and adjacent cheek, annular form at the penile shaft and acute paronychial forms at the nail folds and fingers. CL cases also mimicked erysipelas, rosacea, hydroa vacciniforme, eczema, leg ulcer, sarcoidosis, discoid lupus erythematosus, leprosy, drug eruption, lupus vulgaris, basal cell carcinoma and squamous cell carcinoma (Akman et al., 2001).It has also been reported that the cases of cutaneous leishmaniasis micked squamous cell carcinoma clinically(Akcali et al., 2008; Oetken et al., 2017).

1.9 Epidemiology and Geographical Distribution of Leishmaniasis

1.9.1 World Distribution of Visceral Leishmanisis

(VL) is the second most deadly parasitic disease in the world following malaria, and is responsible for an estimated 20,000-40,000 deaths worldwide each year. Its distribution is highly localised, with over 90% of the 200,000-400,000 estimated annual cases occurring in just 6 countries: Bangladesh and India in the Indian sub-continent (ISC); Ethiopia, Sudan and South Sudan in Africa; and Brazil.

Status of endemicity of visceral leishmaniasis worldwide, 2015

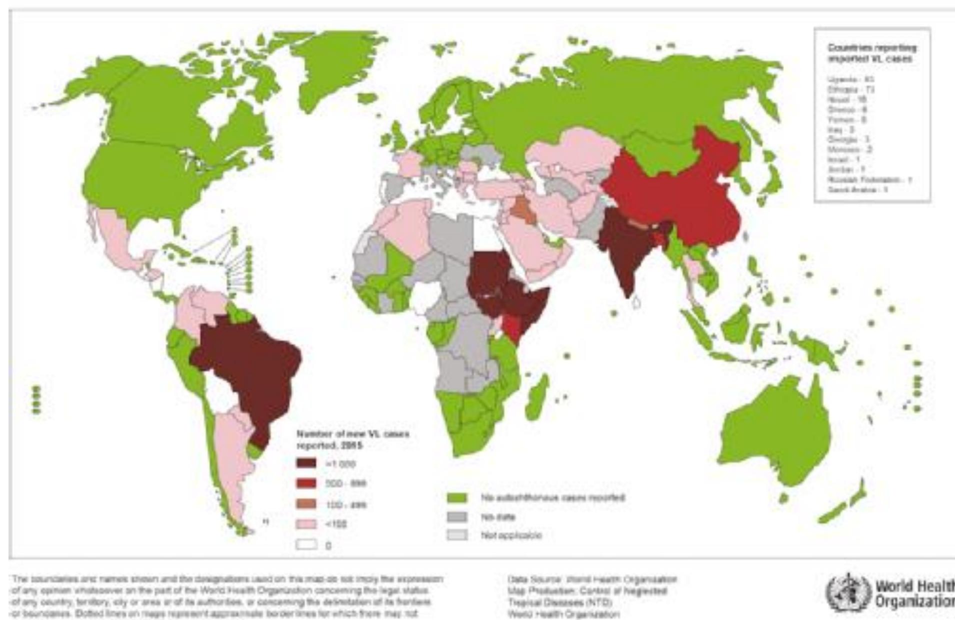


Fig 1.5: Geographical distribution of Cutaneous Leishmaniasis (Source: World Health Organization, WHO, 2015)

1.9.2 World Distribution of Cutaneous Leishmaniasis

CL is the most common form of leishmaniasis and causes skin lesions, mainly ulcers, on exposed parts of the body, leaving life-long scars and serious disability. Cutaneous leishmaniasis has a wide distribution, spreading from the Indian subcontinent, across Central and South-Western Asia, to the Mediterranean Basin, the northern half of the African continent, and Central and South America. About 95% of CL cases occur in the Americas, the Mediterranean basin, the Middle East and Central Asia. In 2015 over two thirds of new CL cases occurred in 6 countries: Afghanistan, Algeria, Brazil, Colombia, Iran and the Syrian Arab Republic. It is estimated that between 600 000 to 1 million new cases occur worldwide annually and most of them affecting children, and only very few receive treatment (WHO fact sheet, 2018).

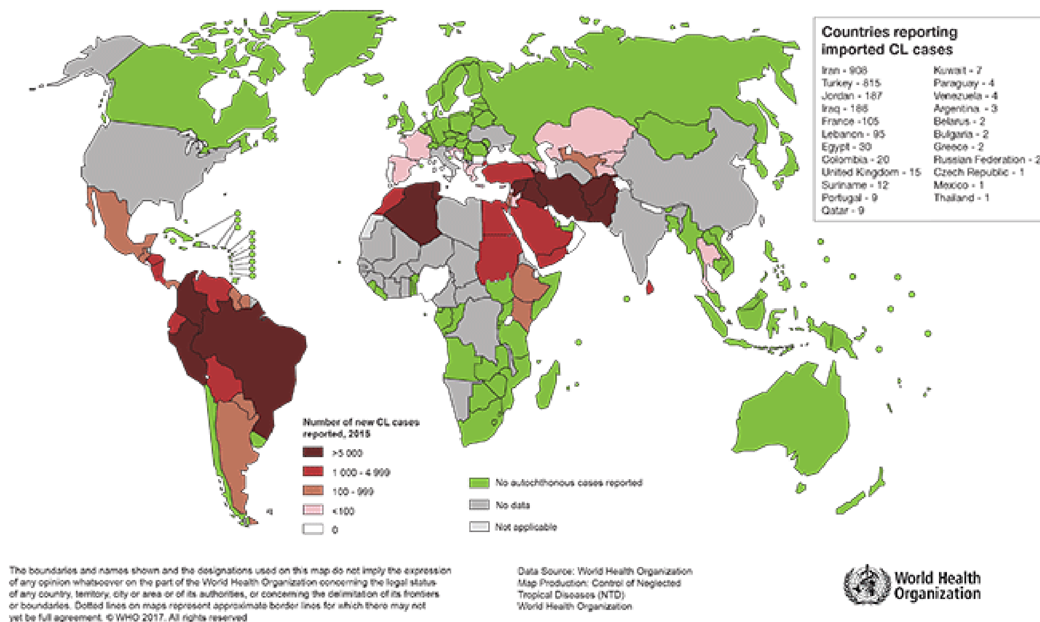


Fig 1.6: Geographical distribution of Cutaneous Leishmaniasis (Source: World Health Organization, WHO, 2015)

1.10 Cutaneous Leishmaniasis in Nepal

Nepal is an endemic zone for visceral leishmaniasis usually the terai region (Joshi et al., 2006) and *L. donovani* is endemic in south Asian countries like Nepal, India, Bangladesh and in east African countries like Ethiopia, Kenya and Sudan (Zijlstra et al., 2003). Primary cutaneous leishmaniasis is not common disease in Nepal, and only a handful of cases were reported from Terai region of Nepal (Jha and gurung, 2013, Pandey et al., 2006).The first imported case of CL was reported by Parija et al., 1998. The first reported case of CL in Nepal was in 2006 by Pandey et al. Four cases of CL of the year 2006 was also reported in the year 2008 by Neupane et al. A case of CL in Nepal caused by *L. major* was confirmed by Kumar et al, 2008. Majority of the previously reported cases were from the people who were suspected to contract the disease during their abroad stay where CL was endemic or had been reported(Neupane et al. 2008,Kumar et al., 2008). Recently, Ghimire et al reported a largest collection of CL cases (33) over the six years period from January 2012 to November 2017.Cutaneous leishmaniasis is in increasing trend in Nepal (Ghimire et al., 2018).There is no experience about MCL and there are no reports published in the literature from Nepal.

1.11 Transmission

Sandfly is only the vector known to transmit infection. It may obtain parasite either directly from the infected skin or by ingesting the circulation blood of the infected host. There are various factors that influence the transmission of the disease. Some are the following: Proximity of residence to sandfly breeding and resting sites (Werneck et al., 2003), Type of housing, Occupation and extent of exposure to sandfly bites, Natural resistance (genetic or acquired) to infection, Virulent of the parasite species, Zoonotic or anthroponotic reservoirs, Density, seasonality, longevity and flight range of sandfly populations and The vectorial capacity, which is defined as the number of infective bites delivered per human per annum (Degu, 2006). Although small number of leishmaniasis is anthroponotic, majority of them are zoonotic which involve either wild or domestic animals as reservoir. Some 70 animal species, including humans, have been found as natural reservoir hosts of *Leishmania* parasites (WHO). 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). Occassionally, sandflies are not involved in transmission,CL also can occur after accidental occupational (laboratory) exposures to *Leishmania* parasites (CDC,2018)

1.12 Major risk factors

i) Socioeconomic conditions

Poverty increases the risk for leishmaniasis. Poor housing and domestic sanitary conditions may increase sandfly breeding and resting sites, as well as their access to humans. Sandflies are attracted to crowded housing as these provide a good source of blood-meals. Human behaviour, such as sleeping outside or on the ground, may increase risk.

ii) Malnutrition

Diets lacking protein-energy, iron, vitamin A and zinc increase the risk that an infection will progress to kala-azar.

iii) Population mobility

Epidemics of both cutaneous and visceral leishmaniasis are often associated with migration and the movement of non-immune people into areas with existing transmission cycles.

iv) Environmental changes

The incidence of leishmaniasis can be affected by changes in urbanization, and the human incursion into forested areas.

v) Climate change

Leishmaniasis is climate-sensitive and affect the epidemiology of leishmaniasis in a number of ways. Changes in temperature, rainfall and humidity can have strong effects on vectors and reservoir hosts by altering their distribution and influencing their survival and population sizes.

Source: http://www.who.int/news-room/fact-sheets/detail/leishmaniasis

1.13 Treatment

Following antileishmanial drugs are used for the treatment of cutaneous leishmaniasis.

i) Pentavalent antimonials

This drug has been used as the first line drug since the early 1940s (Berman, 1997). These are non-covalent chelates of Sb^V with improved solubility and uptake property as compared to trivalent antimonials. World Health Organisation (WHO) recommends a dose of 15-20mg Sbv/kg of body weight per day for 21-28 days, injected intramuscularly or intravenously. There are issues of drug resistance and in Nepal, SSG resistant cases of visceral leishmaniasis were documented (Rijal et al., 2007). So far, this issue was not reported from Nepal in cutaneous leishmaniasis.

ii) Amphotericin B

Amphotericin B, a polyene macrolide, is strongly recommended as a second line of drug for the treatment of leishmaniasis. Amphotericin B 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 or on alternate day. Amphotericin B binds with greater affinity (in parasitic cell membrane) to ergosterol forming intimate binary complex. This binary complex and Amphotericin B induced lipid peroxidation of cell membrane leads to cellular dysfunction and eventually cell lysis(Brajtburg et al., 1985).

iii) Miltefosine

Miltefosine, also called hexadecylphosphocholine, has been developed as an oral anticancer drug. It 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. It has low therapeutic ratio, but with 90% yield cure rate.

iv) Prophylactic vaccines

There is no vaccine for general use against leishmaniasis. However, intradermal inoculation of live virulent *L. major* promastigotes from a fresh culture has been used intermittently for many years to protect against L. major infection. (Adhikari and Shah, 2017).

1.14 Prevention and control

Prevention and control of leishmaniasis requires a combination of intervention strategies because transmission occurs in a complex biological system involving the human host, parasite, sandfly vector and in some causes an animal reservoir host. Key strategies for prevention are listed below:

- **Early diagnosis and effective treatment** reduces the prevalence of the disease and prevents disabilities and death. Early detection and prompt treatment of cases help to reduce transmission and to monitor the spread and burden of disease. Currently there are highly effective and safe anti-leishmanial medicines particularly for visceral leishmaniasis. Access to these medicines has significantly improved thanks to a WHO-negotiated price scheme and a medicine donation programme through WHO.
- **Vector control** helps to reduce or interrupt transmission of disease by controlling sandflies. Control methods include insecticide spray, use of insecticide–treated nets, environmental management and personal protection.
- **Effective disease surveillance** is important to promptly monitor and take action during epidemics and situations with high case fatality rates under treatment.
- **Control of animal reservoir hosts** is complex and should be tailored to the local situation.
- **Social mobilization and strengthening partnerships** – mobilization and education of the community with effective behavioral change interventions must always use locally tailored communication strategies. Partnership and collaboration with various stakeholders and other vector-borne disease control programs is critical.

http://www.who.int/news-room/fact-sheets/detail/leishmaniasis

1.15 Research Plan and Design:

1.15.1 Research hypothesis

1. CL has been spread in different parts of Nepal.
2. Species of *Leishmania* parasite responsible for VL is also responsible for causing CL in Nepal
3. The immune status of the CL patients is different to that of healthy people and there is difference in population of immune cells (T cells and B cells) in CL patients and the healthy people.

1.15.2 Research Objectives

General Objective:

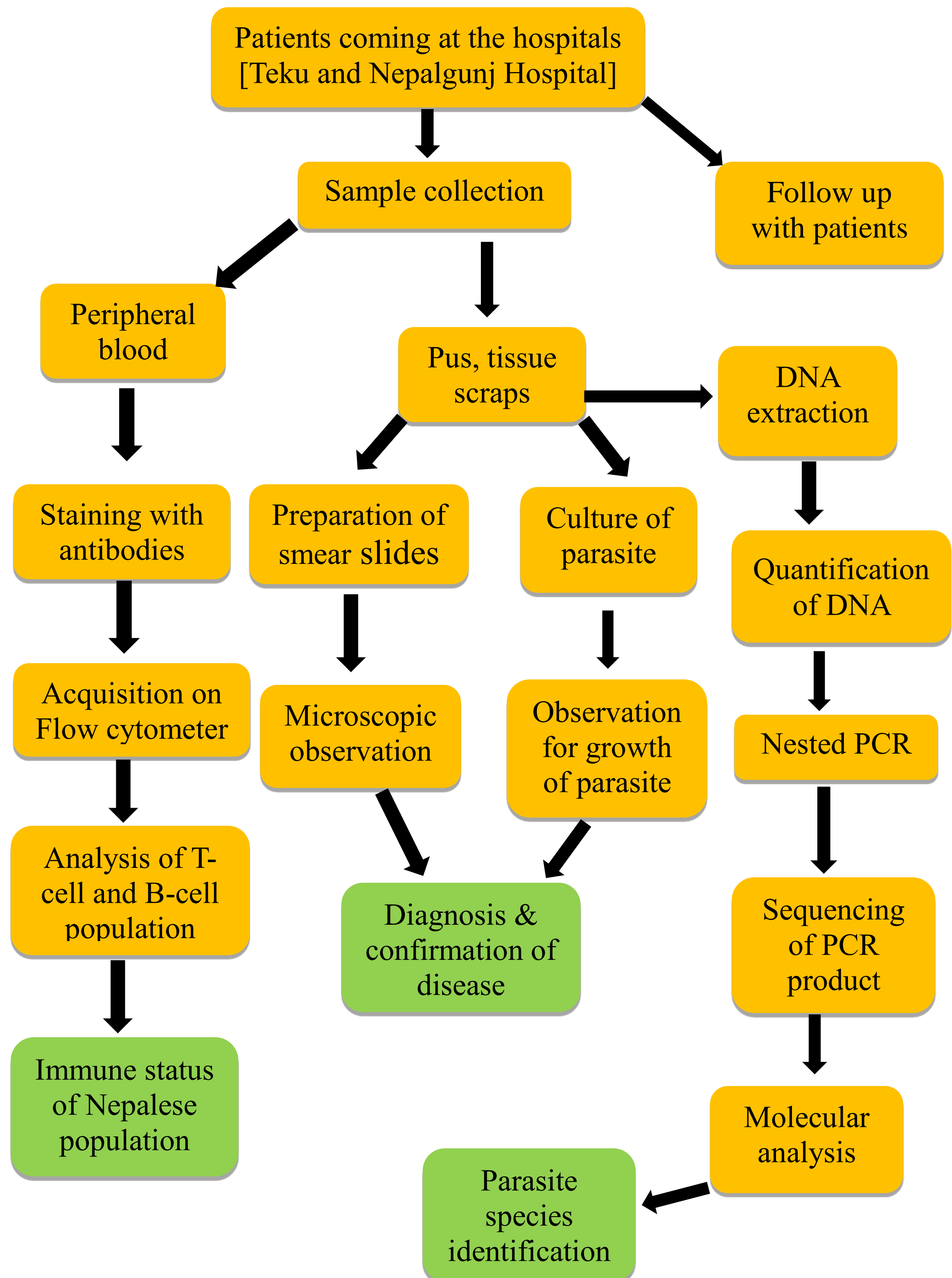
Epidemiology and immune-molecular analysis of the Cutaneous Leshmaniasis prevalent in Nepal.

Specific objectives

1. Clinical diagnosis of CL.
2. Microscopic and cultural confirmation of Leishmaniasis from the pus and tissue specimen collected from acute patient.
3. Nested PCR based verification of *Leishmania* parasite infection and species identification.
4. Sequencing the amplicon of nested PCR II to validate isolation and species identification.
5. Comparison of the cellular immune status of CL patients and healthy controls by flow cytometry (T and B cell population analysis in peripheral blood circulation).

1.15.3 Research Plan

With the view to run the research smoothly, flow chart was made to meet the claimed objectives.



1.15.4 Rationale

Nepal is endemic to visceral leishmaniasis (Kala-azar) disease that is mostly prevalent in Terai belt of Nepal. The other form of leishmaniasis, expressed in skin, is not a common disease issue. The cases of cutaneous leishmaniasis are found mostly in tropical climate. However, limited cases have been identified on the people visiting to hospitals and health centers from the year 1998 and the latest case report was in 2013. Most of the cases were detected from the people who have been to the Arab countries. Some frequent cases have been reported from Sukraraj Tropical and Infectious Diseases Hospital. The cases so far seen in Nepalgunj Medical College and Teaching Hospital. The trend is very much alarming for the stakeholders of health sector in Nepal. Country need to get the data of the disease at the earliest and prepare its strategy to control it beforehand it becomes an additional endemic disease as a national burden. In case of Nepal, the cases have also been found in the high lands people who has no history of travelling to abroad and even in Terai region of Nepal. The case history is much more alarming since it may turn up to epidemic in Nepal like low profile economic status country. The disease also gets misdiagnosed as lupus vulgaris and other diseases due to lack of proper diagnostic facility with improper medications applying topical steroid and antibiotics which further deteriorate the disease. Hence, it is very essential to develop an efficient diagnostic tool that can detect these cases at the earliest and also understand the underlying changes occurring in the vector and host dynamics that has resulted in the increased incidence of Cutaneous Leishmaniasis in Nepal.

Chapter II

LITERATURE REVIEW

Cutaneous leishmaniasis is a public health problem, affects millions of individuals worldwide. This disease displays distinct clinical manifestations from self-healing skin lesions to chronic disfiguring disease(Scott & Novais, 2016). The World Health Organization considers CL a severely neglected disease and a category 1 emerging and uncontrolled disease(de Vries et al., 2015). Primary cutaneous leishmaniasis is not common disease in Nepal and only few cases were reported   However, majority of the previously reported cases were from the people who were suspected to contract the disease during their abroad stay where CL was endemic (Kumar et al., 2008; Neupane et al., 2008). And now, cutaneous leishmaniasis is in increasing trend in Nepal (Ghimire et al., 2018). The patients with cutaneous leishmaniasis present with a papule or nodule at the site of inoculation, followed by formation of crusts. Differential diagnoses of cutaneous leishmaniasis include variety of skin diseases, inflammatory like impetigo, eczema, or granulomatous like sarcoidosis, lupus vulgaris, to skin tumor like basal cell carcinoma & squamous cell carcinoma. Rapid diagnosis of cutaneous leishmaniasis (CL) and identification of *Leishmania* species is highly important for the disease management. The review of most important diagnostic methods of CL and the Host immune response has been presented.

2.1 Disease diagnosis

The diagnosis of CL is based on clinical features (supported by epidemiologic data) and laboratory testing. Numerous diagnostic methods have been described with a huge variation in diagnostic accuracy, including direct parasitologic examination (microscopy, histopathology, and parasite culture and molecular diagnostics) and/or indirect testing with serology (Goto & Lindoso, 2010).

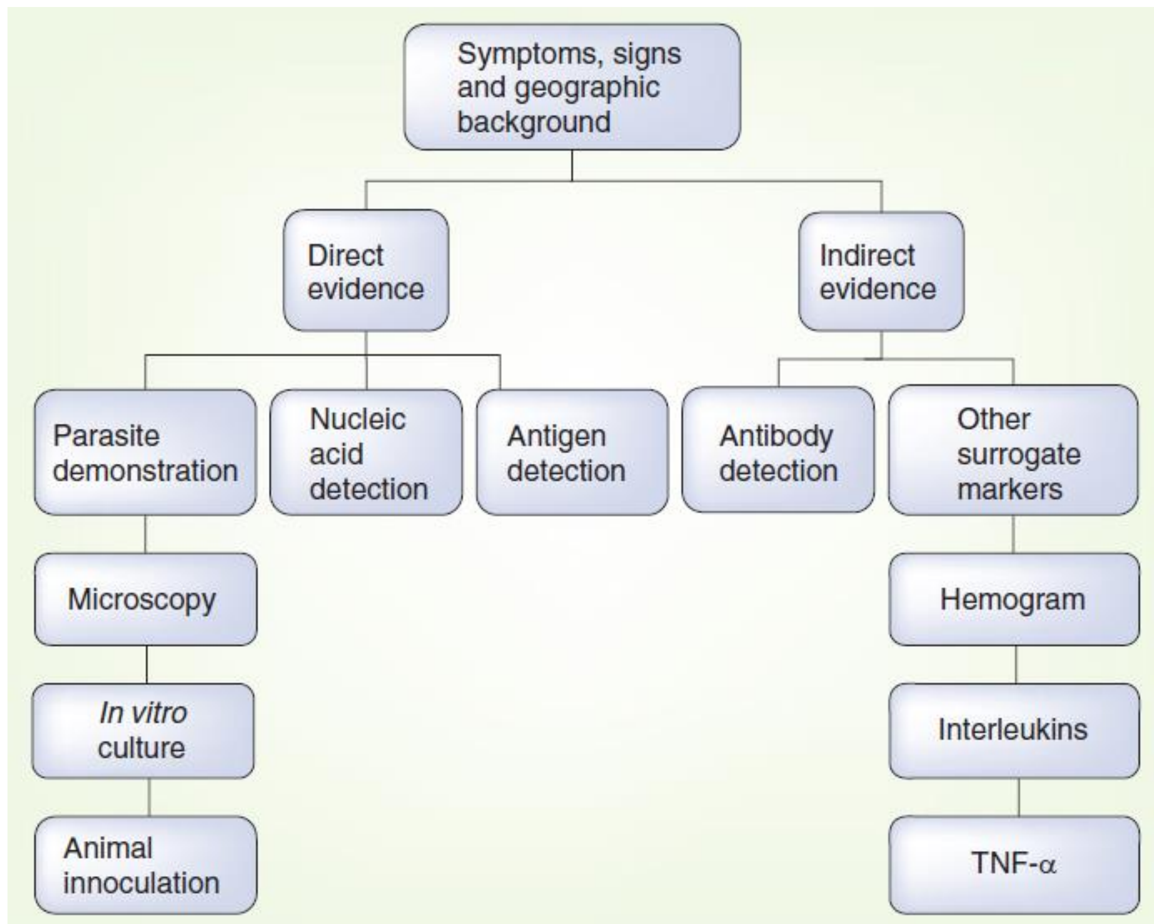


Fig.2.*1*: Summary of the methods commonly used for diagnosing Leishmaiasis (Singh *et al.,* 2005)

2.1.1 Microscopic demonstration of parasite

Parasitologic diagnosis is still considered the gold standard in leishmaniasis diagnosis because of its high specificity. Microscopical diagnosis of CL is performed by the direct identification of amastigotes in Giemsa-stained lesion smears of biopsies, scrapings, or impression smears. Amastigotes appear as round or oval bodies, about 2–4 lm in diameter, with characteristic nuclei and kinetoplasts.

For the microscopic examination of the parasite, thin smears of the specimens are prepared in the clean glass slides, stained with suitable dye and visualized under microscope.

There are various procedures and laboratory techniques used to diagnose leishmaniasis (Adhikari & Shah, 2017).

Slit skin smear test: The affected area of the skin is cleaned and squeezed firmly between the index finger & thumb to give 3-4 mm incision of 3 mm depth. Then slit smear is made for Giemsa staining and demonstration of leishmania species.

Touch imprint test: This technique is suitable for ulcerative lesion and imprints are prepared directly from ulcer after cleaning it. Then Giemsa staining is performed to examine leishmania.

Skin punch biopsy: This is most widely used technique to diagnose cutaneous leishmaniasis in Nepal. In some cases, the entire dermis is diffusely infiltrated by lymphocytes & histiocytes with ill-formed granulomas, while discrete granulomas may be encountered in other cases. Differential diagnoses include other granulomatous disease of the skin like lupus vulgaris, sarcoidosis, fungal infection, leprosy etc. However presence of amastigotes rules out most of these diseases. The spores of Histoplasma capsulatum may mimic amatigotes of leishmania. PAS stain is used to differentiate them as spores of Histoplasma are PAS positive and amastigotes of leishmania are PAS negative. Immunohistochemistry can be used for confirmation of the diagnosis.

Fine needle aspiration cytology (FNAC): Nodular and indurated lesions are subjected to fine needle aspiration and cytological examination may reveal amastigotes of leishmania. There are several case reports emphasizing the role of FNAC in diagnosis of cutaneous leishmaniasis (Mallik, et al., 2001, Jha & Gurung, 2013, Adhikari & Shah, 2017). Cytology smears reveal granuloma with intracellular as well as extracellular amastigotes of leishmania. The following system of grading of parasites used for splenic punctures (Chulay & Bryceson, 1983) can be used for cytology smears if required. Authors have used this grading system to assess parasitic load in punch biopsy sections.

Table 2.1: Grading of *Leishmania donovani* amastigotes in splenic aspirate smears (Chulay and Bryceson, 1983).

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

*Using 10X eye piece & 100X oil immersion lens.

Although microscopy is sensitive and accurate test highly skilled and experienced medical physician, well-equipped hospital, experienced laboratory technician and quality reagents are required in suitable diagnosis of CL.

2.1.2 In vitro culture

In vitro culture of parasite is one of the diagnostic methods of leishmaniasis with 100% specificity. Different 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 (cRPMI1640) and allowed to grow the parasite in BOD incubator at 26ºC 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). Parasite culture in tubes containing Novy-MacNeal-Nicolle medium from suspected lesions is difficult, requires significant technical expertise, is prone to contamination, and is time consuming (Boggild, et al., 2008). The sensitivity of culture tends to be low and highly variable(Hofwegen, et al., 2003).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.

2.1.3 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 for inoculating clinical aspirate for animal model infection. Golden hamster is the animal of choice for maintaining *Leishmania donovani* complex (Sundaar and Benjamin, 2003). The development of disease can be scrutinized through signs like hepatosplenomegaly, cutaneous or metastatic leisons 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 than month 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.

2.1.4 Immunological Diagnostic Methods

Current serologic tests for CL are mainly based on formats such as indirect fluorescent antibody, enzyme-linked immunosorbent assay (ELISA), western blot, lateral flow assay, and direct agglutination test. However, these formats are not widely employed for the diagnosis of CL, because of the poor humoral response provoked by the infection and the consequential low sensitivity (Goto, et al., 2010; Kar 1995). Recent developments in diagnostic methods suggest that incorporation of specific purified antigen preparations or recombinant Leishmania antigens for serologic diagnosis would increase the operational characteristics of these tests. This is following the success of the rK39 antigen for the sero-diagnosis of VL (Maia et al 2012). Heat shock proteins (HSPs), and in particular HSP83, have appeared as potential candidates. An ELISA based on recombinant HSP83 has shown good performance in the diagnosis of CL, next to ML and VL, compared with an ELISA-based crude L. major antigen in terms of sensitivity and specificity. Furthermore, the use of chemiluminescent ELISA to measure levels of anti-a-galactosyl antibodies in human sera, it was found that individuals infected with either *L.tropica* or *L. major* had significantly elevated levels (up to 9-fold higher) of anti-a-Gal IgG compared with healthy control individuals. This assay is found to have higher sensitivity than microscopy analysis. Interestingly, up to 2 years following confirmed CL cure, individuals had 28-fold higher levels of anti-a-Gal IgG compared with healthy volunteers. Monitoring levels of anti-a-Gal antibodies is proposed as both a diagnostic tool and as a biomarker of a cure of Old World CL (Al-Salem et al., 2014). An innovative diagnostic test that is currently under evaluation is the CL DetectTM Rapid Test, which is a qualitative membrane-based immunoassay for the detection of all clinically relevant species of the genus Leishmania that cause CL in skin samples. The test is currently being evaluated (http://clinicaltrials.gov/show/NCT01865032).

2.1.5 Leishmania Skin Test

A marker of cellular immune response, the Leishmania intradermal skin test (LST) also known as Montenegro skin test (MST) are occasionally used in CL diagnosis (e.g., in epidemiologic surveys and vaccine studies) because of its simple use and because of its high sensitivity of 86.4 % up to 100 % (Antonio et al., 2014). 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. Delayed-type hypersensitivity skin reactions to LST >_5 mm are considered positive and<5 mm are considered negative. Patients with negative LST and diagnostic confirmation by other tests are more prone to relapse or treatment failure (Antonio et al., 2014; Passos et al., 2000). The main disadvantages of the LST or MST are that it requires culture facilities to produce the MST antigen, that different antigen preparations impact test sensitivity, and that the test does not distinguish between past and present infections. There is

evidence that when LST data are supported with information on the production of antigen-specific interferon- c (IFN-c), this could better assist in determining whether a suspected case has been exposed to a Leishmania infection In contrast, it is reported that the LST is significantly more sensitive than IFN-c levels in persons who have been cured of CL (Alimohammadian et al., 2012;de Vries et al., 2015).

2.1.6 Molecular Diagnosis of the disease

For the diagnosis of infectious disease, molecular methods and tools is progressively becoming pertinent. Information on DNA and RNA sequences are being extensively exploited for the development of PCR bassed assays. Among all the molecular advances, gene amplification techniques have been the most rewarding as far as the diagnosis and disease management is concerned. Gene amplification through PCR is most sensitive, rapid and ability to be performed with a broad range of clinical specimens (Tavares et al., 2003; Pourmohammadi et al., 2010)

DNA-based assays should be performed, especially if other diagnostic testing is unrevealing. They are emerging as the most sensitive assays for the diagnosis of leishmaniasis(Aronson et al., 2016)

Many molecular diagnostic tests have been developed for the diagnosis of CL, as these are assumed to have better sensitivity and specificity than traditional diagnostic methods and allow the use of less invasive sampling for diagnosis. In particular PCR, either as a single test or in a nested format or as a quantitative assay, has been widely exploited. Numerous tests targeting many different gene sequences have been developed over the last decades, with the ribosomal DNA internal transcribed spacer 1 (ITS1) sequence or sequences within the kinetoplast DNA of Leishmania genus as the main targets (Satow et al., 2013). Bensoussan et al.,2006 found the kinetoplast DNA (kDNA) PCR with highest sensitivity (98.7%) of any assay, correctly diagnosing 77/78 of the confirmed positive samples, followed by the rRNA gene internal transcribed spacer 1 (ITS1) PCR (71/78 positive, 91.0% sensitivity) and then the spliced leader mini-exon PCR (42/78 positive, 53.8% sensitivity). Next to this, several other PCR-like assays, such as a high-tech fluorescence resonance energy transfer based on a real-time assay (Tsukayama et al ., 2013) requires staining with SYBR-green I dye or hybridization with fluorogenic probes (e.g., TaqMan or fluorescence resonance energy transfer [FRET]) . In this case, assays are performed with a single all-in setup, and the detection of fluorescence is done within a closed tube, decreasing the risk of laboratory contamination by amplicons. Applications are rapid and of high throughput, but equipment is comparatively expensive, and working costs remain high (Reithinger & Dujardin, 2007).Other assays based on HSP70 or tryparedoxin peroxidase gene targets (Khosravi et al ., 2012; de Vries et al., 2015) amongst many others, are under evaluation.

2.1.6.1 Nested – PCR

Nested polymerase chain reaction (Nested PCR) is a modification of polymerase chain reaction which is intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites. It involves the use of two sets of primers, used in two successive runs of polymerase chain reaction. In the first PCR, one pair of primers is used to generate DNA products, which may contain products amplified from non-target areas. The products from the first PCR are then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

Nested PCR increase the specificity of DNA. amplification. Two sets of primers are used in two successive reactions. Nested PCR is often more successful in specifically amplifying long DNA products than conventional PCR,

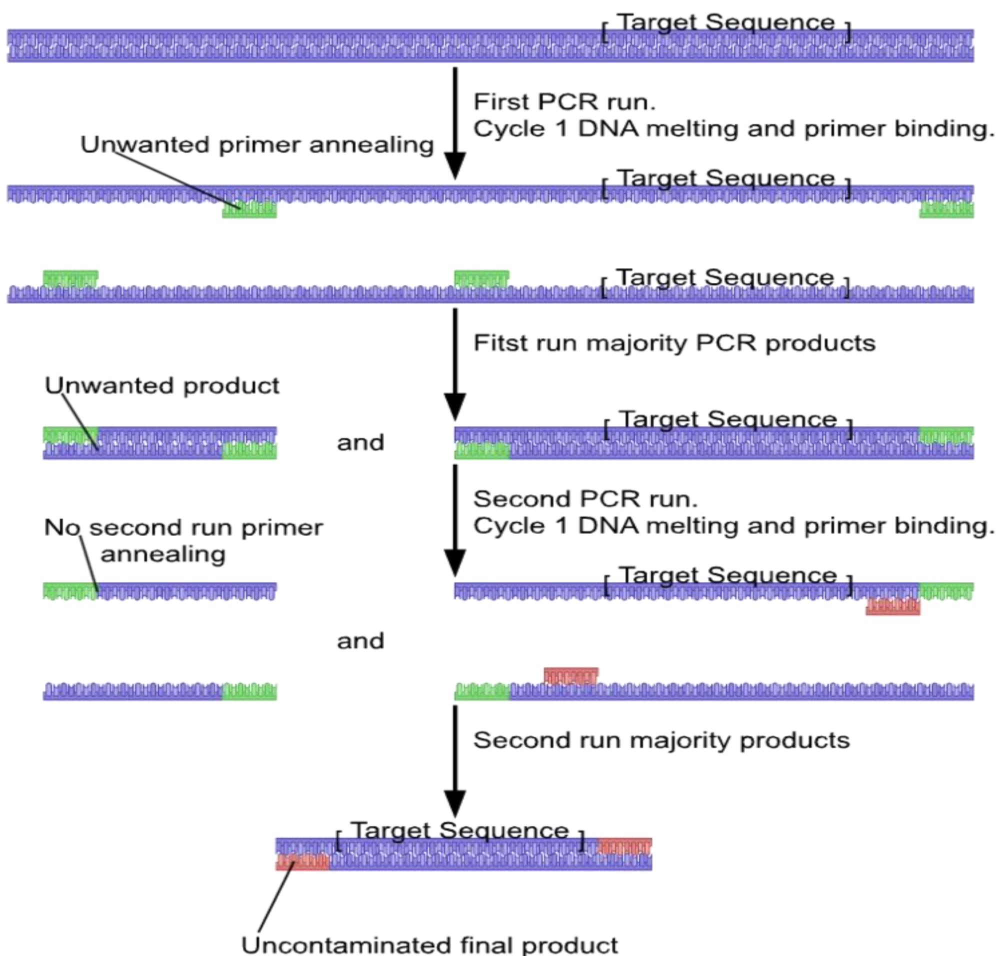


Fig 2.2: Diagram showing the process of Nested PCR (Wheeler, 2005)

*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*. The abundance of these molecules has made them the target for a number of diagnostic Tests. Kinetoplast minicircles code for guide RNAs that are involved in editing the mitochondrial genes of trypanosomatids. 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. 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. These conserved sequence blocks are ideal targets for PCR primers which can amplify all known minicircle classes from all *Leishmania* species. 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(Noyes et al., 1998).

There are three highly conserved blocks (CSB) within CRs: 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 (Ashford et al., 1995). 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 kinetoplastminicircles of all Leishmanai 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).

2.1.7 Limitations of molecular diagnosis

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 Host immune response

Cutaneous leishmaniasis has the wide range of clinical manifestations associated with this disease, which, similar to tuberculosis and leprosy, is dictated largely by the type and magnitude of the immune response of the host. As in most infections, the immune response to cutaneous leishmaniasis depends on many host factors, as well as on the differences between the infecting *Leishmania* spp.

The immunological spectrum observed in patients with leishmaniasis ranges from individuals with a strong T cell response, characterized by delayed-type hypersensitivity (DTH) and high levels of interferon-γ (IFNγ), to individuals who lack a DTH response but may have high levels of antibodies. Because *Leishmania* spp. are killed by IFNγ-activated macrophages and are not neutralized by antibodies, individuals with a strong DTH have few parasites in their lesions, whereas those with only a humoral response are unable to control the parasite load. As expected, patients without a T cell response exhibit a severe disease called diffuse cutaneous leishmaniasis. At the other end of the spectrum, patients with an exaggerated immune response also develop a severe disease phenotype known as mucosal leishmaniasis, which is driven by immunopathology. Between these extremes are patients who develop lesions that may self-heal or become chronic, with intermediate levels of T cell and antibody responses(Scott & Novais, 2016).

2.3 Host parasite interaction:

Entry of metacyclic promastigote in vertebrate host rapidly adapt in hostile environment by changing to amastigote. This complicated process involved the immune response of infected individuals. Through the bite of infected sandfly (*Phlebotomus* spp, *Lutzmoyia* spp) the metacyclic promastigote are inoculated in the dermis that infect macrophage and/or dentritic cells (Langerhans cells) (Titus et al., 1993;Moll et al., 1996). Promastigote were found to interact with sera from infected patient but amastigote were protected

from antibodies and other circulating substances. Finally amastigote evades from immune response through phagocytosis that might protect host cell from apoptosis (Moore and Matlashewski, 1994).

Parasite multiplication is reduced in early infection by generating reactive oxygen and nitrogen intermediates. Nitric oxide synthase (NOS)-2 gene regulation is also marked point for parasite control. Inducible isoform of nitric oxide synthase (iNOS, NOS-2) of macrophage produce nitric oxide. Macrophage also produces reactive oxygen species (O_2^-, H_2O_2) which are generated by NADPH oxidase and superoxide dismutase of phagocytes. Although, passive parasite protection is provided via collective interaction of suppression of the synthesis of reactive oxygen or nitrogen intermediates, modulation of the host cytokine response, inhibition of antigen presentation and T cell stimulation and inhibition and expansion of counter protective T helper cells (Bogdan and Rollinghoff, 1998).

Both humoral and cell mediated immune response plays pivotal role and their proper tracing are important for the success of proper diagnosis, treatment and also for the study of prophylactic action.

2.4 Humoral response:

The mammalian host with entry of *Leishmania* tries to defense through its immune response and in return the parasite escape to avoid destruction. In the process of invasion to macrophage, the parasite encounters the immune system consisting natural immunoglobulin, complements and certain cytokines. Polyclonal immunoglobulins are the major factors that determine the fate of humoral immune response in Leishmaniasis. (Tiwari et al., 1995). Hypergammaglobulinaemia (IgG and IgM) against various non-specific protein and haptens are the remarkable features in Leishmaniasis (Awasthi et al., 2004). The sera of all vertebrate taxa have been known for possessing natural anti-leishmanial antibodies since long time (Rezai et al., 1975;Ulrich et al., 1986).

In the early period of invasion, serum cytotoxicity activity against promastigote contributes significantly as 90% promastigote are found destroyed within 2.5 minutes after serum contact (Dominguez et al., 2002). This strong selective pressure on parasite by complements proteins and glycoproteins leads parasite to enter a permissive monocytes/ macrophage escaping the activated complement lysis.

With the entry of promastigote in serum, opsonins (antibodies and complements) are adsorbed by parasite then binds with Fc or 3Cb receptors of macrophage for promastigote opsonization but metacyclic promastigote escapes from complement lysis and use the process in own favor for internalization in macrophage (Mosser and Edelson, 1987; Da Silva et al., 1989; Descoteaux and Turco, 1999). Metacyclic stage is resistance to complement lysis due to increased thickness and structural modification of

lipophosphoglycan (LPG) (Sacks, 1989; Puentes et al., 1990). Complement lysis of *L. donovani* promastigote is disturbed as C3bi cannot bind to surface and fail to function in C5 convertase formation (Puentes et al., 1989; Descoteaux and Turco, 1999). *Leishmania* phagocytosis by macrophage tissues and langerhans cells is enhanced by ligands like Fc, C3b, fibronectin receptors, gp63 and LPG on the parasite surface (Russell and Talamas-Rohana, 1989) The cunning nature of parasite may cause detrimental effect on host since antibodies as humoral response has no effect on *Leishmania* since they reside in phagolysosome of macrophage (Cunningham et al., 2001). Even though the humoral response cannot be under estimated as the *Leishmania* specific antibodies rise soon after infection and before the cellular immunological abnormalities development.

2.5 Cell mediated immunity (CMI):

The wide range of clinical spectra of disease along with high rate of asymptomatic stage is associated with strong cell-mediated immunity. Both innate and adaptive immune responses are essential for antileishmanial immunity through effective activation of macrophages, neutrophils, dentritic cells, monocytes and antigen-specific $CD4^+$ and $CD8^+$

T cells (Alvar et al., 2006). Although macrophages are the primary host cell for *Leishmania* parasites, monocytes, dendritic cells (DCs) and neutrophils that are recruited to the infection site can become infected and have important and distinct roles in shaping the immune response to infection .

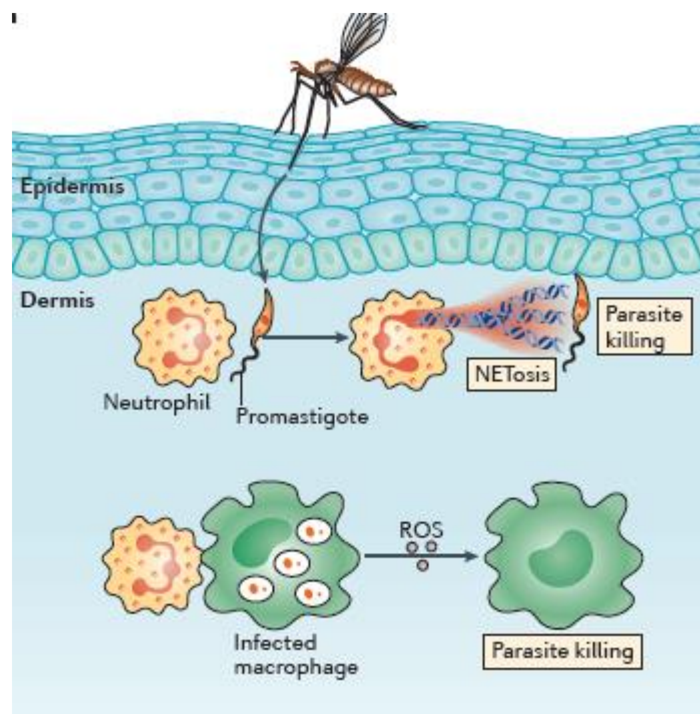


Fig:2.3 The involvement of innate cells in parasite control

Neutrophils are rapidly recruited to the site of a *Leishmania* infection, but their role here is complicated; they may kill the parasites or protect them depending on the parasite species and the host. For example, *Leishmania amazonensis* promastigotes are killed by neutrophil extracellular traps (NETs) however, salivary proteins from the sand fly can protect the parasites against neutrophil-mediated death. Thus, it remains unclear whether NETs have a protective role *in vivo*. Neutrophils can also contribute to the control of *Leishmania braziliensis* and *L. amazonensis* by interacting with infected macrophages (Novais et al., 2009). By contrast, uptake of apoptotic neutrophils by macrophages and DCs after *L. major* infection can limit the activation of macrophages and DCs, leading to better parasite survival (Savill et al., 2993; van Zandbergen et al., 2004). Inflammatory monocytes and DCs are also recruited to the site of infection, and over the first few days become the dominant cells infected with *Leishmania* parasites. Even within the first few hours of infection, some DCs and monocytes are infected with the parasites (Ng et al., 2008). The consequence of monocyte infection is markedly different from infection of macrophages; monocytes exhibit a strong respiratory burst upon infection, leading to early parasite control, whereas macrophages need to be activated by IFNγ to kill the parasites (Goncalves et al.,2011;Scott & Novais, 2016).

T lymphocytes are critical for cure and generation of a protective immune response, also being responsible for the disease persistence and pathology of leishmaniasis. Human leishmaniasis infection resolution is dependent primarily on the events of T cell subtypes and cytokine production profile.(Brelaz-de-Castro et al., 2012). The control of *Leishmania* infection is dependent on cellular immune mechanisms, and evidence has shown that CD4 and CD8 T lymphocytes play different roles in the outcome of leishmaniasis. Although the presence of CD4 T cells is important for controlling parasite growth, the results in the literature suggest that the inflammatory response elicited by these cells could contribute to the pathogenesis of lesions. However, recent studies on CD8 T lymphocytes show that these cells are mainly involved in tissue damage through cytotoxic mechanisms(da Silva Santos & Brodskyn, 2014)

CD4+ and CD8+ T cells act producing biologically relevant cytokines for the activation of monocytes and macrophage. The cytokine profiles produced by T cells are asso- ciated with the healing process or with the development of disease, as well as protective mechanisms (da Silva Santos & Brodskyn, 2014). The production of IFN-c, TNF-a and IL-12, has been associated with the infection control, by macrophage ctivation and parasite destruction. On the other hand, cytokines like IL-4, IL-10 and TGF-b, favor parasite multiplication, inhibiting NO production by IFN-c activated macrophages. These cytokines are also able to inhibit T cell differentiation into a type 1 profile and the subsequent production of IFN-c and TNF-a. Therefore, the quality of the immune response can

influence disease susceptibility and development, with specific activation of T cells (especially multifunctional T cells) contributing to it (Macedo et al., 2012). However, even though there are studies that helped to clarify the immune mechanisms of disease development and pathology, no clear data is available about what immune response is necessary to control the disease and what is the difference between patients that develop the disease and achieve clinical cure (with or without treatment) (Brelaz-de-Castro et al., 2012).

2.6 Cytokine response:

Different strains of murine model such as BALB/c are available immune-model for CL although the exact immune response produced by them on infection does not match human beings. In most intracellular parasite infections, macrophage produces IL-12 along with other cytokines (IL-6, IL-18, IFN-γ and TNF-α) for Th1 type immune response. CD^{+} T cell population plays crucial role in disease progression through IL-4 and prevention through IFN- γ. IL-10 is also produced by CL patients and is responsible for down-regulating inflammatory responses, mainly those induced by IFN-g (42–44). IL-10 is produced by a variety of cells, including macrophages, regulatory T (Treg) cells, Th1 cells, and CD8 T cells. The presence of Treg cells (natural and inducible) in the lesions from CL patients as well as IL-10 and TGF-b production has already been described(da Silva Santos & Brodskyn, 2014). These cytokines are responsible for the control of the immune response in CL patients but also for the pathology of disease, deactivating the mechanisms of macrophage killing and leading to parasite persistence A down- regulation of IL-10 receptor was demonstrated in lesions from ML patients, which can partly explain the lack of IL-10 response and the absence of inflammatory process down-regulation. IL-27,a cytokine with close structural and functional similarity to the IL-6/IL-12 family, is also expressed at high levels in the peripheral blood and tissues of ML and CL patients .Despite the induction of Th1 differentiation during the first steps of the immune response,IL-27 has been shown to promote an attenuation of inflammatory responses, Improving IL-10 production by Th1 CD4 T-cells This function precludes inflammation and subsequent tissue damage in the late phase of the immune response. However, Oliveira et al. showed that the addition by IL- 27 of PBMCs from CL and ML patients did not enhance IL-10 production by these cells, suggesting that IL-27 did not have an effect on regulating the strong inflammatory response observed in human CL patients (Oliveira et al., 2014).Major cytokines like IFN-γ produced by NK cells and IL-12 by macrophage influence Th1 development *in vitro* in various system and IL-4 induces Th2 development (Hsieh et al., 1993).

Chapter III

MATERIALS AND METHODS

3.1 Study site

Nepalgunj of province number 5 and Kathmandu of Province number 3 were defined as the sample collection provinces as the hospitals of the two provinces represents health sectors of Nepal. Two hospitals; Sukraraj Tropical and Infectious Disease Hospital (STIDH), Teku, Kathmandu and Nepalgunj Medical College and Teaching hospital (NGMCTH), Koholpur, Banke were chosen for the collection of blood samples and the tissue scraps, pus samples from the lesion present in the patients' body[Fig. 1]. These two hospital sites are the major government hospitals which cover all the cases from the Eastern to the Western parts of Nepal.

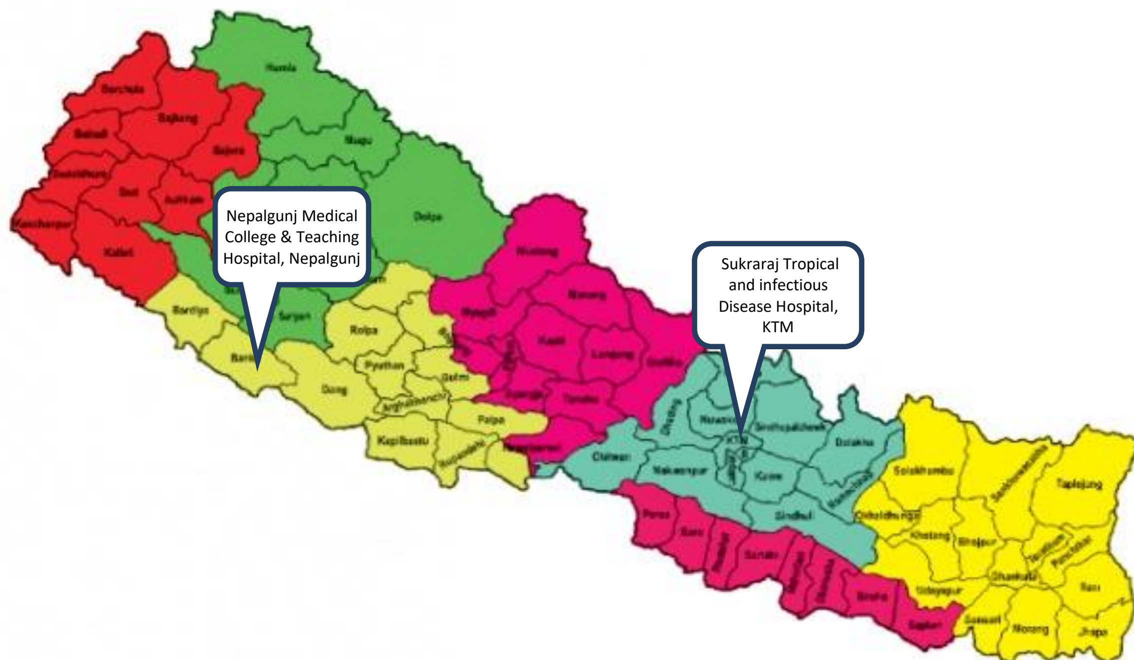


Fig.3.1: Location of Hospital sites for sample collection (a) Shukraraj Tropical and Infectious Disease Hospital (STIDH), Teku, Kathmandu, Province# 3 (b) Nepalgunj Medical College and Teaching hospital (NGMCTH), Koholpur, Banke, Province# 5

3.2 Collection of Bio-specimen

Samples were collected from those patients who visited our collection sites with complaints of non-healing wounds. The cutaneous leishmaniasis suspected patients were clinically diagnosed by the medical officer in respective hospitals. The patients with lesion on the surface of the body with non-healing wounds for a long time were identified and signed informed consents were taken from each.

Questionnaire to get information as below were recorded.

- Demographic and epidemiologcal information
- Clinical symptoms
- Age (In case of below 18 years, signed consents were taken from the parents)
- Gender
- First onset of disease,
- Number
- Position of lesion
- Medicine used before
- Travel history to endemic leishmaniasis regions (outside and within Nepal)

Blood and pus samples were collected from the patients attending at STIDH and NGMCTH. The sampling was done under sterile condition. Lesions and the adjacent normal-looking skin around them were cleaned and sterilized with disinfectant. The sterile hypodermic syringe needle was inserted into the nodule or around ulcers and rotated gently several times. Some tissue aspirate and freed tissue were withdrawn. The syringe was removed from the lesion followed by dispensing

⇒ 1/2 drops in two clean slides for microscopy
⇒ Little contents into tubes containing RPMI medium for culture
⇒ Remaining in an Eppendorf for DNA extraction.

For the collection of peripheral blood, 5 mL syringe with 22 gauze needle was used and about 5 mL of the blood was drawn and collected in 6mL EDTA collection tube from each patients.

After the collection of samples, the samples were transported to laboratory at Central Department of Biotechnology, Tribhuvan University under ambient condition for further processing.

3.3 Microscopy

3.3.1 Preparation of Giemsa stained slides

During collection of sample some of the aspirates was dropped on the clean and grease free glass slides and a thin smear of it was made with the help of edge of another glass slide. The smear was left to dry at room temperature. Then the slides were fixed in 100% methanol for 30 seconds and rinse with water. After that it was stained with 10% Giemsa stain for 30 minutes. The slides were rinsed off with tap water and dried thoroughly at room temperature. The slides were then viewed under objective.

3.3.2 Preparation of media and culture of parasite

Bacto agar (10g) and NaCl (9g) was dissolved in 1000mL of distilled water and pH was adjusted to 7.2. Then the mixture was autoclaved at 15 lb pressure for 15 minutes. Blood collection tube containing 1/5 of glass beads was also autoclaved. Human blood was drawn and transfer to collection tube. It was shaken vigorously and rolled the vials between the palms which defibrinate the blood. Melted agar media (70%) and 30% of the defibrinated blood was mixed by pipetting and 1mL of the mixture was transferred to culture tube. The tubes were positioned on slant bottom side till it solidifies. After that 200 µL complete Roswell Park Memorial Institute medium (cRPMI) was overlaid to each tubes in laminar hood and seal with parafilm. One to two drops lesional aspirates was added to a tube from each patient in their respective labelled culture tube. The sample tube was brought to laboratory at ambient temperature and kept in BOD/cooling incubator at constant 26^{0}C for 9 days. The tubes were checked for growth of parasites in inverted microscope taking a drop of media.

3.4 Molecular Diagnosis

3.4.1 DNA extraction

The DNA from the clinical specimen was extracted by using DNA extraction Kit (Quick-DNA™ Universal Kit, Zymo Research) according to the manufacturer's instructions. For this, 200µl of the sample, 200µl of BioFluid and Cell buffer and 20µl of proteinase K (Provided with kit) was added in an Eppendorf tube followed by vortexing for thorough mixing. The tubes were incubated at 55ºC for 10 minutes in water-bath. Then 420 µl of genomic binding buffer was added to the digested product and mixed thoroughly. After that the mixture was transferred to a zymo-spin™ IIC-XL column in a collection tube. It was then centrifuged at 12,000 rcf for 1 minute. The collection tube with flow through was discarded. Then 400µl of DNA pre-wash buffer was added to the column in a new collection tube and centrifuged at the same rcf as before for 1 minute. The collection tube was made empty. After that 700µl of g-DNA wash buffer was added and centrifuged as before and again the collection tube was made empty. Again 200µl of g-DNA wash buffer was replenished and centrifuged for 1 minute. The collection tube with flow through was discarded. For the elution of DNA, the column was transferred to a clean Eppendorf tube and 40µl of DNA elution buffer was added and incubated for 5 minutes and then centrifuged for 1 minute at 12000 rcf. The DNA was kept at −20°C until PCR processing.

3.4.2 Quantification of DNA by Nanodrop

DNA was eluted in DNA elution buffer. So the same elution buffer was used for the measurement of blank. Then, 1µl of the extracted sample was put in a nano drop (SHIMADZU BIOTECH BioSpec-nano) and absorbance was measured in the wavelength 260nm and 280nm for quantification of extracted DNA.

3.4.3 Nested PCR

For molecular identification of the parasite, specific primers for the variable region of the kinetoplast minicircles were amplified. The primers and the protocols were adapted from Noyes et al. (Noyes et al. 1998).

First round PCR (PCR1)

In order to carry out the PCR1, the work station was wiped with 70% ethanol and exposed to UV for 15 minutes. The first round PCR was performed with 2 µL of template DNA and the forward and reverse primers.

Forward Primer (CSB2XF)–C/GA/GTA/GCAGAAAC/TCCCGTTCA

Reverse Primer (CSB1XR)– ATTTTTCG/CGA/TTTT/CGCAGAACG

The reaction mixture and the reaction condition were performed as shown in the table (Table 1 and Table 2).PCR-grade water was used as negative control. The 5x FIREPol master mix (in 1x) from Solis BioDyne composed of DNA polymerase, 0.4M Tris-HCl, 0.1M $(NH_4)_2SO_4$, 0.1%w/v Tween-20, 2.5mM $MgCl_2$ and 0.8mMdNTPs.

Table 3.1: Reaction Composition for PCR1 and PCR 2

PCR1		**PCR2**	
Reagents	**Vol. Per reaction**	**Reagents**	**Per reaction**
Master mix (5x)	5µl	Master mix (5x)	5µl
CSB2XF	1µl	13Z	1µl
CSB1XR	1µl	LiR	1µl
Template DNA	2µl	Diluted PCR 1	1µl
DNA grade water	16µl	DNA grade water	17µl
Total	**25µl**	**Total**	**25µl**

Table 3.3: Thermal cycling condition for PCR1 and PCR 2

Steps	PCR 1			PCR 2		
	Cycles	**Temp.**	**Time**	**Cycles**	**Temp.**	**Time**
Initial denaturation	1	94ºC	2 min	1	94ºC	2 min
Denaturation		94ºC	0.5min	40	94ºC	0.5min
Annealing	40	54ºC	1min		56ºC	1min
Extension		72ºC	1.5min		72ºC	40 sec
Final extension	1	72ºC	10 min	1	72ºC	10 min
Hold		4ºC	Until PCR2		4ºC	Indefinitely

Second Round PCR (PCR2)

In the second round PCR, the amplicon of the first round PCR was diluted to 1:10 ratio and 1µl of the diluted PCR1 was used as template together with the primer sets (13Z – ACTGGGGGTTGGTGTAAAATAG and LiR – TCGCAGAACGCCCCT). The reaction mixture and condition is shown in the table (Table 3.1 and Table 3.2). The PCR products were resolved on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. PCR-grade water was used as negative control.

3.4.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to resolve the PCR products for the confirmation of amplification. For this 1.5% of agarose gel was prepared in 1X TAE (Tris base, acetic acid and EDTA) buffer and casted of gel electrophoresis tank. Three microliters of PCR2 product with 1 µl of loading dye were loaded along with suitable molecular weight DNA ladder (100bp, Solis BioDyne). Then the gel was run at 80 volts for 1 hour. Finally the PCR products on agarose gel, stained with ethidium bromide was visualized under UV Trans Illuminator and gel doc (MS major science UVDI) to confirm the amplified fragment and photographed to keep the record.

3.4.5 Sequencing

The PCR amplicons were sent to Xcelris Pvt. Ltd., Ahmedabad for bidirectional sequencing using the primer sets LiR and 13Z. The sequences obtained were analyzed using the software MEGA7 and compared using BLAST with existing database of National Center for Biotechnology Information (NCBI).

3.5 Immunology by flow cytometry (BD FACS Calibur)

3.5.1 Preparation of antibody stained cells

Fresh blood was collected from clinically diagnosed CL patients and healthy controls. Fifteen microliter of T cell panel antibody cocktail (BD Biosciences, USA) and 15 µl of B cell panel antibody cocktail panel (BD Biosciences, USA), were mixed with 200 µl of fresh blood and incubated in dark for 15 mins at room temperature. Then 2 mL of 1:10 diluted BD FACS lysing solution (BD Biosciences, USA) was added, vortexed briefly and incubated for 12 mins at ambient temperature. After that, the tubes were centrifuged at 1200 rpm for 5 mins. The supernatant was discarded and 2 mL of 1X Phosphate buffer saline (PBS) was added and mixed and centrifuged at 1200 rpm for 5 minutes. Again the same procedure was repeated, supernatant was discarded and the cells were resuspended in 400 µl PBS. For unstained control the same procedure was followed except the addition of antibodies.

Cell markers and tagged fluorochromes of the T and B cell panel used are as following:

T cell panel: CD3-FITC, CD8-PE, CD45-PerCP and CD4-APC and

B cell panel: CD3-FITC, CD56/16-PE, CD45-PerCP and CD19-APC.

Table 3.4: Channels and Panel in Flow cytometry

Channel	Fluorochromes	T-Cell panel	B-Cell Panel	Negative control
FL1	FITC	CD3	CD3	-
FL2	PE	CD8	CD56/16	-
FL3	PerCP	CD45	CD45	-
FL4	APC	CD4	CD19	-
Blood		200µl	200µl	200µl

3.5.2 Compensation of FL Channels in flow cytometer

The four FL channels in the BD FACS Calibur machine are FL-1, FL-2, FL-3, FL-4 which collects the emission wavelength from the flurochromes FITC, PE, PerCP and APC respectively. The compensation of the channels were done by using BD calibrate beads. Compensation was done by aligning the cells in their respective quadrant by manipulating the voltages, current and compensation of different channels. When compensation was performed properly the further processing of samples were done in the same condition.

3.5.3 Acquisition of stained cells and Analysis

After the completion of compensation of FL channels, the processed cells were acquired. First the unstained cells were acquired followed by the acquisition of stained cells of different panels. After completion of acquisition the results were analysed using FlowJo software ver.10

3.6 Ethical Approval

As the research includes involvement of human participants, the research protocol was approved from ethical review board of Nepal Health Research Council (NHRC) (Reg. no.45/2018) before the sample collection [Annex I]. No information on the patients has been presented in this research.

Chapter IV

RESULTS

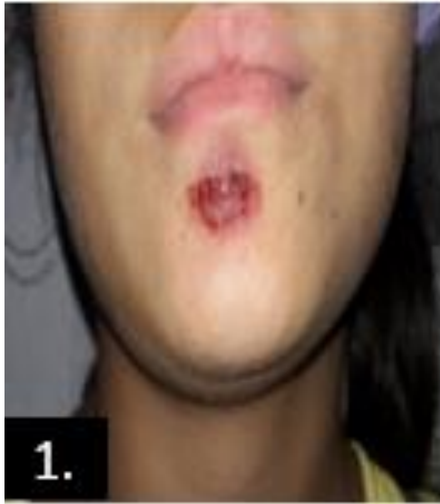
4.1 Clinical features of CL patients

The new emerging thirteen cutaneous disease infected patients were diagnosed clinically by the characteristics of lesions. The lesions were found in various parts of the body like face, neck and limbs. Most of the patients (64.70%) had lesions on their face followed by neck (17.64%), lower limb (711.76%) and upper limb (7.69%) [Fig. 4.1, Table 4.1]. In the facial region different parts were found infected like cheek [Fig 4.1 (8, 12 & 15(a&b)], upper and corner lip [Fig 4.1(11a), 16], upper nose [9], around eyes [Fig 4.1 (2)], lobular ear [Fig 4.1(3)], neck [Fig. 4.1(4, 5&14)], chin [Fig 4.1 (1)] developed lesions.

The patients who have initial infection stage showed acne like eruption at the site of sandfly bite [Fig 4.1(1&2)]. After the incubation of sometime the acne found to grow and become crusted associated with oozing [Fig. 4.1(1)] and the lesions were mildly pruritic sometime. The size of the crusted part were also found to increase in size (3.5cm) as in a case who has at the region of ear lobe [Fig 4.1 (3)]. The crusting were found erupted with oozing as is seen in neck, lip and upper arm [Fig. 4.1(4, 5 & 11a)], There were crusted erythematous plaque with induration of size (1.5X 1cm) as seen upper cheek and chin [in Fig.4.1 (1&2)].There were ulceration cases with raised borders at the site of the vector bite as seen in the forearm of hand and shank of leg [Fig 4.1 (6&7)]. There was no history of any illness. CL were found to produce single to multiple numbers of skin lesions on exposed parts of the body. Fourteen patients had single lesion on their body parts mainly face, three patients had multiple lesions; two patients had 2 lesions and one patient had 3 lesions (Table 4.2). The disease has been misdiagnosed in many of the cases. Five patients used to take anti TB drug before they were diagnosed as CL (Table 4.3) while others took skin ointments, antibacterial and antifungal drugs.

Table 4.1: Distribution of Lesion in patients

Site of Lesion	Frequency	Percentage (%)
Face	11	64.70
Neck	3	17.64
Upper limb	1	7.69
Lower limb	2	11.76
Total	**17**	**100**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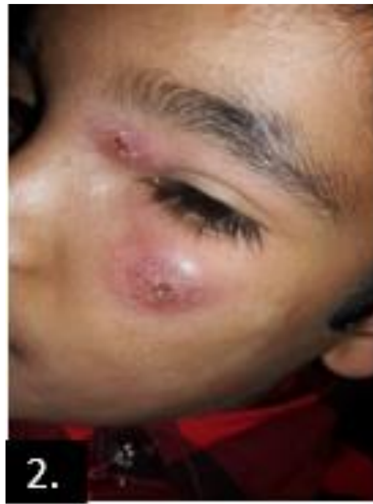
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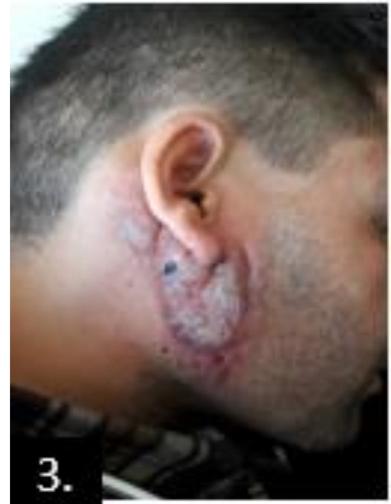
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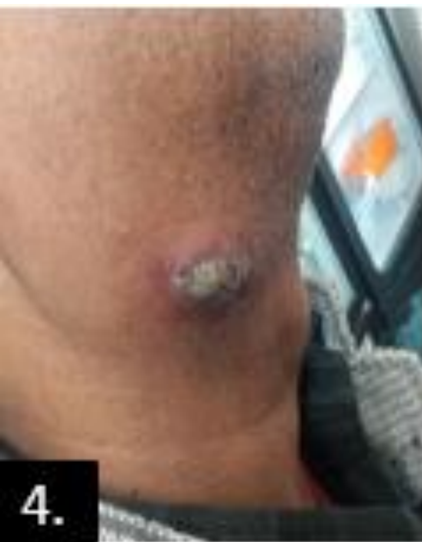
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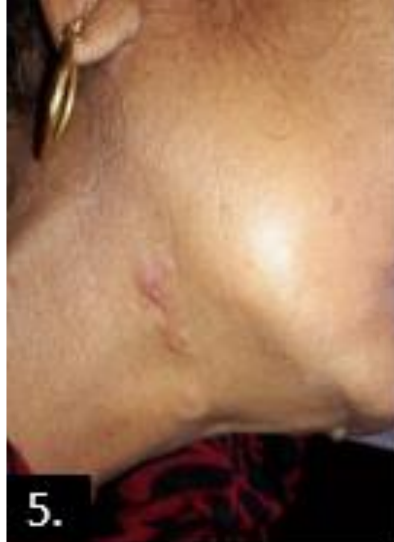
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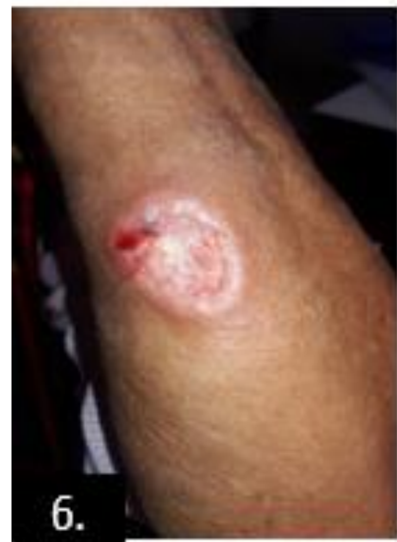
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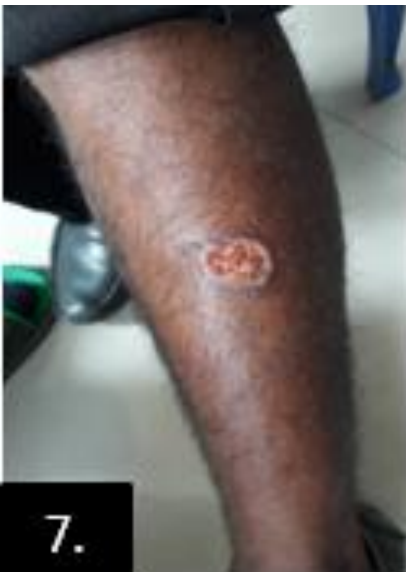
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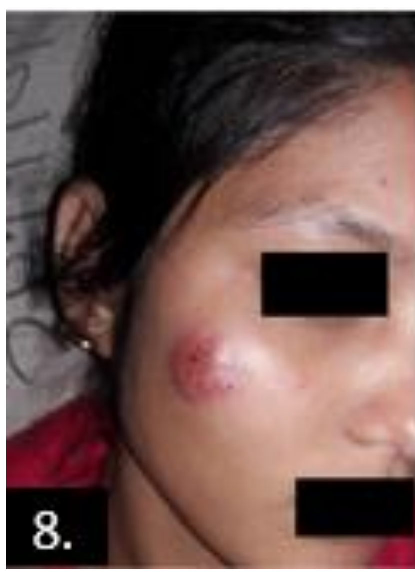
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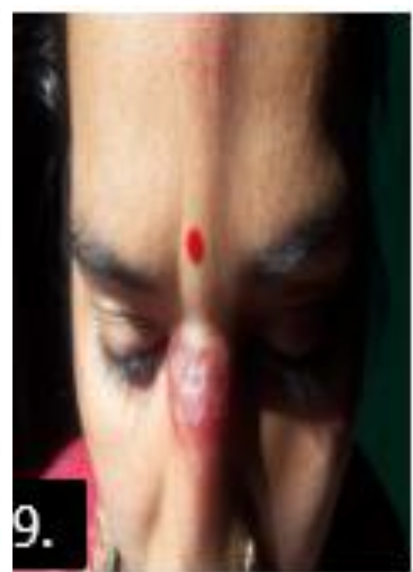
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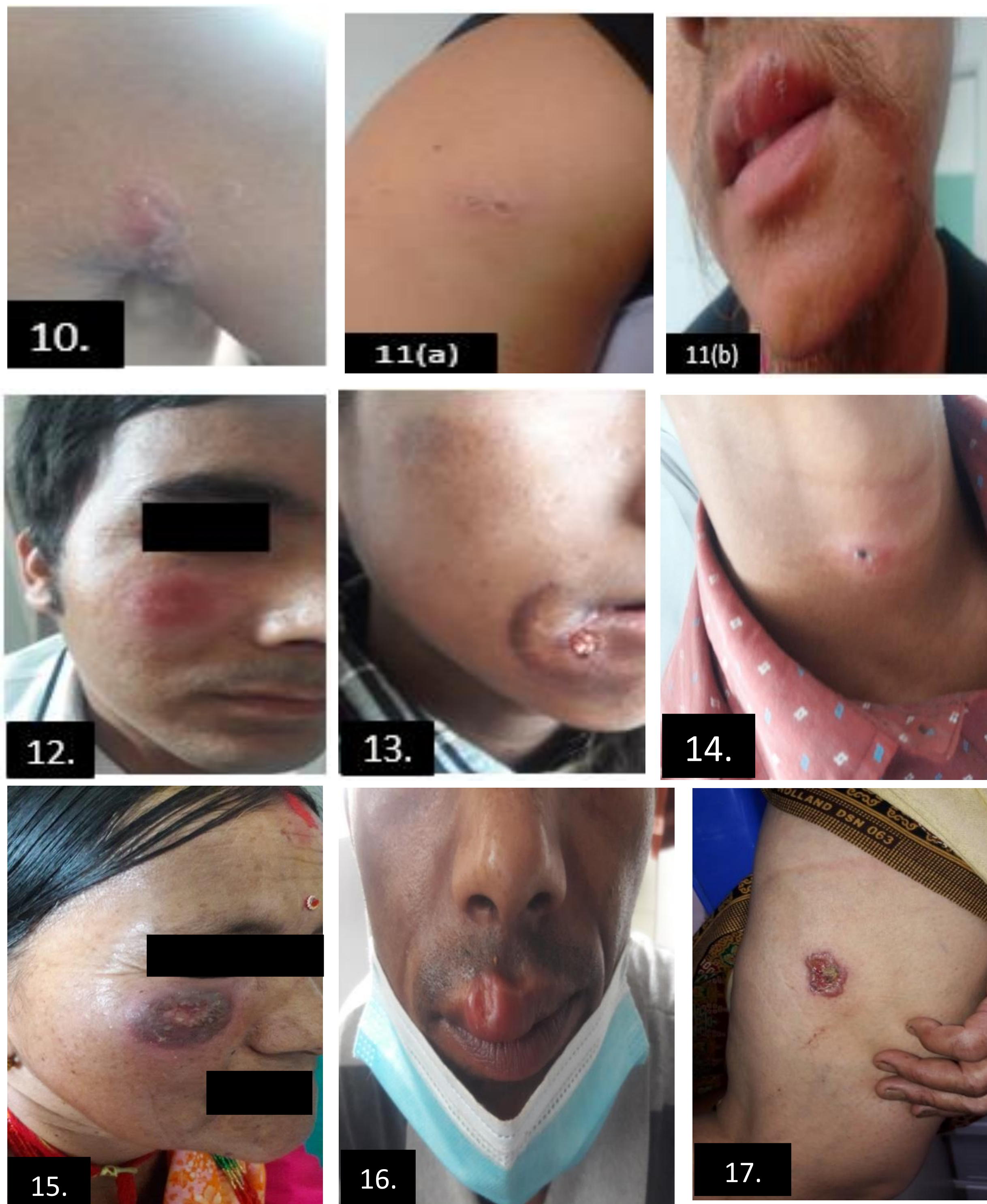

Fig 4.1 Clinical Feature of CL patients of the study based on duration of lesion appearance and characteristics of lesions.

Characteristics of lesions in the Patients. (1) Acne with induration in chin. (2) Multiple induration and ulcerated lesion. (3) Inflammatory lesion on below right ear lobe. (4) Non healing ulcer. (5) Non healing ulcer.(6) Ulcerated lesion on fore limbs with central

depression. (7) Ulcerated lesion on hind limbs with central depression. (8) Nodulated lesion on cheek of female. (9) Nodulated lesion on upper nose. (10) Induration with oozing. (11a)Nodulo-ulcerated lesion on arm with raised indurated border. (11b)Central depressionon healing lesion on upper lip.(12) Towards healing. (13) Ulcerated lesion on near lower lips. (14) Non healing ulcer (15) multiple nodulo ulcerated lesion on face (16) Nodulated lesion of upper lip (17) Ulcerated lesion of right thigh with central depression.

Table 4.2: Mono and multilesions in the studied cases

No. of lesion	No. of patients	Percentage	Location of lesion
1	1	82.35	Cheek, Chin, upper lip, nose, behind ear lobe, front neck, limbs, thigh
2	2	11.76	front neck, hand, arm
3	1	5.88	right and left cheek, chin

Table 4.3 Misdiagnosis of disease

No. of patients	Medicine used before
5	Anti-Tuberculosis
1	Fusid acid cream (anti-bacterial)
2	Sodium stibogluconate
1	Cetrizine, mupirocin
6	Skin ointment
2	Itraconazole (antifungal)

4.2 Epidemiology and demography of cutaneous leishmaniasis in Nepal

4.2.1 Distributionof CL during the project period

The new emerging disease was found 12 districts, of Nepal including all the provinces of Nepal except province 1. Mostof the patients involved in the study were from Kalikot [Fig. 4.2]. Incidence of the CL were found in the Rautahat District of Province 2, Ramechhap of Province 3, Tanahun, Gorkha and Syangja of Province 4, Rolpa and Palpa1 of Province 5 and Kalikot and Humla of Province 6, and Kailali, Kanchanpur and Baitadi of Province 7. Many numbers of cases were found in Province 7 (n=5) and Kalikot District of Province 6 (n=4,).

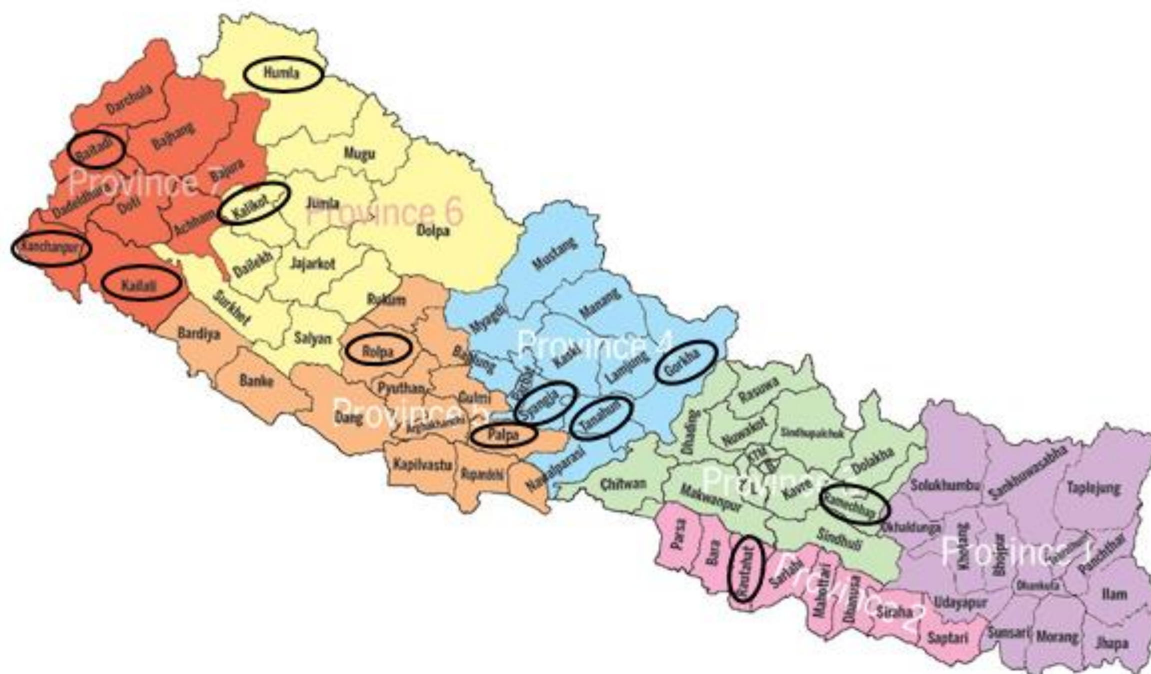

Fig 4.2. Incidence of CL cases in the Districts and Provinces of Nepal

Table 4.4: Distribution of Patients according to District and Provinces.

S.No.	District	Altitude(asl)m	Province no.	Frequency	Percentage
1.	Rautahat	122-244	2	1	7.69
2.	Ramechhap	1000-4848	3	2	15.38
3.	Syangja	1088	4	1	7.69
4.	Tanahun	1437	4	1	7.69
5.	Gorkha	300-6400	4	1	7.69
6.	Rolpa	1547	5	1	7.69
7.	Palpa	300-6600	5	1	7.69
8.	Kalikot	738-4790	6	3	23.07
9.	Humla	1000-5000	6	1	7.69
10.	Kailali	179-1957	7	1	7.69
11.	Baitadi	2439-6936	7	3	17.64
12.	Kanchanpur	270	7	1	7.69
	Total			**17**	**100.00**

4.2.2 CL patients visited to the hospitals

During the period of 10 months (December 04, 2017 to September 06, 2018), 17 clinically diagnosed CL samples were collected from two hospitals. Most of the samples were form STIDH (88.23%).

Table 4.5: Total number of samples collected from two hospitals

SN	Hospital Site	No. of Participants	Percentage (%)
1.	STIDH	15	88.23
2.	NGMCTH	2	11.76
	Total	17	100

4.2.3 Gender wise distribution of Patients

The study cohort consisted of 58.82 %(n=10) males and 41.17 %(n=7) females. The male population was found to be higher than the female population. Male:female ratio was found to be 1.4:1.The lesions appeared in the body of the male and female showed that there is no correlation of the lesion sites specific to the sex.

Table 4.6: Distribution of research participants gender wise

Gender	Frequency	Percentage
Male	10	58.82
Female	7	41.17
Total	**17**	**100**

4.2.4 Distribution of Patients according to age

A total of 17 patients ranging from 8 to 85 years with mean age 36±22.05 years were involved in the study. Most of the patients infected were within the age group of 40 with the highest frequency seen among the age group 21 – 40 year-age group (35.29%) followed by below 20 years old (29.41%)

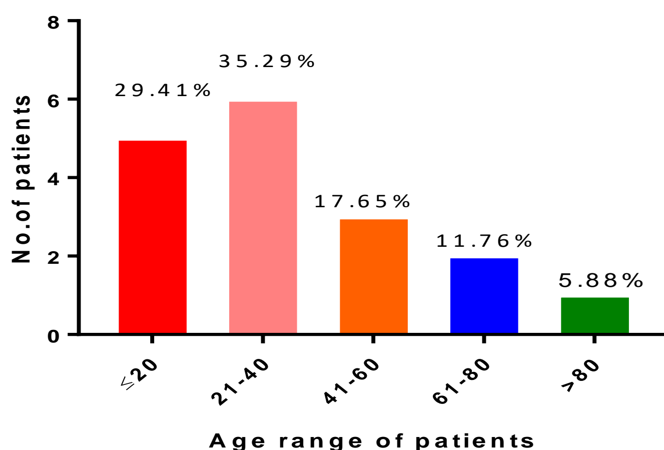

Fig. 4.3 Distribution of research participants into different age groups

4.2.5 Skin lesion eruption and sample collection

CL diagnosis was negatively associated with the duration of lesions prior to sampling. It was found that most of the patients visit hospital very late after 4-5 months of lesion eruption. None of the patients take the acne on their body as cutaneous leishmaniasis until 2 months and mostly CL infected patients (52.94%) visit hospital only after 8 months showing the trend of visiting hospital for recovery from CL as the number of days increase.

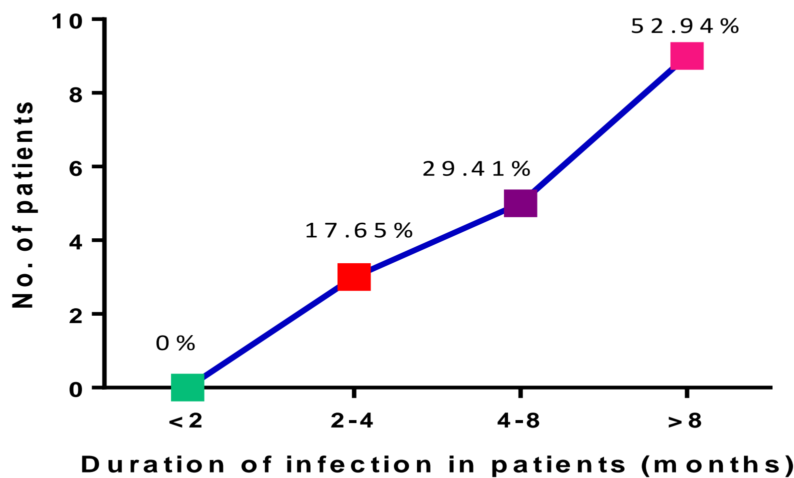

Fig. 4.4 Time duration of hospital visit after skin lesion appeared

4.3 Diagnosis:

4.3.1 Microscopic study

Out of 13 clinically diagnosed as CL cases, 2 biopsies were found to be positive by microscopy. The amastigote stages of *Leishmania spp.* (LD bodies) were observed in the slides of two patients confirmed by the presence of two dot stained nuclear material of nucleus and kinetoplast as seen clearly due after giemsa stainning. In the given first microscopy picture, there are multiples number of LD bodies in bunch with high scores of around 4+ positive[Fig. 4.5(A)]. While in the next microscopy picture there were 2 parasites after searching 100 fields showing the score of +1 positive [Fig 4.5(B)].

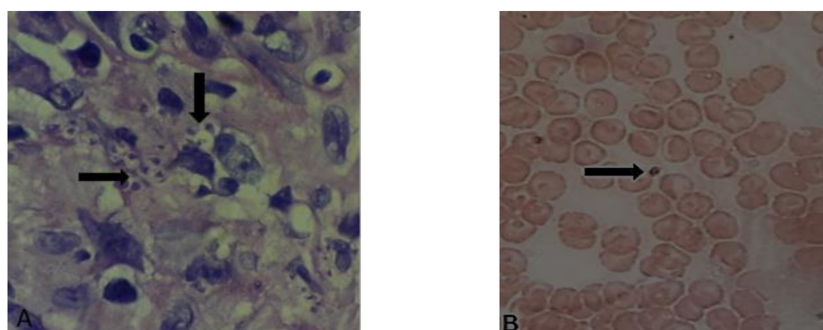

Fig4.5 Smears showing the LD bodies under 100X shown by arrows.

4.3.2 Parasite Culture

After 7 – 9 days of inoculation of samples into biphasic NNN media, a drop of culture was put into clean glass slide and observed under inverted phase contrast microscope. The parasites were not seen in any of the culture tubes. Only the RBCs were observed. Since no promastigote form of parasites were observed, further subculture could not be performed.

Figure 4.6 (A) Culture of parasite in NNN media (B) Observation of Culture after 7 days of inoculation

4.3.3 Molecular Diagnosis

4.3.3.1 DNA quantification:

The DNA extracted by kit from the biopsy samples of lesions was quantified using nanodrop and found between 5ng/µl to 300ng/µl, which were satisfactory yield [Fig 4.5]. According to Sambrook and Russel, DNA is pure enough when optical density (OD) ratio at the wave length 260/280 is 1.8 and the observed OD of the samples were varied. The concentration and purity of DNA samples are tabulated below [Table 4.4].

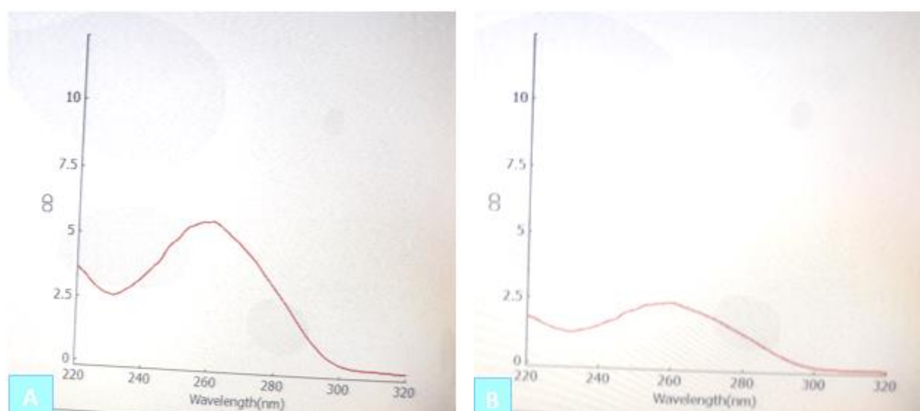

Fig. 4.7 Graph showing the maximum absorbance at 260 nm (A) CL12,- 276 ng/µL (B) CL 13- 123.17 ng/µL.

Table 4.7 Concentration and purity of DNA samples from STIDH and NGMCTH

S.N.	Sample name	Absorbance		Concn. (ng/µl)	Ratio(260/280)
		260nm	280nm		
1	CL1	0.329	0.156	15.07	2.10
2	CL2	0.11	0.042	6.35	2.61
3	CL3	1.807	0.935	89.59	1.93
4	CL4	0.214	0.061	12.45	3.50
5	CL5	0.464	0.26	17.28	1.78
6	CL6	0.224	0.104	10.5	2.15
7	CL7	0.195	0.045	12.3	4.33
8	CL8	0.245	0.122	12.73	2.00
9	CL9	0.765	0.523	24	1.46
10	CL10	0.353	0.202	15.11	1.74
11	CL11	0.346	0.116	19.57	2.98
12	CL12	5.598	2.962	276.85	1.88
13	CL13	2.442	1.267	123.17	1.92
14	CL14	0.384	0.345	5.57	1.54
15	CL15	0.795	0.687	13.82	1.63
16	CL16	0.782	0.683	13.52	1.58
17	CL17	0.421	0.398	6.64	1.57

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

4.3.3.2 Polymerase Chain Reaction (PCR)

Nested PCR

The DNA extracted from the samples collected from STIDH and NGMCTH hospitals were subjected to nested PCR (two step PCR) along with negative control (no template). The step I PCR (PCR 1) electrophoresis showed bands of different sizes in some of samples based on comparison with 100bp DNA ladder. However, in many of the samples did not expressed bands which do not represent whether they are CL positive or negative. In the samples where step I PCR gave products upon visualization, the amplicon sizes ranged from 700bp to 800bp. Similarly, in the step II PCR (PCR 2), the amplicon sizes ranged between 600bp and 720bp. The differences in the amplicon size in PCR 2 were used to indicate different *Leishmania* species prevalent among the study population.

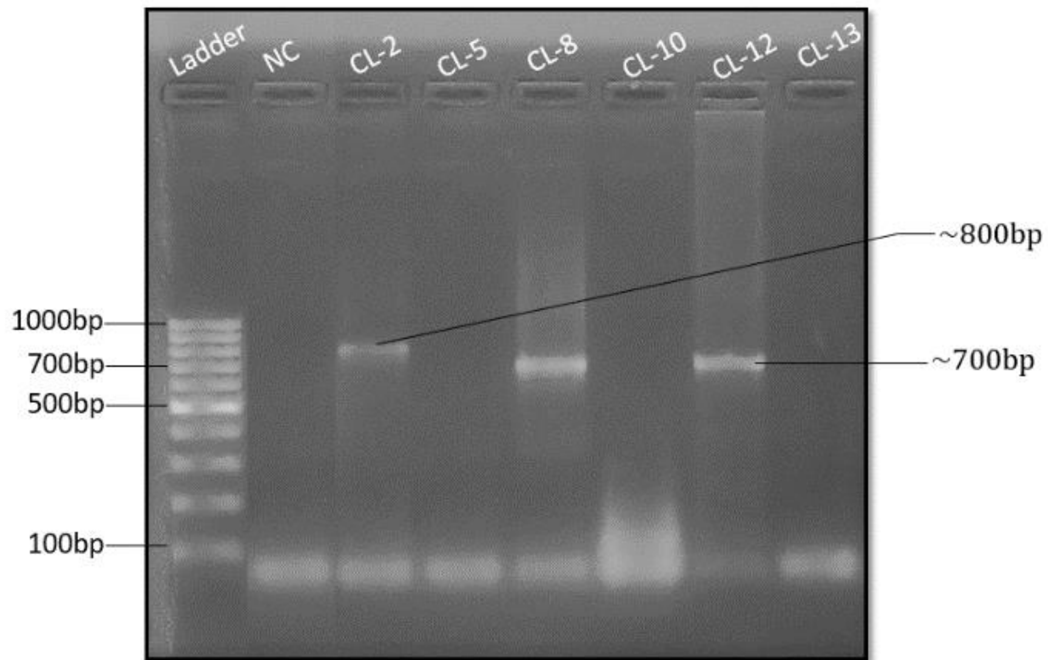

Fig 4.8 PCR 1 amplicon run for gel electrophoresis Lane1- 100 bp ladder, Lane 2- Negative control (NC), Lane 3-8 CL samples, Lane-3, ~800bp PCR amplicon, Lane- 5&7, ~700bp PCR amplicon, Lane 4,6,8- Not visualized

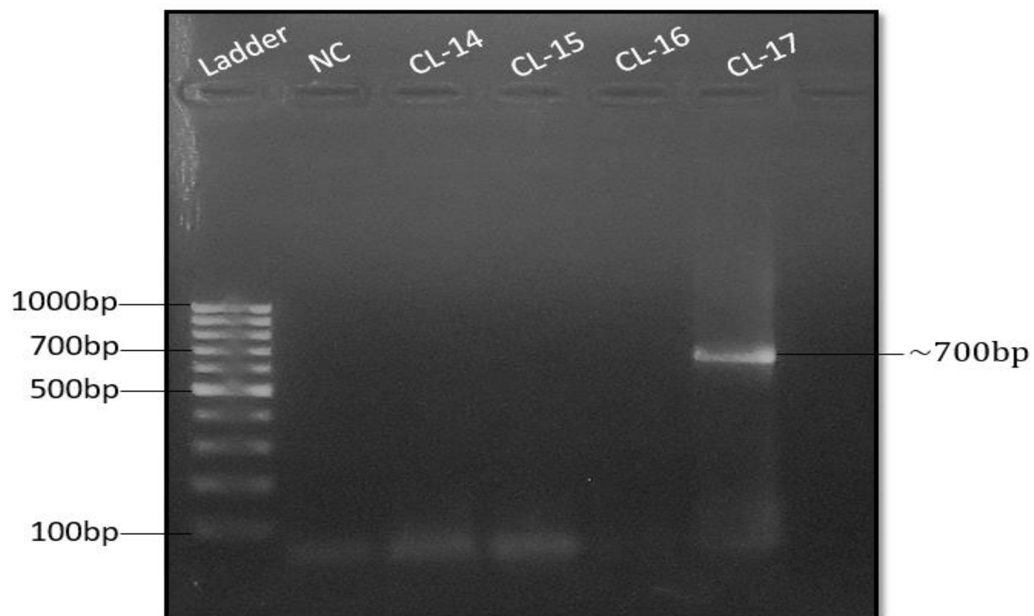

Fig 4.9 PCR 1 amplicon run for gel electrophoresis Lane1- 100 bp ladder, Lane 2- Negative control (NC), Lane 3-6 CL samples, Lane-3-5 not visualized, Lane- 6, ~700bp PCR amplicon

The gel electrophoresis visualization under gel doc of amplicon from PCR 2 expressed clear and/or sharp bands at the base pair of 720 bps and 590bps which confirmed the *Leishmania spp.*

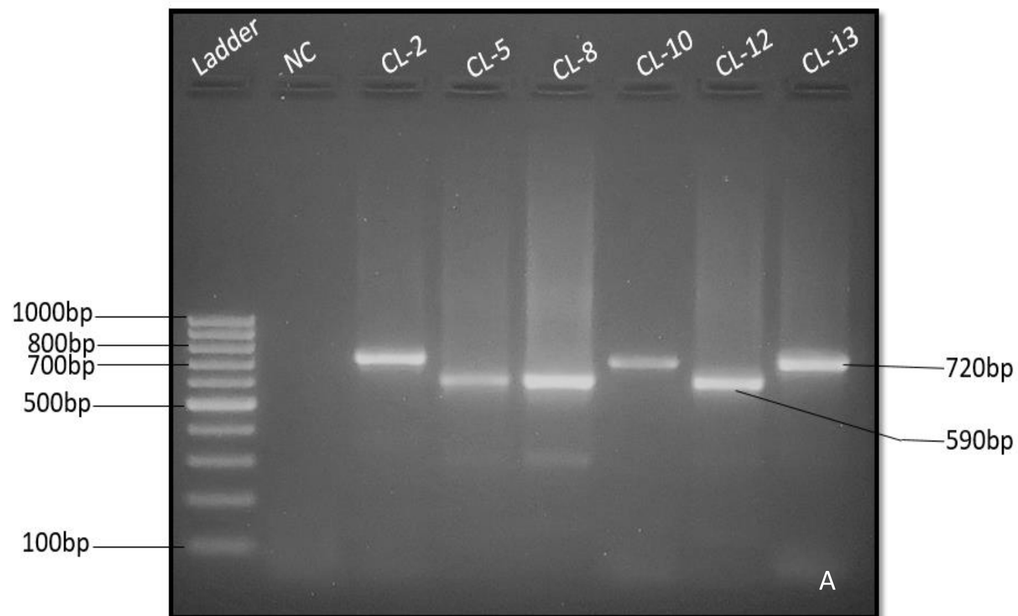

Fig. 4.10 : PCR 2 amplicon 1.5% agarose gel electrophoresis Lane 1- 100 bp ladder, Lane2-Negative control (NC), Lane 3-8 PCR amplicons of CL samples, Lane 3, 6,8- 720bp PCR amplicon, Lane- 4,5,7-590bp PCR amplicon

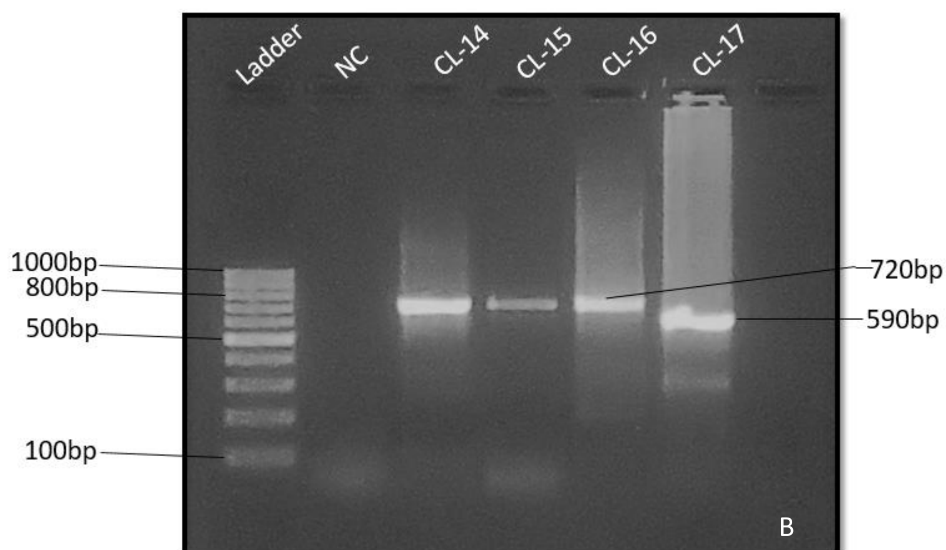

Fig 4.11 PCR 2 amplicon 1.5% agarose gel electrophoresis Lane 1- 100 bp ladder, Lane2-Negative control (NC), Lane 3-8 PCR amplicons of CL samples, Lane 3, 6,8- 720bp PCR amplicon, Lane- 4,5,7-590bp PCR amplicon

Table 4.8: Table showing causal leishmania species

A			B		
Samples	**Amplification**	**Leishmania sps**	**Samples**	**Amplification**	**Leishmania sps**
Ladder	100bp		ladder	100bp	
NC	-		NC	-	
CL2	~720 bp	*L. donovani* complex	CL14	~720 bp	*L. donovani* complex
CL5	~600 bp	*L. major*	CL15	~720 bp	*L. donovani* complex
CL8	~600 bp	*L. major*	CL16	~720 bp	*L. donovani* complex
CL10	~720 bp	*L. infantum*	CL17	~600 bp	*L. major*
CL12	~600 bp	*L. major*			
CL13	~720 bp	*L. donovani* complex			

4.4 Gender based PCR positive CL cases

Out of 17 samples, 58.82% (n=10) were males and 41.17% (n=7) were females. The genderwise disribution of the PCR positive cases in male were 50% and those in female were 71.42% (Table 4.7).

Table 4.9 Gender wise distribution of research participants

Gender	**Frequency**	**Percentage**	**PCR +ve**		**PCR -ve**	
			No.	**%**	**No.**	**%**
Male	10	50.82	5	50	5	50
Female	7	41.17	5	71.42	2	28.57
Total	**17**	**100**	**10**		**7**	

4.5 Age Based PCR positive CL cases

The study subjects were divided based on the age gap of 20 years for evaluation. The data showed that highest (n=3) number fell into age group ≤20 yrs with 60% positive cases and the lowest (n=0) number fell into age groups (41-60) yrs and other groups 21 – 40, 61-80 and >80 each had one positivecase.

Table 4.10: Distribution of research participants into different age groups

Age Group (in Years)	**PCR Positive**		**PCR Negative**	
	No	**%**	**No**	**%**
≤20	3	60	2	40
21-40	3	50	3	50
41-60	2	66.67	1	33.33
61-80	1	50	1	50
>80	1	100	0	0
Total	**10**		**7**	

4.6 Comparison of different diagnostic procedures:

Out of three diagnostic procedures used to validate the clinically confirmed cases, higher positivity results (n=10, 58.82%) was found by nested PCR while it was 11.76% (n=2) by direct parasite examination in microscopy. PCR positivity were found in all of the microscopy positive cases along with 8 additional cases which were negative by microscopy and culture (Table 4.8).

Table 4.11: Results of the different diagnostic process

Microscopy	**Culture**	**Nested PCR**	**Number (n=17)**	**Percentage**
-	-	+	8	47.05
+	-	+	2	11.76
-	-	-	7	41.17

4.7 Travel History:

Out of 10 PCR positive cases only 2 of patient had travel history outside Nepal of which 1 had travel history in leishmaniasis endemic country. Rest of the patients hadn't travelled outside Nepal but some had travelled terai regions of Nepal.

Table 4.12: The travel history of the CL PCR positive cases.

S.N	Samples	Location of patients	Travel history outside Nepal	Travel History Inside Nepal	Duration of Stay
1	CL2	Kalikot	No	Yes (Pyuthan)	1.5 years
2	CL5	Kalikot	No	Yes (Surkhet)	Still there
3	CL8	Tanahun	No	No	-
4	CL10	Syangja	Yes (UK)	No	-
5	CL12	Ramechhap	No	No	-
6	CL13	Rauthat	No	Darchula	Still there
7	CL14	Baitadi	No	No	-
8	CL15	Palpa	Yes (Gujrat, India)	No	3 years
9	CL16	Humla	No	No	-
10	CL17	Gorkha	No	No	-

4.8 Sequencing Analysis

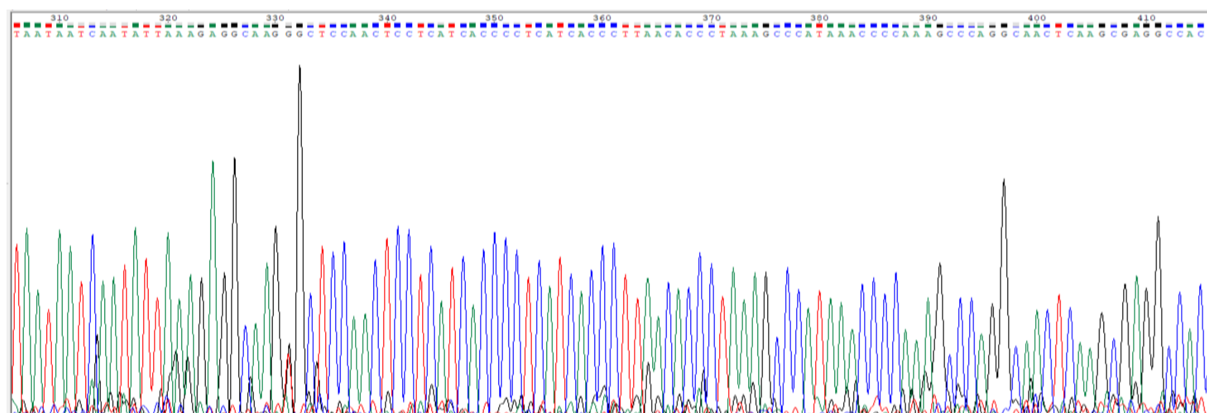

Fig. 4.12: Chromatogram of Sequence

Two PCR amplicons of approx. size 720 bp indicating positive result as shown in Fig. 4.10 were sent for sequencing which corresponds to CL2 and CL10 patient samples.

Sequencing of the PCR amplicons were performed and further analyzed using softwares MEGA 7 and online tools such as BLAST and T-coffee for multiple sequence alignment and phylogenetic tree generation. Upon analysis using the software MEGA 7 and performing sequence comparison using BLAST tool present in NCBI, it was observed that both the samples belonged to the Leishmania spp. Multiple sequence alignment and phylogenetic analysis revealed CL2 as being closest to the *Leishmania donovani complex* (Fig. 4.13).

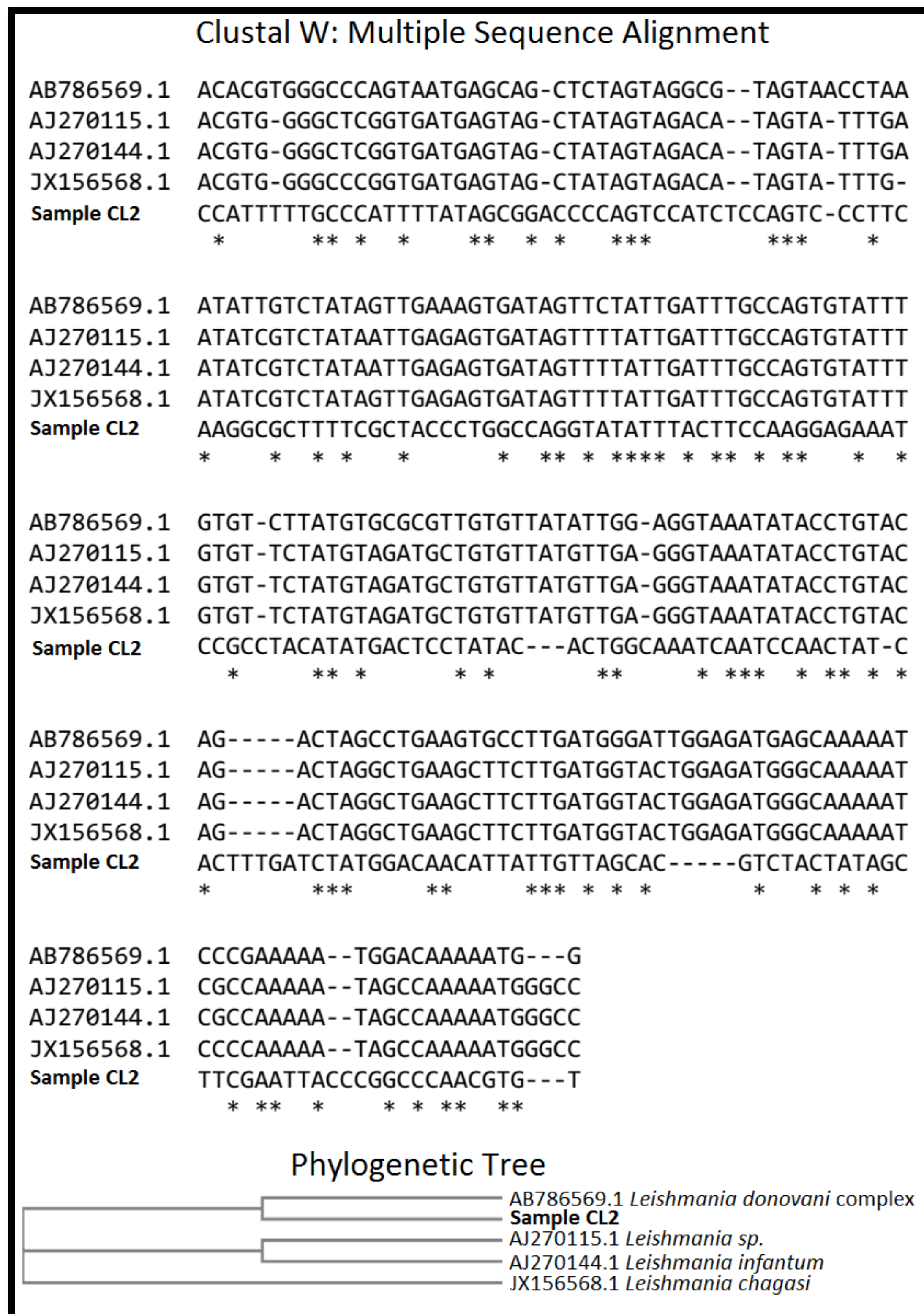

Figure 4.13: Multiple Sequence Alignment and Phylogenetic tree representation for the sample CL2.

Similarly, for the sample CL10, multiple sequence alignment and phylogenetic analysis showed sequence similarity to *Leishmania infantum* (Fig. 4.14).

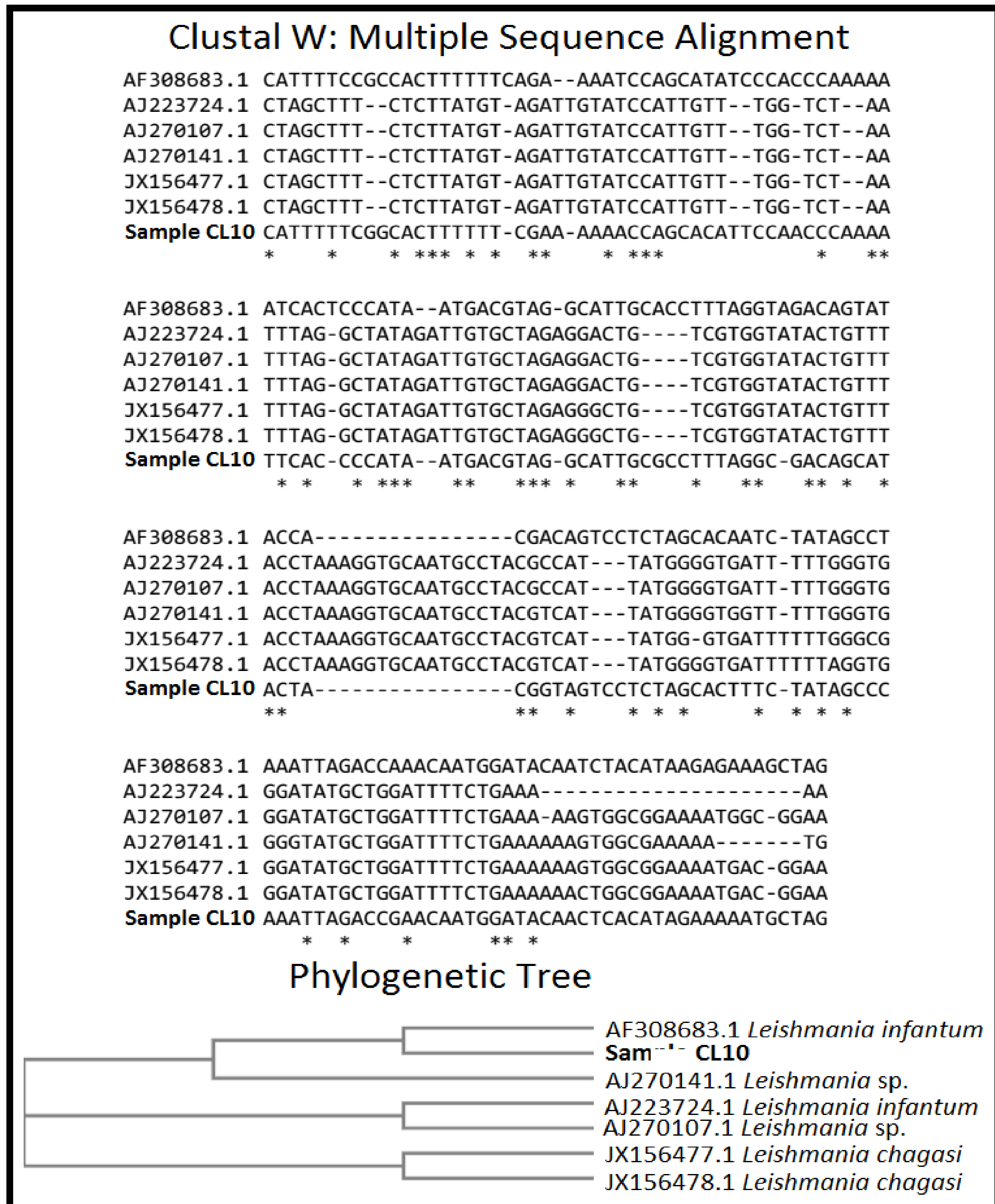

Figure 4.14. Multiple Sequence Alignment and Phylogenetic tree representation for the sample CL10.

4.9 Follow up response:

We followed two patients, one after a week of treatment and it was found that there was progression on healing of lesions (Fig: 4.15 A)and another after 6 months and there was complete healing of lesion (Fig 4.15 B) after treatment.

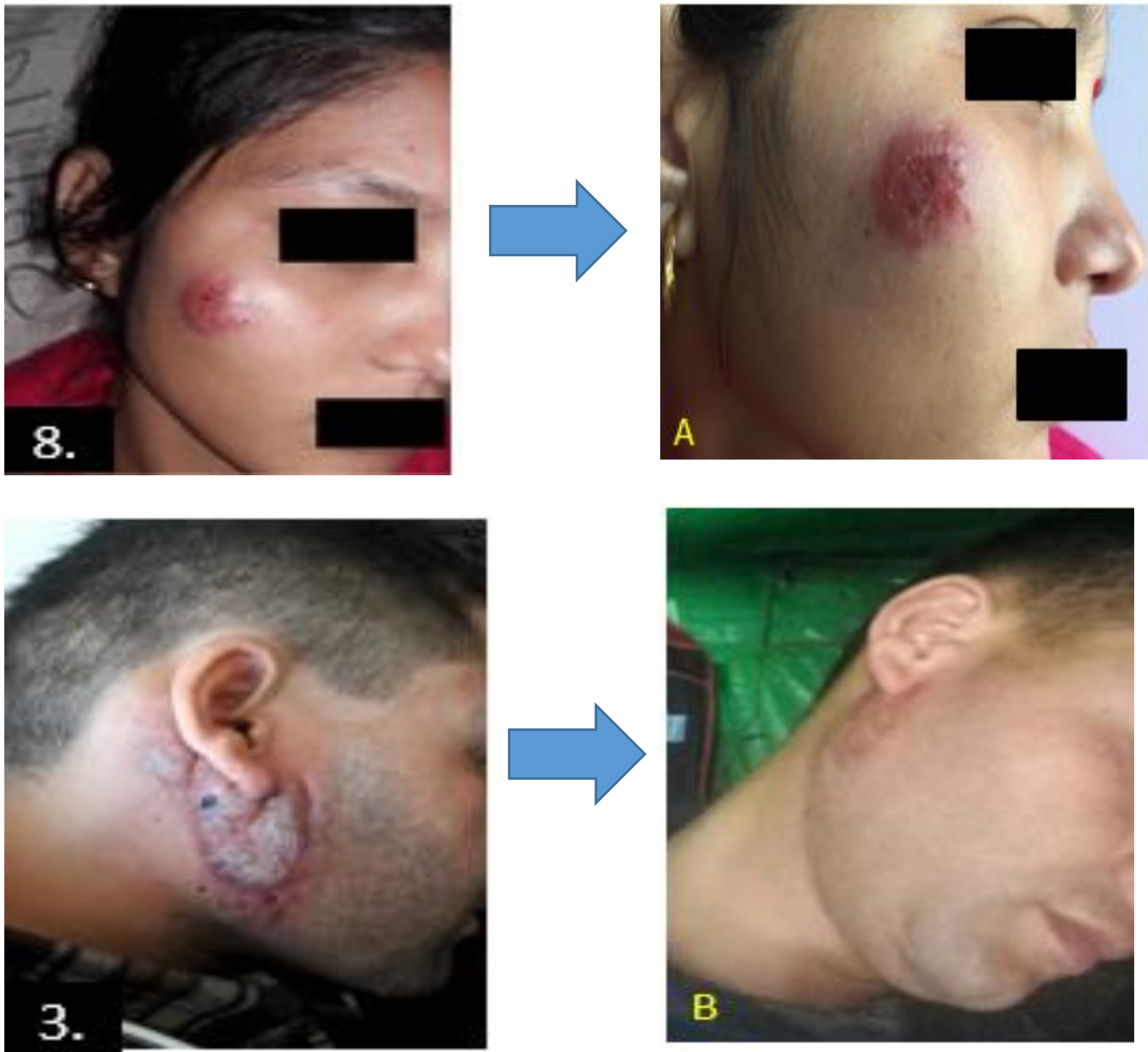

Fig4.15: (A) Follow up of patient no.8 after 1 week of intravenous injection of Liposomal Amphotericin B, still in progression of the lesion (B) Patient no. 3 after 6 months, healing of lesions

4.10 Analysis of T-Cell and B-Cells population: A flow cytometry study

4.10.1 T-Cell population:

Fresh blood samples collected and stained with T and B cell panel from 12 CL patients and the gated mean lymphocyte population of the CL patients in the FSC vs. SSC plot was 21.92±7.73%. The cells positive for CD45 and CD3 were identified as T cells and mean percentage were of 60.49±9.61%. The gated population of the T cells were further categorized to T sub-type cells into CD4+ and CD8+ T cells, the mean percentage of which were respectively 52.00±7.17% and 37.59±6.06% [Table 4.11]. Representative acquisition of flow cytometry was as below in Fig 4.16.

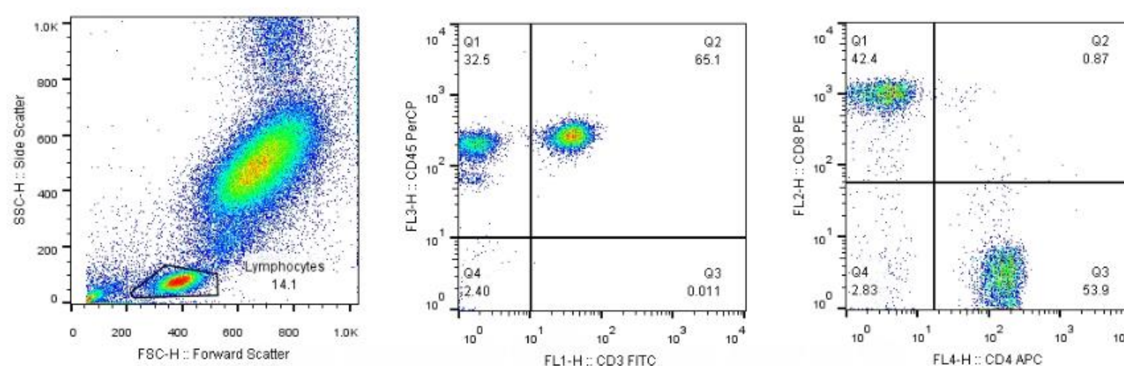

Fig: 4.16: Gating strategy for identification of T cells, CD4+ and CD8+ cells from peripheral blood sample.

Table 4.13: Mean frequencies for different immune cells in peripheral blood of CL patients and HC.

Cell population	CL patients	HC
T lymphocytes	60.49±9.61%	69.43±6.32%
CD4+ T cells	52.00±7.17%	46.83±12.98%
CD8+ T cells	37.59±6.06%	45.7±13.24%
B lymphocytes	9.39±4.21%	8.85±3.54%
NK cells	14.19±7.38%	17.09±7.99%
NKT cells	3.57±2.57%	3.62±1.35%

Table 4.14: Frequency of T lymphocytes in the fresh blood samples drawn from CL patients

Sample No.	PCR	Freq. of Lymphocytes	Freq. of T lymphocytes	Freq. of CD8+ T cells	Freq. of CD4+ T cells	Ratio (CD4/CD8)
CL5	+	36	65.5	33.6	61.9	1.84
CL8	+	16.4	45.2	35.9	53.5	1.49
CL10	+	26.2	40.9	49.9	40.2	0.81
CL12	+	32.6	67.6	31.8	54.6	1.72
CL13	+	11.7	54.2	35.4	52.7	1.49
CL14	+	13.55	65.1	35.6	55.5	1.57
CL15	+	13.53	71.1	41.1	46.2	1.12
CL16	+	26.2	61.4	42.9	50.4	1.17
CL17	+	21.5	58.7	34.8	46.1	1.32
CL4	-	18.8	58.5	27	65.5	2.43
CL9	-	20.2	69.7	41.7	52.7	1.26
CL11	-	26.4	68	41.4	44.7	1.08
Mean		21.92	60.49	37.59	52.00	1.44
St Dv		7.73	9.61	6.06	7.17	0.43

Similarly the fresh blood samples collected and stained from 7 healthy controls with T cell and B cell panel antibodies showed the 69.4% frequency of T cells and the relative proportion of CD4+ and CD8+ cells among the T cells in healthy control population were 46.8±13% and 45.7±13.24% respectively.

Table 4.15: Frequency of T lymphocytes in the fresh blood drawn from Healthy controls.

Sample no.	Freq. of Lymphocytes	Freq. of T lymphocytes	Freq. of CD8+ T cells	Freq. of CD4+ T cells	Ratio (CD4/CD8)
HC1	13.1	75.6	34.7	57.3	1.65
HC2	20.1	67	45.4	42.6	0.94
HC3	14.1	65.1	42.4	53.9	1.27
HC4	14.4	78.2	24.2	67.7	0.36
HC5	18.5	70.2	55.6	37.7	1.47
HC6	10.3	70.4	56	34.4	1.63
HC7	17.5	59.5	61.6	34.2	1.8
Mean	15.43	69.43	45.70	46.83	1.30
St Dv	±3.42	±6.32	±13.24	±12.98	±0.50

The data were plotted as Mean±SEM (Graphpad Prism v.7) and the difference was considered significant when p value was less than 0.05. As depicted in Fig.4.17, quantification of the relative proportion of T lymphocytes out of total lymphocytes in the peripheral blood of CL vs. HC was performed. The T lymphocytes in CL patients were found to be lower compared to those in HC (60.49±9.61% vs. 69.43±2.39%, *p=0.0431*). We infer the reduction in T cell compartment was contributed by the reduction in $CD8^+$ T cells however there was increment in the population of $CD4^+$ T cells. The percentage of $CD4^+$ T cells was not significantly different among the CL patients or HC. However, there was approximately 25% reduction in $CD8^+$ T cells in CL patients compared to HC (37.59±6.06% vs. 45.70±13.24%, *p=0.0230*).

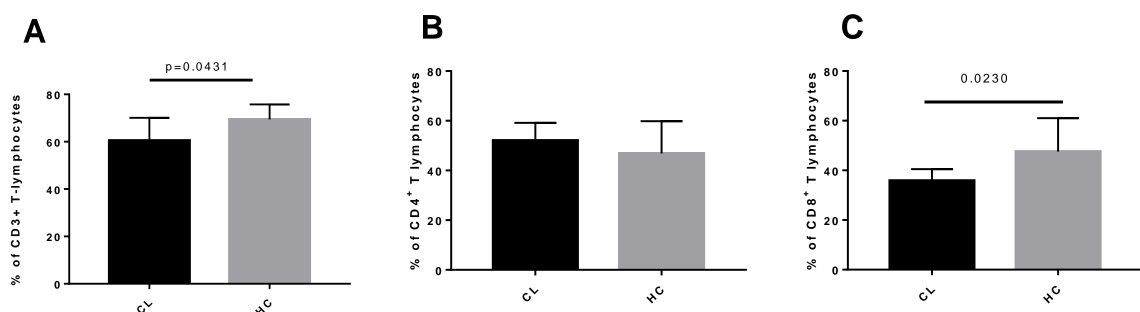

Fig. 4.17: Comparison of T lymphocytes and its subsets in the peripheral blood of CL patients and Healthy Controls (HC). Peripheral blood samples were stained with T cell antibody panel and the data was acquired in BD FACS Calibur. Percentage of T lymphocytes staining positive for $CD45^+CD3^+$ were calculated out of the total lymphocyte gate. Similarly, percentages of $CD4^+$ and $CD8^+$ T lymphocytes were calculated relative to the gated population of T lymphocytes. (n=12 for CL, n=7 for HC)

4.10.2 B-Cell Population:

The lymphocytes plotted in the FSC vs. SSC further identified as the population of leukocytes as CD45+ cells positive cells (Fig 4.18). The frequency of T cells (59.41±6.05%), B cells (9.39±4.21%), NK cells (14.19±7.38%) and NKT cells (3.57±2.57%). B cells ($CD19^+CD3^-$) and T cells ($CD19^-CD3^+$) were identified in CD19 vs. CD3 plot. Likewise, the NK cells and NKT cells in the gated lymphocyte population was identified as NK cells ($CD56^+CD3^-$) and NKT cells ($CD56^+CD3^+$) in the CD56 vs. CD3 plot.

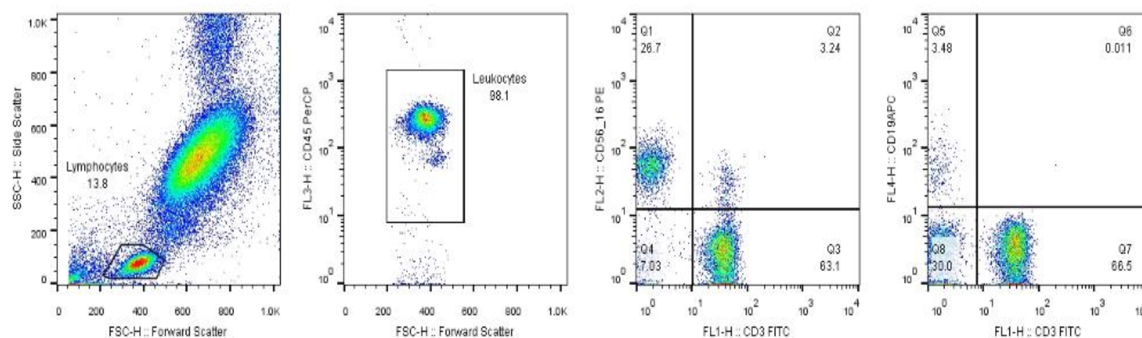

Fig. 4.18: Gating for identification of T cells, B cells, NK cells and NKT cells.

Similarly, fresh blood samples from 9 CL patients and 6 Healthy control (HC) were stained for B cell panel antibodies. Table 4.13 and 4.14 show the frequency of T cells, B cells, NK cells and NKT cells in CL patients and HC respectively.

Table 4.16: Frequency of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from CL patients

Sample No.	PCR	Freq. of NK cells	Freq. of NKT cells	Freq. of T cells	Freq. of B cells
CL5	+	8.8	1.1	69.4	18.2
CL8	+	29	5.14	47.9	8.21
CL11	-	17.2	7.79	57.2	6.3
CL12	+	6.01	2.59	58.5	9
CL13	+	20.7	7.08	55	5.7
Cl14	+	6.98	3.95	63.3	9.41
CL15	+	13.2	1.61	62.9	6.17
CL16	+	15.4	1.59	62.1	7.22
CL17	+	10.4	1.26	58.4	14.3
Mean		14.19	3.57	59.41	9.39
St Dv		7.38	2.57	6.05	4.21

Table 4.17: Frequency of T cells, B cells, NK cells and NKT cells in the fresh blood samples drawn from Healthy Controls

Sample No.	Freq. of NK cells	Freq. of NKT cells	Freq. of T cells	Freq. of B cells
HC1	16.2	4.07	67.8	9.5
HC2	26.1	3.18	61.9	3.42
HC3	12.7	6.11	66.8	6.58
HC4	13.6	3.16	69.7	9.22
HC5	6.81	2.98	69	10.6
HC6	27.1	2.23	53	13.8
Mean	17.09	3.62	64.70	8.85
St Dv	±7.99	±1.35	±6.36	±3. 54

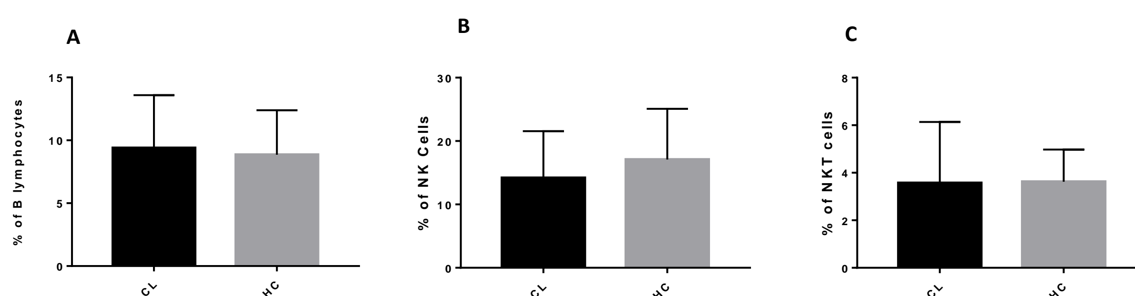

Fig4.19: Comparison of the immune cells in the peripheral blood of CL patients and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of (A) B lymphocytes ($CD3^-CD19^+$) (B) NK cells ($CD3^-CD16/56^+$) and (C) NKT cells ($CD3^+CD16/56^+$) were calculated out of the total lymphocyte gate. (n=9 for CL and n=6 for HC)

With a limited number of samples, we were not able to find significant differences in percentages of B cells, NK cells or NKT cells between the CL patients and the HC (Fig 4.19). The average percentage of B cells in CL patients (9.39±4.21) was not significantly different from those in HC (8.85±3.54). Similarly, the NK cells constituted 14.19±7.38% of CD3+ T cells in CL patients which was not statistically different from 17.09±7.99% in the HC. The NKT cells constituted a small portion with an average of 3.57±2.57% in the CL patients and 3.62±1.35% in the HC.

4.10.3 Comparison of cell population between PCR +ve and PCR –ve samples

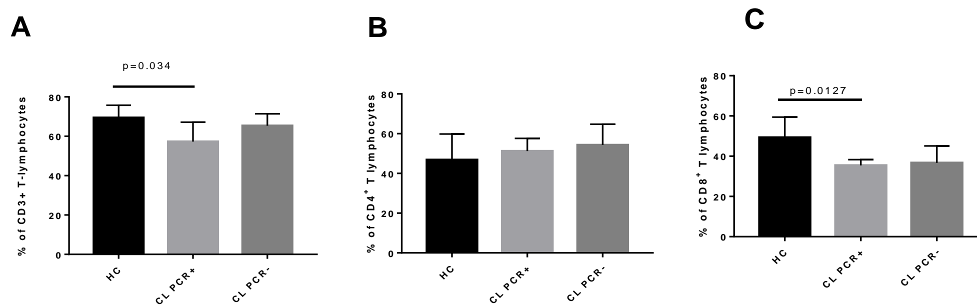

Fig 4.20: Comparison of T lymphocytes and its subsets in the peripheral blood of CL patients with PCR positive (CL PCR +) and PCR negative (CL PCR -) and Healthy Controls (HC). Peripheral blood samples were stained with T cell antibody panel and the data was acquired in BD FACS Calibur. Percentage of T lymphocytes staining positive for $CD45^{+}CD3^{+}$ were calculated out of the total lymphocyte gate. Similarly, percentages of $CD4^{+}$ and $CD8^{+}$ T lymphocytes were calculated relative to the gated population of T lymphocytes. (n=9for CL PCR +, n=3 for CL PCR -, n=7 for HC)

Interestingly, when we further analyzed the relative proportion of T cell subsets based on PCR positivity for *Leishmania spp.* DNA, there was a significant reduction of T lymphocytes in patients who tested positive for *Leishmania spp*. DNA by PCR compared to healthy controls. We could infer that reduction in T lymphocytes was contributed by the reduction in $CD8^{+}$ T lymphocytes but not by $CD4^{+}$ T lymphocytes (Fig 4.20).

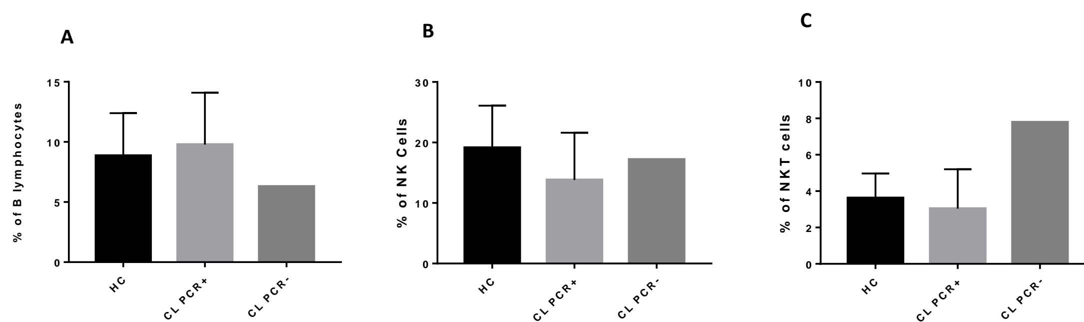

Fig.4.21: Comparison of the immune cells in the peripheral blood of CL patients with PCR positive (CL PCR +) and PCR negative (CL PCR -) and Healthy Controls (HC). Peripheral blood samples were stained for B cell panel the data was acquired in BD FACS Calibur. The relative percentages of (A) B lymphocytes ($CD3^{-}CD19^{+}$) (B) NK cells ($CD3^{-}CD16/56^{+}$) and (C) NKT cells ($CD3^{+}CD16/56^{+}$) were calculated out of the total lymphocyte gate. (n=8 for CL PCR +, n=1 for CL PCR -, n=5 for HC)

As observed previously when the patient samples were analyzed without subdivision into PCR +ve or PCR –ve, no significant difference could be observed in the relative proportion of B cells, NK cells or NKT cells between HC, CL PCR + and CL PCR – groups.

Chapter V

DISCUSSION

Cutaneous leishmaniasis is a neglected public health problem transmitted by sandfly. The disease is endemic in places with dry and hot environment, which is favorable for breeding of sandfly. Till date only few cases have been reported in Nepal. Despite having a rare incidence in Nepal, cases of CL is has been reported from India where *L. tropica* has been known to be the major causative agent (Hepburn, 2003). Similarly, *L. donovani* and *L. infantum* are both known to be the etiologic agents for visceral leishmaniasis with *L. infantum* further implicated to be the antecedent for cutaneous leishmaniasis as well (BenSaid et al., 2006). However, in the recent years, there have been several reports of *L. donovani* causing cutaneous leishmaniasis (Siriwardana et al., 2007).

Some case reports have been published starting from 1998 to till date. Majority of the previously reported cases of Nepal were from the people who were suspected to contract the disease during their abroad stay where CL was endemic or had been reported (Neupane et al., 2008). In our study, cases of CL were seen in some of the districts of hilly region, like Rolpa, Baitadi, Ramechhap, Tanahun, Syangja, Kalikot Palpa, Humla and Gorkha. This shows that the vector dynamics at higher altitudes is progressively changing with slow but steady rise of temperature at this region (Dhimal et al., 2014). Hence, it has become imperative for the concerned authorities to take quick and dedicated actions if they intend to eradicate the disease by 2020. Infected sandflies carrying *L. donovani* have been found at altitudes up to 1200 m above sea level (asl) in Nepal (Ostyn et al., 2015). Similarly, visceral leishmaniasis has been reported from hilly regions of Nepal (Pandey et al., 2011). Furthermore, cutaneous leishmaniasis occurring in South America have been reported from altitudes of 2438 m asl (SCHMIDT, 1950).

In the present study, highest number of CL patients (35.29%) was in the age group of 21-40, which is similar to study by Sharma et al (40.37%) (Sharma et al., 2005, Ghimire et al., 2018). In contrast, Gurel MS et al (70%) found higher incidence(27%) found a in patients of 5-9 years age group (Gruel et al., 2002). However, some other authors have found a higher incidence (27%) of cutaneous leishmaniasis in the 10-19 years age group (Yemisen et al., 2012, Aara et al., 2013). The number of patients decreased with advancing age which may be due to acquired immunity (Ghimire et al., 2018).The disease was more common in males with male to female ratio 1.4:1, male (58.82%) and female (41.17%), similar to Aara et al., 2013) male (55%) and Female (45%). In our country Nepal, females are mostly involved in household works and males are involved in outdoor works. This may have predisposed the males to the bite of sandfly (Ghimire et al., 2018).

Most of the patients had single lesion and the lesions were most common in the facial parts. Lesions were also seen in neck, upper and lower limbs. Involvement in upper limb was common in the studies conducted by other authors (Galgamuwa et al., 2017; Samaraj et al ., 2009). These findings suggest that the exposed body parts are prone to the bite of sandfly. In our study, it was found that the patients visited the hospital very late after the eruption of the lesion. Even the medical officers in the health centres are unaware of the prevalence of CL because of which there is high chances of misdiagnosis and improper treatment at initial phase. This misdiagnosis makes the disease more severe. Hence there was delay in the diagnosis of the disease. Also the patients are mostly from the rural places of Nepal and hence take longer time to travel to seek medical advice. Diagnosis of CL is difficult because of the varied symptoms and the different species involved (de Monbrison et al., 2007, Bailey et al., 2007, Pourmohammadi et al., 2010). In our study, for the diagnosis of cutaneous leishmaniasis, direct microscopy, culture of parasite and molecular diagnosis by PCR were performed.

Microscopy was done for the visualization of LD bodies in the clinical specimen. Different factors are involved, as microscopy requires highly skilled and experienced medical physician, well-equipped hospital, experienced laboratory technician, quality reagents high power microscope and density of LD bodies in specimen. The sensitivity of direct microscopy is not high as reported previously elsewhere (Safaei et al., 2002, Pourmohammadi et al., 2010). The sensitivity of microscopy is less in comparison to other diagnostic techniques like PCR (Culha et al., 2006).We were able to observe LD bodies only in two cases (11.76%).Many authors have reported that recovery of parasites in culture is rarely more than about 70% efficient even with easily cultured parasites. *Leishmania braziliensis*is frequently difficult to isolate, and in Tunisia, *Leishmania infantum* parasites causing cutaneous lesions have never been successfully cultured in the standard NNN blood agar medium used for the isolation of *Leishmania* (Noyes et al., 1998, Ben-Ismail et al., 1992). Our culture result was also not so good and is not consistent with other researchers' findings. It may be affected by some technical problems such as the type of materials applied in the media and fungal or bacterial contaminations that sometimes occurred in this study, Pourmohammadi et al., 2010).

It has been indicated that the PCR technique has a higher sensitivity as compared to other microscopical techniques (Culha et al., 2006; Medeiros et al., 2002). In our study PCR positive cases was 58.82% (n=10). PCR was found positive in the cases which were negative in microscopy and culture. It has been reported that PCR was 92% sensitive (Safaei et al.,2002) However PCR was found more sensitive then microscopy and culture. Moreover the PCR approach makes possible the fast identification at the *Leishmania* species and subspecies level (Vega-López, 2003).

On comparing the PCR band size with 100bp DNA ladder, it was found that the band size of 720 bps was comparable with that of *L. donovani complex* (Howard et al., 1991), 680 ~ 700 bps of *L. infantum* (Bensoussan et al., 2015), 560-590 bps of *L. major*(Oryan et al., 2013). All these species are known to cause CL in human (Haddad et al., 2016).The samples were further confirmed as *Leishmania* spp. through sequencing. Sequence analysis showed that a sample had sequence similarity to *Leishmania donovani* complex while next had sequence similar to *Leishmania infantum,* which corresponds with existing literature. Both *Leishmania infantum* as well as *Leishmania donovani* have been known to be the cause of cutaneous lesihmaniasis (Bensaid et al., 2006; Siriwardana et al., 2007; Kumar et al., 2015).

It has been shown that the host immune cells are involved in the progression or healing of the CL lesions primarily based on the types of the immune cells activated. Th1 mediated immune response is considered protective while Th2 mediated immune response support parasite growth and dissemination (Ajdary et. al., 2000). Th1 mediated arm of adaptive immune response involve the potent cytokines such as IFN-g produced by Th1 cells and $CD8^+$ cells which activate macrophages to phagocytose the parasites effectively. The mechanisms by which $CD8^+$ cells limit bacterial and viral infection is well understood. However, their effector roles in parasitic diseases like cutaneous leishmaniasis is gradually being unfolded (Novais, 2015). In our study, we reported decrease in $CD3^+$ T lymphocytes in patients with active CL disease by almost 10% compared to healthy controls. In a study done by Campanelli et. al.(2006), there were not significant differences in CD3+ T cells, CD4+ T cells or CD8+ T cells in the peripheral blood of CL patients and the healthy control. In another study done in Brazil (Almedia et al., 2011), it was shown the T cells decreased in active CL cases decreases by approx. 3% compared to control patients which later increased upon chemotherapy. In the same study, the authors did not find significant differences in the $CD4^+$ and $CD8^+$ T cells between active CL cases and controls, which contrasts with our findings. However, in the same study upon chemotherapy, both $CD4^+$ and $CD8^+$ cells were found to be increased which suggest of protective functions of $CD4^+$ and $CD8^+$ T cells. In our study, we did not find difference in $CD4^+$ T cells but $CD8^+$ T cells were significantly decreased in CL patients with active lesions. This observation further strengthens the possibility of protective function of $CD8^+$ cells in cutaneous leismaniasis. Discrepancy in the composition of immune cells as found in our study compared to other studies might be because of inherent pathological mechanism associated with different *Leisnmania spp*. that cause cutaneous leishmaniasis. In the above studies (Almeida et. al., 2011, Campanelli et. al., 2006), *L. vianna brazilensis* was implicated in the active CL cases while in our study we found the CL cases could be attributed to infection by *L. donovani complex* or *L. major* or *L. infantum*.

To the best of our knowledge, this is the first study to study the immune cells in peripheral blood from cutaneous leishmanias in patients from Nepal by the application of flow cytometry.

Nepal is an endemic to the visceral leishmaniasis. An implementation of a three country based (India, Bangladesh and Nepal) collaborative project, recently Nepal has achieved to reduce the cases to less than 1 in 15000 cases. Now, the CL has been seen in the Nepalese scenario and the cases are increasing many more beyond the expectations. If the trend goes on, the disease may become additional endemic keeping huge economic burden. Based on this preliminary report, the concern authorities of Nepal Government should think about the strategic plan at the earliest.

Chapter VI

SUMMARY

Cutaneous leishmaniasis (CL) is neglected vector borne disease caused by *Leishmania spp* of protozoa. Globally, the disease is endemic in more than 98 countries and about 350 million people are at risk of contracting the pathogen (McGwire & Satoskar, 2013). Approximately 1 million cases are reported annually with up to 30,000 deaths (WHO, 2017) The severity and clinical symptoms caused due to the infection depends upon the species of the *Leishmania spp* as well as the host response (Kane & Mosser, 2000). Cutaneous leishmaniasis (CL) is the most prevalent form, endemic to tropical and neotropical regions (Reithinger et al., 2007). Nepal has reported the first case of CL in 1998, however, the case was imported case from Saudi Arabia as the patient was returned from the country (Parija et al., 1998). Nepal saw very few cases of this disease, although, recently increased the number of cases are coming up which indicates the indigenous circulation. The patients with cutaneous leishmaniasis present with a papule or nodule at the site of inoculation, followed by formation of crusts. Differential diagnoses of cutaneous leishmaniasis include variety of skin diseases, inflammatory like impetigo, eczema, or granulomatous like sarcoidosis, lupus vulgaris, to skin tumor like basal cell carcinoma & squamous cell carcinoma. As misdiagnoses and irrelevant treatment have been reported, this research aims to establish timely, cheap, and more sensitive PCR based diagnosis.

During the period of short period of time, seventeen CL cases were studied molecularly and immunologically. Microscopic examination, culture, PCR was performed. Along with demographic and clinical details like site, duration of disease onset, travel history etc. was recorded. Nested PCR and sequencing of the amplified product of the parasite was performed to confirm the identity of the parasite. In addition, immunological profiling of the patient using flow cytometer was also performed. Statistical analysis was done using Graph Pad prism ver.7

A total of 17 patients with age ranging from 8 years to 85 years were included in the study. Mean age was 36±22.05 years. Most patients were in the age group 21-40 years. Male: Female ratio was 1.4:1. And it was also found that majority of the patient visit hospital after 6-8 months. Microscopy confirmed 11.76% as positive and 58.82% positive by PCR, resulting *Leishmania donovani complex, Leishmania infantum, L. major* as the causative agent. Sequencing of the two amplified product of the parasite confirmed *Leishmania donovani* complex and *Leishmania infantum* as the causative agent. The relative proportion of different immune cells in peripheral blood such as T cells and its sub-types, B cells, NK cells and NKT cells were also compared between CL patients and healthy

controls. T cells were found to be relatively lowered in CL patients. Specifically, the reduction in T cells was contributed by the significant reduction of CD8+ T cells in CL patients which were 25% lesser than in healthy controls.

Chapter VII

CONCLUSION

Cutaneous leishmaniasis; though referred as imported and least common disease of Nepal, our study found it to be alarming ailment. We found a total of 17 clinically diagnosed cutaneous leishmaniasis cases within a short period of 9 . Most of the cases have been misdiagnosed as other skin diseases because; even the health workers are unaware of this disease. The patients visit to hospitals at least after 2 months of their first lesion eruption looking for some other treatments which indicates the unawareness about this emerging disease; cutaneous leishmaniasis among the people. The CL is known to occur at tropical and subtropical regions but most of the cases were coming from the hilly parts of the country and within the non–travelers, showing that the disease is spreading even in temperate zones and is indigenously circulating among Nepali people.

From this work, it was found that the infected patients were mostly of age group 21-40 yrs followed by ≤20 years. It is huge burden to Nepal as the most economically viable age-group of the country is being affected by this disease. Also, the disease was more common in males who are mostly doing outdoor works in comparison to females doing household works. It causes long time non-healing dermal lesions and disfiguring of the body parts of patients if not treated promptly. Many times, the medicine for this disease is not easily available and the best effective drug, Liposomal Amphotericin-B is very expensive. So, concerned stakeholders and organization need to bring a special awareness programmes and efforts should be made to make PCR platforms more user-friendly and cost-effective, especially in remote areas where leishmaniasis is endemic.

This research unveiled that during the CL infection there is down regulation of T cells due to suppression of cytotoxic T cell (CD8 T cells). Such condition is prone to the other secondary infection for the patients. Therefore, patient can opt for better treatment to boost their immune status and get recover from the CL infection.

LIMITATIONS OF STUDY

- As CL is recently emerging ailment, the work could be carried out in limited number of samples.
- Many times patients denied to give samples (clinical specimens) from their leisons and blood samples so, microscopic slides and flow cytometry work could not be performed of all cases.
- If we could have used M119 culture media, there might good chances of parasite growth which could not be done due to unavailability of media in the Nepali market.
- Due to limitation of time we were not able to sequence all PCR positive amplicons Because of not getting good purified amplicon, the sequencing is needed to be repeated in future.

RECOMMENDATION

This research work has revealed some of novel aspects of cutaneous leishmaniasis which is a tropical vector born disease and emerged recently as indigenous circulation in Nepalese population. The trend of increasing number of cases is havoc and once could be epidemic and endemic, so we should step forward to

- Coordinate the Ministry of Health and Population, Government of Nepal to develop a strategic plan to screen the CL cases at root level.
- Preparation of epidemiological data of Cutaneous leishmaniasis in Nepal in order to implement effective programs to control the disease
- Circulate information of prevalence of CL to all the media for making people aware of the diseases.
- Launch public and health workers' awareness/orientation program focused to the districts of CL reported cases.
- Bring the vector control program effectively.
- Facilitate the diagnostic tools like PCR to the representative health centres.
- Extensive research programs need to be conducted based on the CL disease.

Further, we recommend continuing the research work with increased numbers of sample sizes to get more significant results. Study of Follow-up of patients to compare the immune response before treatment and the status after treatment will let us understand the role of host immune system for causing the disease. Study of other cellular markers and cytokines like IL-10, IL-12, IFN- γ etc. in CL patients will help in treatment of the ailment.

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Appendix I

Composition of NNN media

Agarose solution:

Bacto Agar 10 gm
NaCl 9 gm
Triple distilled Water (TDW) 1000 mL

Procedure-

1. Bacto-Agar and NaCl in TDW are mixed thoroughly by warming at 60-70^0 C.
2. Adjusted pH to 7.2.
3. Autoclave the mixture at 15 lb pressure for 15 minutes.
4. Store at 4^0 C until use.

Collection of blood

1. Autoclave Blood collection tube (containing 1/5 part glass beads).
2. Wipe the dorsal part of ear with ethanol (70%).
3. Shave the region.
4. Swab the ear region with alcohol.
5. Using disposable syringe, draw blood and transfer to collection tube.
6. Shake the tube vigorously and roll the vials between the palms which defibrinate the blood.
7. Store at 4^0 C until use.

Preparation of NNN media

1. Transfer the blood to fresh 15 ml tube and calculate the required volume of agar media
   Agar Media – 70 %
   Blood- 30 %
   Ex. If , Blood volume = 6 mL
   Agarose medium = 14 mL
2. Add autoclaved melted agarose solution (50^0 C) to the blood and mix by pipetting.
3. Take Mc Cartney tubes(NNN tube) and transfer 1 mL mixture in each tube
4. Positioned the NNN tube on slant bottom side.
5. Store at 4^0 C until use.

Composition of Giemsa stain

Giemsa stock solution		Giemsa Buffer	
Giemsa powder	1gm	Na_2HPO_4	9.5g/ml
Glycerol	60ml	KH^2PO^4	9.07g/ml
Methanol	66ml		

Giemsa Stain: 10% giemsa stock in giemsa buffer

Appendix II

Ref. No.: 2180.

13 March 2018

Mr. Krishna Das Manandhar
Principal Investigator, Tribhuvan University (TU)

Ms. Srijan Shrestha
Principal Investigator, Tribhuvan University (TU)

Ref: **Approval of thesis proposal** entitled **Immuno-molecular study of cutaneous leishmaniasis, an emerging disease of Nepal**

Dear Mr. Manandhar and Ms. Shrestha,

It is my pleasure to inform you that the above-mentioned proposal submitted on **29 January 2018** (**Reg. no. 45/2018**) has been approved by Nepal Health Research Council (NHRC) National Ethical Guidelines for Health Research in Nepal, Standard Operating Procedures Section 'C' point no. 6.3 through Expedited Review Procedures.

As per NHRC rules and regulations, the investigator has to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure, data management and budget that may be necessary in course of the implementation of the research proposal can only be made so and implemented after prior approval from this council. Thus, it is compulsory to submit the detail of such changes intended or desired with justification prior to actual change in the protocol. Expiration date of this proposal is **January 2019.**

If the researcher requires transfer of the bio samples to other countries, the investigator should apply to the NHRC for the permission. The researchers will not be allowed to ship any raw/crude human biomaterial outside the country; only extracted and amplified samples can be taken to labs outside of Nepal for further study, as per the protocol submitted and approved by the NHRC. The remaining samples of the lab should be destroyed as per standard operating procedure, the process documented, and the NHRC informed.

Further, the researchers are directed to strictly abide by the National Ethical Guidelines published by NHRC during the implementation of their research proposal and **submit progress report in between and full or summary report upon completion.**

As per your thesis proposal, the total research budget is **NRs 2,00,000** and accordingly the processing fee amounts to **NRs 1,000.** It is acknowledged that the above-mentioned processing fee has been received at NHRC.

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

Thanking you,

Prof. Dr. Anjani Kumar Jha
Executive Chairperson

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

IMMUNO-MOLECULAR STUDY OF CUTANEOUS LEISHMANIASIS, AN EMERGING DISEASE OF NEPAL

सम्बन्धि अध्ययनमा सहभागीको मन्जूरीनामा फाराम

मिति: २०७४-१२-२८

तपाईंलाई यस अध्ययनमा सहभागी हुनलाई आह्वान गर्नुको कारण तपाईंको घाउबाट लिइएकोथोरै कोषको नमूनामा कालज्वर परजीवि छ की छैन, यदी छ भने कुन प्रकारको परजीवि छ भनी अध्ययन गर्नु हो र तपाईंबाट लिइएको रगतबाट तपाईको रोग प्रतिरोघात्मक क्षमताको बारे अध्ययन गर्नु हो । यस मन्जूरीनामापत्रको उद्देश्य तपाईंलाई यस अध्ययन सम्वन्धि पर्याप्त जानकारी दिनु हो ता की तपाईं यस अध्ययनमा सहभागी हुने नहुने सहि निर्णय लिन सक्नु हुनेछ । तपाईंलाई प्रष्ट नभएका कुनै पनिप्रश्नहरु सोध्न सक्नु हुनेछ । तपाईको घाउबाट सानो कोषका टुक्रा र रगत लिइने छ । संकलित नमूनाहरुबाट जैविक प्रविधि केन्द्रिय विभाग, त्रिभुवन विश्वविद्यालयमा विश्लेशण गरिने छ । संकलित नमुना हरुलाई गोप्य संख्यामा लेखिनेछ ताकी तपाईंको व्यक्तिगत परिचय गोप्य रहन सकोस् । तपाईंको व्यक्तिगत परिचयलाई गोप्य राखिने छ । झिकिएको कोषको टुक्राले तपाईंलाई कुनै किसिमको हानीहुने छैन ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाईं वा तपाईंको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनबाट प्राप्त जानकारी मार्फत भविष्यमा तपाइ उक्त रोगको जोखिमबाट बच्न सक्नु हुने छ र यस रोगबारे सर्तकता अपनाउन सक्नु हुन्छ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतीजा प्रकाशित गर्न सकिनेछ, तर त्यसमा तपाईंको नाम तथा परिचय उल्लेख हुने छैन ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैले आफ्नो इच्छाले विना जरिवाना, डर, विनात्रास अनुसन्धानकर्ता समक्ष पूर्व सुचना बिनानै कुनै पनि बेला यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई पढेर सुनाइएको छ, मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फारममा सही गरको छु ।

सहभागीको हस्ताक्षर:
अभिभावकको हस्ताक्षर:
(यदि सहभागी १८ वर्ष भन्दा मुनीको भए)

सहभागीको नाम: लिला ब. मगर
उमेर: ८५
ठेगाना: तनहुँ, गजुरकोट

सम्पर्क नं. : 9817990547

IMMUNO-MOLECULAR STUDY OF CUTANEOUS LEISHMANIASIS, AN EMERGING DISEASE OF NEPAL

INFORMED CONSENT FORM

Questionnaire format

Code no.: 8 Date: 2074-12-28

Name of Patient: Lila Bahadur Magar

First name: Lila Middle name: Bahadur Last name: Magar

Age(inyears) 85 Caste: Ethnicity:

Sex : Male

Address: Tanahun , Gajurkot, Jharpati

Travel history to abroad: Yes ☐ No ☑
If yes, where
When
Duration of stay

Travel history inside Nepal: Yes ☐ No ☑
If yes, where
When
Duration of stay

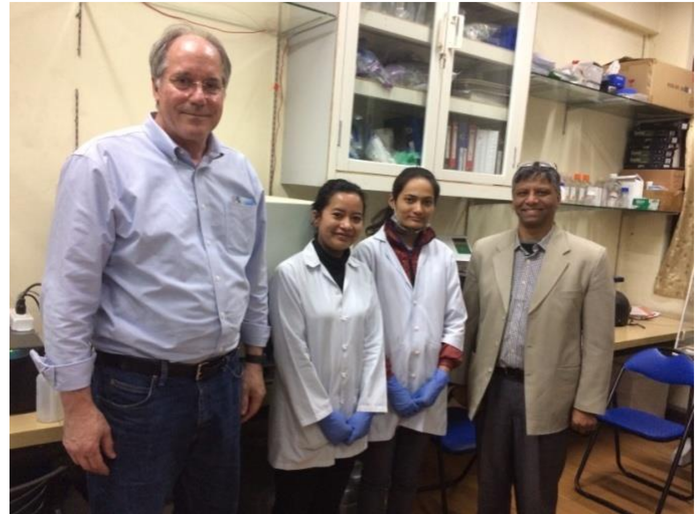
Type of leishmaniasis:

1. Cutaneous ✓
2. Muco cutaneous
3. Visceral

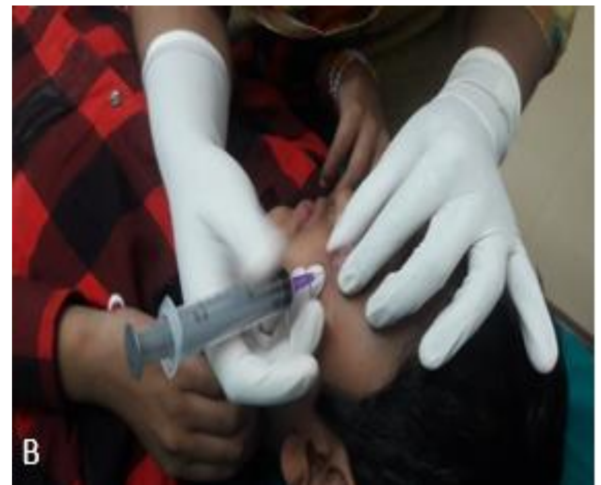
First onset of disease: Ashoj, 2074

Position of lesion: Left hand,

Number of lesions: One Ulcerated lesion with central depression



During flow cytometry training with Dr. William Telford, Prof. Krishna Das Manandhar and Madan Pandey, senior lab technician (Bharatpur Cancer Hospital), Prof. Dr.Paul Wallace.

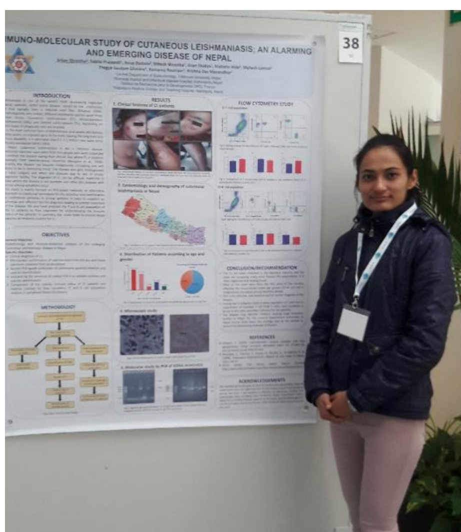


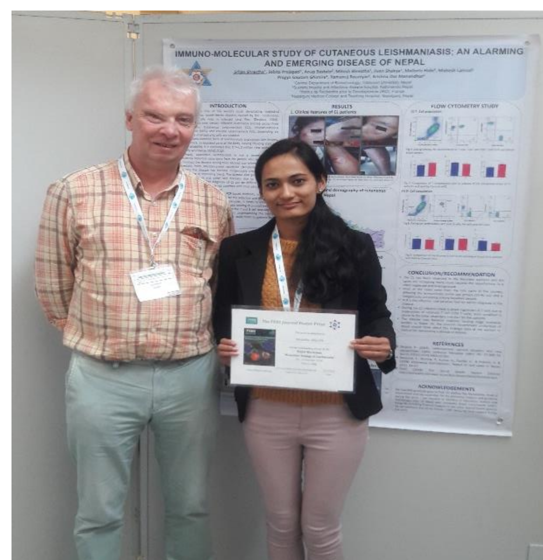
Sample drawing from CL patients at STIDH and NGMCTH

At Nepalgunj Medical College and Teaching Hospital with Dr. Pragya Gautam Ghimire and Ramesh G.C, Lab technician

Doing Flow Cytometry work

Holding best post presenter prize on one day symposium on revolutionizing life sciences





Presenting and holding best poster award with Dr. Lawrence Banks at ICGEB workshop on Molecular Biology of Leishmania-2018 (22-24 oct, 2018) atTrieste, Italy

Holding best poster award on ICGEB workshop on Molecular Biology of Leishmania-2018,

Participants of ICGEB workshop on Molecular Biology of Leishmania-2018, at Trieste, Italy

The FEBS Journal Poster Prize

The prize is awarded to

Shrestha SRIJAN

For an outstanding poster at the

ICGEB Workshop

"Molecular biology of Leishmania"

22 - 24 October 2018

Trieste, Italy

Lawrence Banks and Greg Matlashewski — Conference organisers

24/10/2018 — Date

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1 Case Report: Cutaneous Leishmaniasis at High Altitudes of Nepal

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57 **Abstract**

58 **Background:**

59 Cutaneous Leishmaniasis is the most common form of the leishmaniasis, however, it
60 is considered to be a rare disease in Nepal incomparison to the visceral leishmaniasis.

61 **Case Presentation:**

62 Here we report a case of cutaneous leishmaniasis in a 32 years old man who had been
63 residing at Dunai village of Dolpa,Nepal (~2000 m above sea level) prior to case
64 diagnosis and became symptomatic on February, 2016. The patient was previously
65 misdiagnosed aslupus vulgaris, however was later identified as suffering from
66 cutaneous leishmaniasis. The patient had erythematous plaque with induration of size
67 6 X 5 cm covering major part of left side of the nose and extending to a part of the

68 left cheek while the lesion at the center of left cheek measured 3 X 3 cm size with
69 crusting and induration. Histological microcopy showed presence of amastigotes
70 with positive rK39 test in blood. Nested PCR also showed presence of *Leishmania*
71 DNA in the patient's tissue lysate. The patient was started on Liposomal
72 Amphotericin B (LAMB - 3mg/kg for 7 days) intravenously in January 2017. After
73 seven days of LAMB, the lesions started regressing. He was then continued with two
74 weeks of Itraconazole 100mg twice a day for 2 weeks following seven days of LAMB
75 and miconazole 1% cream for continue application. He continued to apply
76 miconazole following two weeks of Itraconazole. The skin lesions regressed after six
77 months.

78 **Conclusion:**

79 This case presents the first case of cutaneous leishmaniasis by *Leishmania* spp.from
80 mountain region of Nepal. Cutaneous Leishmaniasis is a rare diagnosis in Nepal.
81 Furthermore, this case signifies a unique disposition in its occurrence at higher
82 altitude and colder climatic condition.

83 **Keywords:** Cutaneous Leishmaniasis, *Leishmania donovani*, *Leishmania infantum*,
84 Nepal

85 **Background**

86 Infected Sandflies (*Phlebotomus* spp. and *Lutzomyia* spp.) carry protozoan parasites
87 of the genus *Leishmania* which are responsible for various forms of leishmaniasis.
88 Globally, the disease is endemic in more than 98 countries and about 350 million
89 people are at risk of contracting the pathogen [1].Approximately 1 million cases are
90 reported annually with upto 30,000 deaths [2].The severity and clinical symptoms
91 caused due to the infection depends upon the species of the *Leishmania* spp as well
92 as the host response [3].Three major clinical manifestations in humans caused by
93 different species of *Leishmania* include localized cutaneous leishmaniasis (CL),
94 cutaneous leishmaniasis with mucosal involvement (MCL) and systemic visceral
95 leishmaniasis (VL) [4].Among these, CL is the most prevalent form, endemic to
96 tropical and neotropical regions [5,6]. The clinical features of CL include varying
97 number of skin ulcers, satellite lesions or nodular lymphangitis [4].

98 Although visceral leishmaniasis is common in the terai plains of Nepal, limited
99 number of cases has been documented for cutaneous leishmaniasis. The first reported
100 case of CL in Nepal dates back to the year 1998 followed by few intermittent cases
101 and the latest case reported was in 2013[7-12]. As of now, one case of VL has also
102 been recently reported from Dolpa, although from a relatively higher altitude [27].
103 However, no case of CL has been yet reported from the district.

104 **Case Presentation**

105 Here, we present a case of Cutaneous Leishmaniasis from patient with no travel
106 history outside of Nepal; however, the patient had been to mountain region of Nepal
107 prior to clinical manifestation of the disease. The patient was a 32 years old male
108 permanent resident of Ramechhap Hiledev-3 (province 3) currently staying in
109 Dhapakhel-9, Lalitpur (province 3) with frequent travel and stay in the Dolpa district
110 for extended period before being infected (province 6) (Figure: 6). In 2013, he stayed
111 in Dunai, the district headquarters of the mountainous district, Dolpa which has an
112 elevation of approx. 2000 m asl, for eight months followed by ten months from May,
113 2015 to February, 2016.The patient then started to develop acne like eruption on
114 upper part of left nasolabial fold in February, 2016. It started to grow and became
115 crusted associated with oozing. The lesion was mildly pruritic sometime. Yet another
116 lesion started on the central part of left cheek (face) and increased in size with crusting
117 and occasional oozing, after 2 months of first lesion. Lesion on the left nasolabial
118 fold increased in the size involving most part of the left side of nose and nearby skin
119 of the cheek. Lesion in left cheek also increased in size with crusting and occasional
120 oozing. There was no history of any illness. He had consulted dermatologist in April
121 2016 and was started antitubercular therapy (ATT) in May 2016 with diagnosis of
122 lupus vulgaris. The patient was subjected to various antibacterial medicines and
123 applied topical steroid and antibiotics, however, showed no improvement locally. He
124 continued ATT for five months without any improvement. He then visited Sukraraj
125 Tropical & Infectious Disease Hospital where biopsy was done and amastigotes were
126 visualized in histology (Figure: 2). Clinically other systems were unremarkable
127 except skin lesions (Figure: 1). There was crusted erythematous plaque with
128 induration of size 6 X 5 cm covering major part of left side of the nose and extending
129 to a part of the left cheek. The lesion at the center of left cheek measured 3 X 3 cm
130 size with crusting and induration. Laboratory tests for sample were positive for rk39
131 RDT however, baseline investigations were within normal limit including whole
132 blood counts, liver function test as well as renal function test.

133 The case was further confirmed by using nested PCR protocol previously described
134 by Noyes et al. in 1998 [16]. Two sets of primer (CSB2XF –
135 C/GA/GTA/GCAGAAAC/TCCCGTTCA and CSB1XR –
136 ATTTTTCG/CGA/TTTT/CGCAGAACG) for the first round and (13Z –
137 ACTGGGGGTTGGTGTAAAATAG and LiR – TCGCAGAACGCCCCT) for the
138 second round was used respectively. The PCR conditions were 94ºC for 2 minutes
139 followed by 94ºC for 30 seconds, 54ºC for 1 minute and 72ºC for 1.5 minutes, for 40
140 cycles for the first round. For the second round, 5 µl was used from the first round
141 was used as a template and the conditions were 94ºC for 2 minutes followed by 94ºC
142 for 30 seconds, 56ºC for 1 minute and extension at 72ºC for 40 seconds, for 40 cycles.
143 The *Leishmania* DNA template for PCR was extracted manually from tissue lysate
144 [13].A band size corresponding to size of approx. 700 bp in the second round PCR

confirmed the presence of *Leishmania* parasite (Figure: 5).The band size corresponds to that typical of *L. infantum* and *L. donovani* [16].

The patient was admitted and started on Liposomal Amphotericin B (3mg/kg for 7 days) intravenously in January 2017. After seven days of LAMB the lesions appeared to be regressing (Figure: 3). The patient was continued with two weeks of Itraconazole 100mg twice a day for 2 weeks following seven days of LAMB and miconazole 1% cream for continue application. He continued to apply miconazole after two weeks of Itraconazole. The skin lesions had degenerated significantly (Figure: 4) after six months.

Discussion

Despite having a rare incidence in Nepal, CL is common in India where *L. tropica* has been known to be the major causative agent [20].Similarly, *L. donovani* and *L. infantum* are both known to be the etiologic agents for VL with *L. infantum* further implicated to be the antecedent for CL as well [21, 22]. However, in the recent years, there have been several reports of *L. donovani* causing CL [23 – 26]. Furthermore, a report showcasing the unusual case of skin ulcer in the patient suffering from VL, due to high parasitic load, has also been reported from Nepal [12]. Infected sandflies carrying *L. donovani* have been found at altitudes upto 1200 m above sea level (asl) from Nepal [14]. Similarly, VL has been reported from hilly regions of Nepal [15].Furthermore, CL occurring in South America has been reported from altitudes of 2438 m asl [17]. However, in Nepal, majority of the previously reported cases were from the people who were suspected to contract the disease during their abroad stay where CL was endemic or had been reported [7 – 9].

There have been only a handful of reported cases of cutaneous leishmaniasis from Nepal [7-12]. This report, to our knowledge, is the first to be reported from a patient residing at higher altitude regions of Nepal prior to his clinical presentation and no previous travel to CL reported or endemic foreign countries. The patient became symptomatic upon arrival to his home and was misdiagnosed several times before getting correct diagnosis for leishmaniasis. The patient must have contracted the pathogen while being at the high altitude since he presented the symptoms upon return. As VL has been reported from Nepal at hilly region; it is not improbable for CL to be present in the surrounding area. Furthermore, *L. donovani* itself could be the underlying element for cutaneous leishmaniasis as observed from the previous studies [12, 15, 23 – 26]. The combined result of rK39 positive test followed by confirmation with the nested PCR points towards the possibility for *L. donovani* as the prime pathogen responsible for causing cutaneous leishmaniasis in Nepal.

The vector dynamics at higher altitudes is progressively changing with slow but steady rise of temperature at this region [18]. Hence, it has become imperative for the

183 concerned authorities to take quick and dedicated actions if they intend to eradicate
184 the disease by 2020 [19].

185 **Conclusion**

186 To our knowledge, this is the first case of CL occurring at a higher altitude. Although
187 sporadic cases have been reported from the lower regions of the country and that too
188 with a travel history to CL endemic countries, this case is particularly unique in a
189 sense that the patient had been residing in the mountainous region prior to his disease
190 presentation. Preliminary study has shown the probability of patient being infected
191 while living in the higher altitude, however, further research on the vector dynamics
192 and disease incidence needs to be carried out for additional confirmation. Lastly, this
193 case study along with others should be an eye opener for the policy makers to
194 establish new norms and values towards rapid diagnosis and training of personnel in
195 efficient and effective management for tropical disease interventions.

196 **Declarations**

197 **Ethics approval and consent to participate**

198 This research work was given an ethical clearance by the Nepal Research Health
199 Council. (Ref No: 2180).

200 **Consent**

201 Written consent was obtained from the patient with regard to the publication of this
202 case report and any accompanying images, which would be available upon request.

203 **Availability of data and materials**

204 All data generated or analyzed during this study are included in this published article.

205 **Competing interests**

206 The authors declare that they have no competing interests.

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210 **Author's Contribution**

211 AB prepared the case report, performed rK39 test, histological microscopy and
212 treatment of the case; MS performed the DNA extraction and PCR for the sample and
213 prepared the manuscript; ML, SS and SP helped with writing of manuscript; AA and
214 BPG helped in manuscript preparation and interpretation of result; LD and BSC
215 helped AB in treatment of case; MH provided the required PCR reagents and,

216 reviewed, edited and gave final touch to the manuscript; KP helped with manuscript
217 preparation and result interpretation; KDM designed and conceived the experimental
218 work, prepared and proof read the manuscript. All authors read and approved the final
219 manuscript.

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305 **Figure Legends**

306 Figure 1. Primary skin lesions on the patient

307 Figure 2. Microscopic Picture (100 X) of hematoxylin and eosin stained
308 amastigotes.

309 Figure 3. Regression of the skin lesion upon treatment by Liposomal Amphotericin
310 B.

311 Figure 4. Recovery from the skin lesions 6 months post treatment.

312 Figure 5. Agarose Gel Electrophoresis for PCR amplicons Ladder – 100 bp Ladder
313 (Cat. No.SM0243). NC – Negative Control; PCR2 – PCR product obtained after
314 2nd round of PCR.

315 Figure 6. Map of Nepal showing the travel history of the patient.

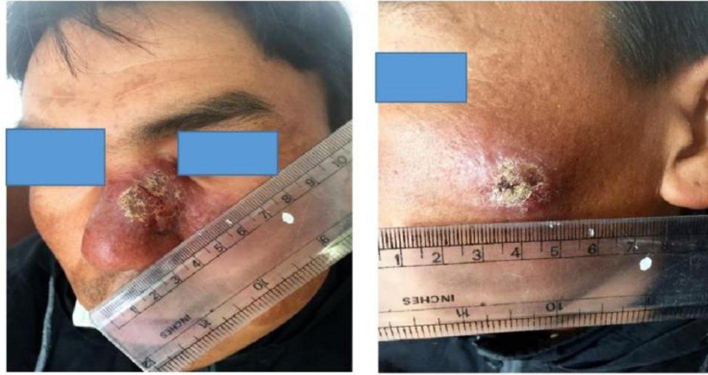
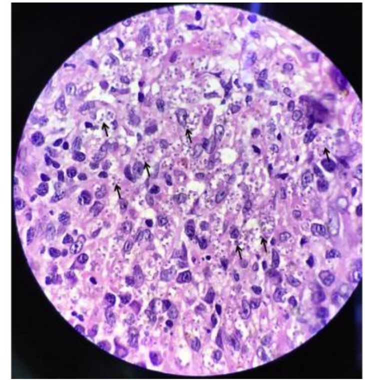

Figure 1

Figure 2

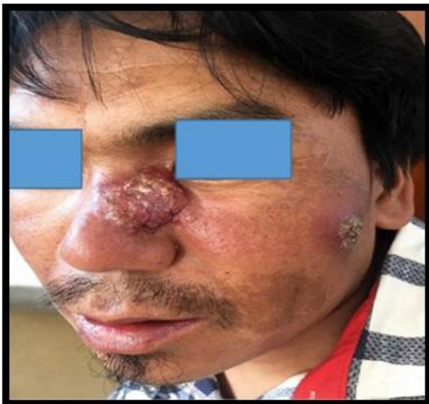
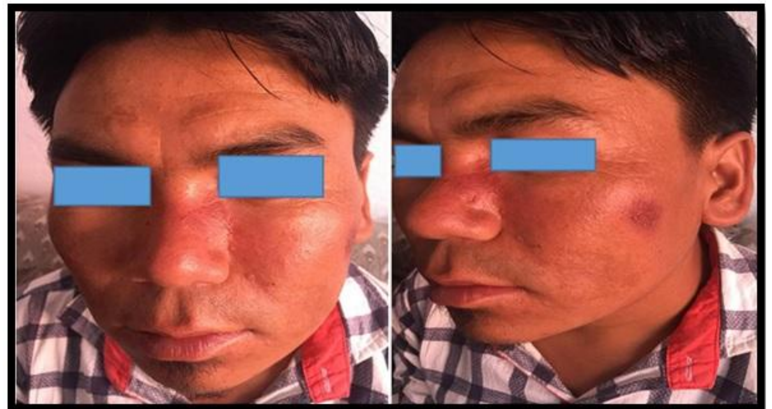

Figure 3

Figure 4

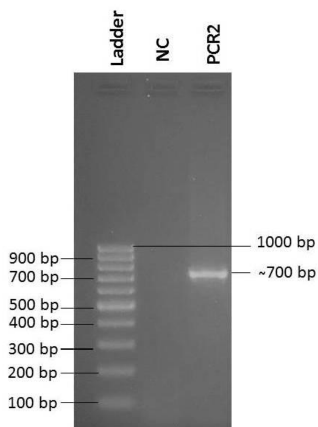

Figure 5

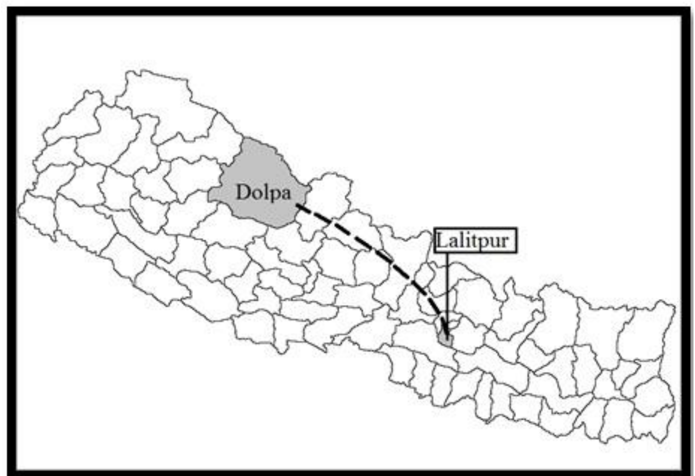

Figure 6

Immuno-molecular Study of Cutaneous Leishmaniasis: an Alarming and Emerging Disease of Nepal

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Abstract

Background: Cutaneous Leishmaniasis is a vector borne, parasitic disease caused by the bite of an infected sandfly. The disease is rare in Nepal with only few cases reported till date. The diagnosis of CL might be difficult, in particular in areas where the disease is uncommon and where other skin diseases with similar clinical symptoms occur.

Objective: To contribute to improve the diagnosis of CL and the identification of Leishmania species and studying the immune response of the patients

Materials and Methods: A total of 13 patients presenting with cutaneous lesions suggestive of CL were sampled for parasitological diagnosis by direct examination (DE), kinetoplast DNA (kDNA) nested PCR (CSB1X/CSB2X and 13Z/LiR primers). Immunological status was analyzed by flow cytometer and compared with healthy controls. The data were statistically analyzed using graph pad prism ver.7

Results: A total of 13 patients with age ranging from 8 years to 85 years were included in the study. Mean age was 34.46 ± 24.01 years. Most patients were in the age group ≤20 years (38.46%) followed by 21-40 years. Male: Female ratio was 1.6:1. LD bodies were observed in (15.38%) of the cases. PCR positive cases was 46.15%. The diagnostic criteria of CL in KDNA -PCR were based on observation of 720bp, 680bp and 560bp for *Leishmania donovani* complex, *Leishmania infantum* and *Leishmania major* respectively. Immunological study showed significant difference in $CD8^+$ T cells in CL patients with active lesions but not in $CD4^+$ T cells and in B cells between the CL patients and the healthy controls.

Conclusion: Cutaneous leishmaniasis is not common in Nepal. So, it is often neglected and misdiagnosed. It is in an increasing trend. The PCR-based assays used increased the speed and sensitivity of the diagnosis of CL as well as in species identification compared to the conventional

BACKGROUND

Leishmaniasis is one of the world's most devastating neglected tropical, parasitic disease caused by protozoa of the genus *Leishmania* which typically resides in infected female sand flies (*Phlebotomus sps* and *Lutzomyia sps*)(Mouttaki et al., 2014) [1,3]. In terms of global burden of disease, the leishmaniases are the third most important vector-borne disease[6], endemic in more than 98 countries , affecting at least 12 million people worldwide. Each year, 2 million new cases arise and 350 million humans are at risk of infection and contracting this disease [7,8]. Morphologically very similar, different leishmania species causes three main clinical forms , Cutaneous Leishmaniasis (CL), Mucocutaneous leishmaniasis (MCL) and Visceral Leishmaniasis (VL), depending on which types of phagocytic cells are invaded (9). CL is the most common form of leishmaniasis with 0.7–1.3 million new cases occurring annually worldwide [9,11].

Skin lesions of cutaneous leishmaniasis may clinically mimic variety of skin diseases, inflammatory like impetigo, eczema, or granulomatous like sarcoidosis, lupus vulgaris, to skin tumor like basal cell carcinoma & squamous cell carcinoma. [12]. Different diagnosis techniques; microscopy, culture, PCR has been used for the diagnosis of of CL. [9,12]. However it takes a lot of time in diagnosis of the disease in the regions where the disease is not prevalent. Different factors are involved, as microscopy requires highly skilled and experienced medical physician, well-equipped hospital, experienced laboratory technician, quality reagents high power microscope and density of LD bodies in specimen [13]. %).Many authors have reported that recovery of parasites in culture is rarely more than about 70% efficient even with easily cultured parasites. *Leishmania braziliensis* frequently difficult to isolate, and in Tunisia, *Leishmania infantum* parasites causing cutaneous lesions have never been successfully cultured in the standard NNN blood agar medium used for the isolation of Leishmania [14,15]. It has been indicated that the PCR technique has a higher sensitivity as compared to other microscopical techniques and also help to identification the causal leishmanial sps. As different leishmanial species shows different immune response [16], so this study helps to to improve the diagnosis of CL and the identification of Leishmania species and studying the immune response of the patients

MATERIALS AND METHODS

Ethics statement

The study was approved by Nepal Health Research Council, Nepal (Reg. no.45/2018). No

Manuscript ready for submission