MOLECULAR CHARACTERIZATION OF *Leishmania spp*. CAUSING CUTANEOUS LEISHMANIASIS IN NEPAL

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GLOSSARY ACRONYMS

BLAST:	Basic Local Alignment Search Tool
BOD:	Biological Oxygen Demand
CD:	Cluster of Differentiation
CDC:	Centre for Disease Control and Prevention
CL:	Cutaneous Leishmaniasis
CMI:	Cell mediated immunity
CPB:	Cysteine Proteinase B
cRPMI:	Complete Roswell Park Memorial Institute medium
CSB:	Conserved Sequence Blocks
CytB:	Cytochrome B
DAT	Direct Agglutination Test
DCL:	Diffuse Cutaneous Leishmaniasis
DCs:	Dendritic cells
DE:	Direct Examination
DMSO:	Dimethyl Sulphoxide
DNA:	Deoxyribonucleic acid
EDTA:	Ethylenediamine Tetraacetic Acid
ELISA:	Enzyme-Linked Immunosorbent Assay
FBS:	Fetal Bovine Serum
FNAC:	Fine Needle Aspiration Cytology
G6PDH:	Glucose 6 Phosphate Dehydrogenase
GP63:	Glycoprotein 63
HSPs:	Heat Shock Proteins
IIFA:	Indirect Immunoflourescence Assay
ITS:	Internal Transcribed Spacer

kDNA:	Kinetoplast DNA
LAMP:	Loop Mediated Isothermal Amplification
LST:	*Leishmania* Intradermal Skin Test
MCL:	Mucocutaneous Leishmaniasis
MEGA7:	Molecular Evolutionary Genetics Analysis Version 7
MLEE:	Multilocus Enzyme Electrophoresis
MST:	Montenegro Skin Test
NCBI:	National Center for Biotechnology Information
NETs:	Neutrophil Extracellular Traps
NHRC:	Nepal Health Research Council
NK:	Natural Killer
NNN:	Novy-McNeal Nicolle medium
PBS:	Phosphate buffer saline
PCR:	Polymerase Chain Reaction
PKDL:	Post Kala-azar Dermal Leishmaniasis
PSG:	Promastigotes Secreting Gel
rDNA:	Ribosomal DNA
RFLP:	Restriction Fragment Length Polymorphism
RPMI:	Rosewell Park Memorial Institute medium
SSU:	Small Subunit
STIDH:	Sukraraj Tropical and Infectious Disease Hospital
TAE:	Tris base, Acetic acid and EDTA
Th1/Th2:	T helper1/ Thelper2
VL:	Visceral Leishmaniasis
WHO:	World Health Organization

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Leishmaniasis is one of the leading vector borne disease to cause death world-wide. It is caused by more than 20 *Leishmania* species in 98 countries in five continents. The disease is categorized as one of the most “neglected tropical diseases'' and has a strong and complex association with poverty. Nepal, was formerly endemic for the visceral type where fewer cases of cutaneous leishmaniasis has been seen. Due to lack of facilities at all medical centers, diagnosing the disease is challenging. The main concern right now to determine the types of *Leishmania spp*. that are currently prevalent in Nepal and identifying whether or not the agent that causes visceral form is also responsible for cutaneous form. Patients with cutaneous lesions were sampled for parasitological diagnosis using direct examination (DE), kinetoplast DNA (kDNA) nested PCR (CSB1X/CSB2X and 13Z/LiR primers). Further, the kDNA positive samples were amplified for the ITS-1 region. The amplified ITS-1 region were subjected to Restriction Fragment Length Polymorphism (RFLP) using enzyme HaeIII. For the validation of the RFLP result Sequencing was performed. The data were statistically analyzed using graph pad prism. Only 22 (55%) were found to be positive for kDNA Nested PCR observed bands were 720bp, 600bp for *Leishmania donovani* complex and *Leishmania major* respectively and 12 (30%) on ITS-1 PCR. Following, ITS1 PCR-RFLP genotyping of ITS-1 with restriction enzyme HaeIII, results in two distinct patterns that clearly distinguished *L. donovani* (50,75,180 bp) from *L. major* (140, 210bp). The RFLP finding was then validated by sequencing the amplified ITS1 PCR products. This study finds that the parasite *L. donovani* which causes visceral form of the disease is also the causative agent for cutaneous form. Two species *L. donovani* and *L. major* are circulating species causing Cutaneous Leishmaniasis. As the CL is in increasing trend in Nepal. PCR based diagnostic facilities might help to prevent misdiagnose the disease. Molecular screening can be done by ITS1 PCR-RFLP followed by sequencing.

Keywords: Cutaneous leishmaniasis, HaeIII, kinetoplast DNA, nested PCR, RFLP, Sequencing.

CHAPTER 1

INTRODUCTION

1.1 Background

Leishmaniasis is a vector-borne disease, caused by obligate intracellular parasite belongs to the order of Kinetoplastida, genus *Leishmania.* Sandflies of the species *Lutzomyia* serve as the vector in the New World, while the *Phlebotomus* species transmit infection in the Old World (Markle, 2004). Over 20 species of *Leishmania* are capable infecting human these includes *L donovani* complex (*L donovani L infantum*), the *L mexicana* complex with 3 main species (*L mexicana*, *L amazonensis*, and *L venezuelensis*), *L tropica*, *L major*, *L aethiopica*, and the subgenus Viannia with 4 main species (*L[V] braziliensis*, *L[V] guyanensis*, *L[V] panamensis* and *L[V] peruviana*) (CDC, 2018). Leishmaniasis presence is reported in 88 countries around the world, and the prevalence of this disease is estimated to be approximately 12 million annually and about 350 million people are at the risk of catching the disease (Murray et.al., 2005).

Leishmaniasis is a severe health problem, endemic tropical and subtropical regions of 98 countries in Africa, Asia, Europe, and the Americas and 350 million people are at risk (Mcgwire & Satoskar, 2014). More than 90% of new cases reported to WHO occurred in 10 countries: Brazil, Ethiopia, Eritrea, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan. In 2019 over 87% of new CL cases occurred in 10 countries: Afghanistan, Algeria, , Brazil, Colombia, Iran (Islamic Republic of), Iraq, Libya, Pakistan, the Syrian Arab Republic and Tunisia. It is estimated that between 600 000 to 1 million new cases occur worldwide annually (WHO fact sheet, 2021). Cutaneous leishmaniasis is more widely distributed, with about one-third of cases occurring in each of three epidemiological regions, the Americas, the Mediterranean basin, and western Asia from the Middle East to Central Asia. The ten countries with the highest estimated case counts, Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru, together account for 70 to 75% of global estimated CL incidence (Alvar et al., 2012).

Leishmaniasis is a disease of the poor, occurring mostly in remote rural villages with poor housing and little or no access to modern health-care facilities (Okwor & Uzonna, 2016). It includes the spectrum of disease from self- healing skin disease, diffuse cutaneous disease, mucocutaneous disease and potentially fetal visceral leishmaniasis (VL) (Bittencourt & Barral, 1991).

Visceral leishmaniasis: Incubation period varies from 3 to 8 months. Features include fever, weight loss, hepatospleenomegaly, lymphadenopathy, pancytopenia and hypergammaglobinaemia. Due to the feature of skin pigmentation it is called kala-azar, black disease.

Cutaneous leishmaniasis: Incubation period 2 weeks to several months. This initially starts as a papule at the site of sand fly bite which the increases the size, crusts and eventually ulcerates. The variations of the cutaneous leishmaniasis are leishmaniasis recividans, which are characterized by tuberculoid lesions around the scars of healed cutaneous ulcers and diffuse cutaneous leishmaniasis with dissemination of skin lesions rarely occurs over the face, hands and feet, revealing high parasite numbers due to poor cell-mediated immune response.

Mucocutaneous leishmaniasis: The incubation period is 1-3 months. It may occur many years after the cutaneous leishmanisis healed. It involves the nose, oral cavity and pharynx causing difficulties with eating, swallowing and increased risk of secondary infection which carries significant mortality.

Post-kala azar dermal leishmaniasis (PKDL): It develops after resolution of visceral leishmaniasis. The skin lesions are macular, maculo-papular or nodular. (Desjeux, 2004).

1.2 History of Leishmaniasis

The existence of *Leishmania*-like species in prehistorical times is documented in two fossil ambers. The first *Leishmania*-like fossil was found in the proboscis and alimentary tract of a blood-filled female of the extinct sand fly *Palaeomyia burmitis* preserved in a 100 million-year-old Cretaceous Burmese amber (Poinar et al., 2004).

The first written reference to the conspicuous symptoms of cutaneous leishmaniasis surfaced in the Paleotropics within oriental texts dating back to the 7th century BC allegedly transcribed from sources several hundred years older, between 1500 and 2000 BC. Due to its broad and persistent prevalence throughout antiquity as a mysterious disease of diverse symptomatic outcomes, leishmaniasis has been dubbed with various names ranging from "white leprosy" to "black fever". Some of these names suggest links to negative cultural beliefs or mythology, which still feed into the social stigmatization of leishmaniasis today (Cox, 2002).

Old World Leishmaniasis or Kala-azar was first noted in Jessore (now in Bangladesh) in India in 1825. In the year following 1858 an epidemic of quinine-resistant fever was reported in the district Burdwan (Gibson M E., 1983). The cause remained unidentified, 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. 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 (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, et al., 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. In Nepal, VL was officially recorded in 1980 from Dhanusha district (Rijal et al., 2010) (Pandey et al., 2011). Henceforth, it is endemic in 13 districts in central and eastern Terai lowlands bordering North Bihar. Recently, the disease has been reported even from the non-endemic districts like Doti and Bardiya of the county (B. D. Pandey et al., 2011). In Nepal, the cases of CL have 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).

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 Shape and form of *Leishmania*

Leishmania have a digenetic life cycle involving both a mammalian host and an insect vector. *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 *Leishmania* have two major different cell morphologies, represented as the promastigote morphology in the sand fly and the amastigote morphology in the mammalian host (Bates PA. 2019). *Leishmania* is included in the family Trypanosomatidae, having several genera and are characterized by the possession of a Kinetoplast, a unique form of mitochondrial DNA (Scherl et al., 2002).

Amastigotes:

Leishmania amastigotes live and multiply within macrophages by binary fission. The rupture of infected cells disperses the parasite and allows invasion of uninfected monocytes and macrophages by free forms. During the sucking of blood, sandflies ingest amastigotes within macrophages. The free amastigotes multiply and divide asexually in the insect's stomach, becomes elongated and develops flagella and transform to metacyclic promastigotes. Within 2 weeks, such infective forms migrate through the lining of the stomach and enter the proboscis of the sandfly, allowing them to be inoculated in

the human host while the sandfly takes a blood meal. Promastigotes are taken up by macrophages, where they become amastigotes by simple fission.

Promastigotes:

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 (Dostálová & Volf, 2012).

Promastigotes can be grown in a variety of media at 22–25°C. A biphasic medium, for example Nicolle's modification of Novy and MacNeal's medium (NNN) is often used. Schneider's *Drosophila* medium supplemented with fetal bovine serum is often more effective in primary isolation from New World cutaneous lesions. When animal or human specimens are being cultured, penicillin and streptomycin should be added with the inoculum to prevent bacterial overgrowth. 5-Fluorocytosine can be used to inhibit fungal contamination, but amphotericin B should not be added as it may inhibit growth of *Leishmania*. Promastigotes are generally found 2–7 days after inoculation of amastigotes into Schneider's medium and after 7–21 days in NNN medium (Magill, 2005).

1.4 Vector of leishmaniasis

Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*. The parasites are transmitted by the bite of a tiny only 2–3 mm long – insect vector, the phlebotomine sandfly.

There are some 500 known *Phlebotomine* species, but only about 30 have been found to transmit leishmaniasis. Only the female sandfly transmits the parasites. Female sandflies need blood for their eggs to develop, and become infected with the *Leishmania* parasites when they suck blood from an infected person or animal. Over a period of between 4 and 25 days, the parasites develop in the sandfly. When the infectious female sandfly then feeds on a fresh source of blood, it inoculates the person or animal with the parasite, and the transmission cycle is completed.

Phlebotomine sandflies are found throughout the intertropical and temperate regions of the world**.**

The female sandfly lays its eggs in the burrows of certain rodents, the bark of old trees, ruined buildings, cracks in the walls of houses, animal shelters and household rubbish, where the larvae can find the organic matter, heat and humidity they need to develop.

In its search for blood (usually in the evening and at night), the female sandfly can cover a distance of up to several hundred metres around its habitat (WHO, 2021).

1.5 Life cycle

Bite by the infected sand fly

When an infected sand fly bites the skin of a person or animal, the *Leishmania* parasites are injected into a new host, which can then lead to the development of disease. Feeding on a person or animal is also how vectors become infected, and these blood meals are important for development of the parasites inside the fly (Bates PA, 2018).

Transformation of promastigotes to Amastigotes

When a *Leishmania*-infected sand fly takes a blood meal, metacyclic promastigotes are deposited into the site of the bite. The damage caused by the sand fly results in the recruitment of macrophages to the bite site, and these are the cells which *Leishmania* infects and resides in allowing them to persist in the host (Arango D & Descoteaux, 2015). Metacyclic promastigotes are highly motile cells, and *Leishmania* are able to migrate through a collagen matrix. The continued movement of the flagellum within the macrophage results in plasmamembrane damage and lack of integrity, which promotes lysosomal exocytosis potentially altering the composition of the parasitophorous vacuole, thereby increasing the chances of the parasite successfully infecting the macrophage (Forestier CL, 2011). Once inside the macrophage, the promastigote differentiates from a motile promastigote form, which has a long flagellum and an elongated cell shape, to an amastigote form that has a short flagellum with only a small bulbous tip extending beyond a now more spherical cell body. reducing the area over which the cell is exposed to the harsh environment of the parasitophorous vacuole, and also a likely reformatting of flagellumuse (Sunter & Gull, 2017).

Amastigotes to promastigotes transition:

After ingestion by the sand fly and release from the macrophage, the amastigote begins to differentiate into a motile promastigote form. The exact cues for differentiation have yet to be established but are likely to be a combination of the change in temperature and pH akin to other parasites and as seen for *Leishmania* as it differentiates from a promastigote to an amastigote in the parasitophorous vacuole (Sunter & Gull, 2017). The first visible step in this process is the elongation of a motile flagellum, which occurs before cell division, and after this first cell division both daughter cells had a motile flagellum. During differentiation into a promastigote form, the cell shape also begins to change from the spherical amastigote to a more elongated ovoid shape (Sunter & Gull, 2017). 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 (Ghazanfar & Malik, 2016). Then they move forwards to the anterior midgut of the host (Killick-Kendrick, 1990). 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 (Lang,T. & Kaye, P. M., 1991)These parasites again will be taken by sandfly to be transmitted to new host and cycle thus repeats.

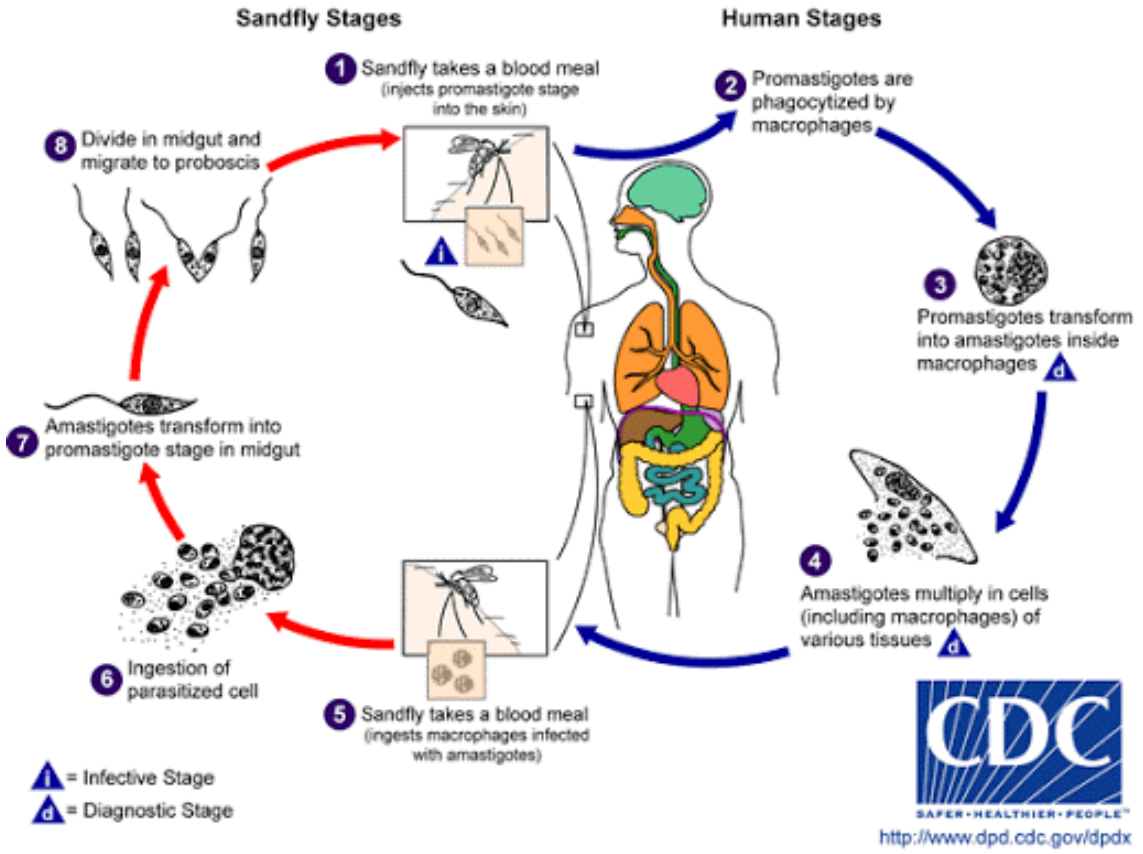


Figure 1: Life cycle of Leishmania

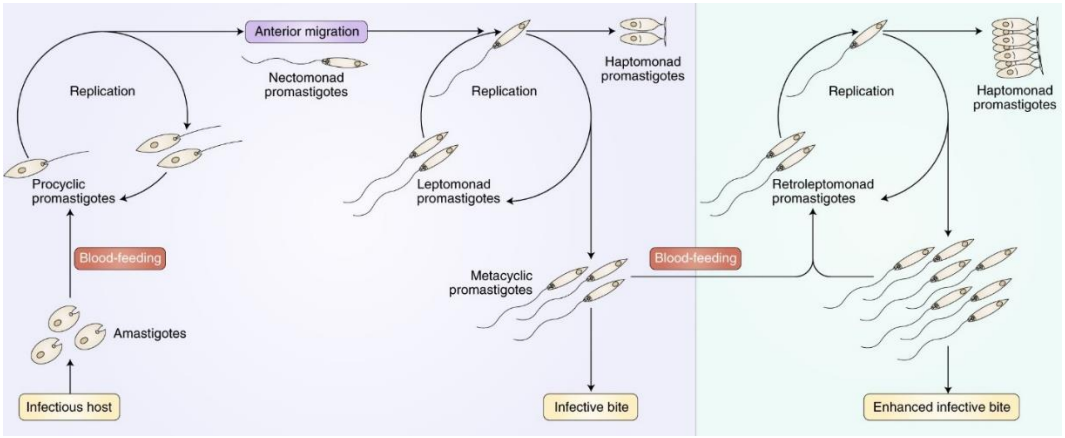


Figure 2: Phases of promastigotes during replication (Bates PA, 2018).

Two replicative phases occur in the first blood meal, involving procyclic and leptomonad promastigotes and leading to the differentiation of metacyclic promastigotes, the infective stage. The new replicative cycle involving retroleptomonad promastigotes is shown on the right, leading to a substantially increased population of metacyclic promastigotes and haptomonad promastigotes, both contributing to enhanced transmission (Bates PA, 2018).

1.6 Clinical presentation

Cutaneous lesions may develop anywhere from a few weeks to months after the sand fly bite, and the lack of travel in the immediate past does not rule out infection. The patient's immune status should be noted. CL lesions are usually found on exposed parts of the body such as the face, arms, and legs. Patients may have one or more lesions; in strict CL, each lesion represents an independent sand fly bite. In rare cases, CL can manifest as a disseminated disease (diffuse CL (DCL)). Lesions are often chronic and unresponsive to attempts at using antibiotics or steroids (Al-Jawabreh et al., 2004), (David CV, 2009). Lesions are often pruritic and are not as painful as they may appear. In most cases, systemic symptoms are absent. However, some systemic symptoms such as lymphadenopathy, fever, and hepatomegaly have been reported to precede *L. braziliensis* ulcerative lesions (Al-Gindan Y et al.,, 1989). Lesions may begin as small red papules (5–10 mm initially). Depending on the species of *Leishmania*, over 1–3 months, they can progress into erythematous nodules, indurated plaques, scaly plaques, or ulcers with raised, rolled dusky borders. Lesions may be dry and crusted or accompanied by exudates. Satellite lesions and local lymphadenopathy are sometimes present. Lesions may leave depigmented retracted scars (Dowlati Y, 1996). Acutely, lesions of CL often are mistaken for furuncles and methicillin resistant *Staphylococcus aureus* infections. The differential diagnosis for patients with chronic lesions like these and a travel history to endemic areas includes deep fungal infections (paracoccidioidomycosis and histoplasmosis),

mycobacterial infections (*Mycobacterium marinum*, cutaneous tuberculosis, and other atypical mycobacteria), syphilis, tertiary yaws, leprosy, sarcoidosis, and cutaneous neoplasms.

1.7 Epidemiology

Approximately 0.2 to 0.4 cases and 0.7 to 1.2 million VL and CL cases, respectively, occur each year. More than 90% of global VL cases occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (Alvar et al., 2012).

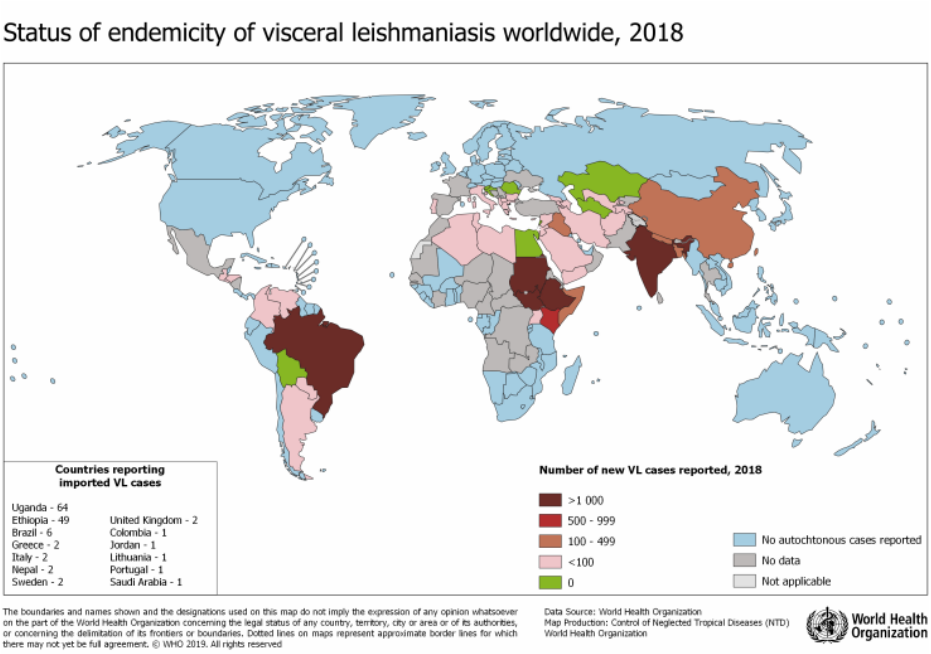


Figure 3: Geographical distribution of Visceral Leishmaniasis (World Health Organization, WHO, 2018)

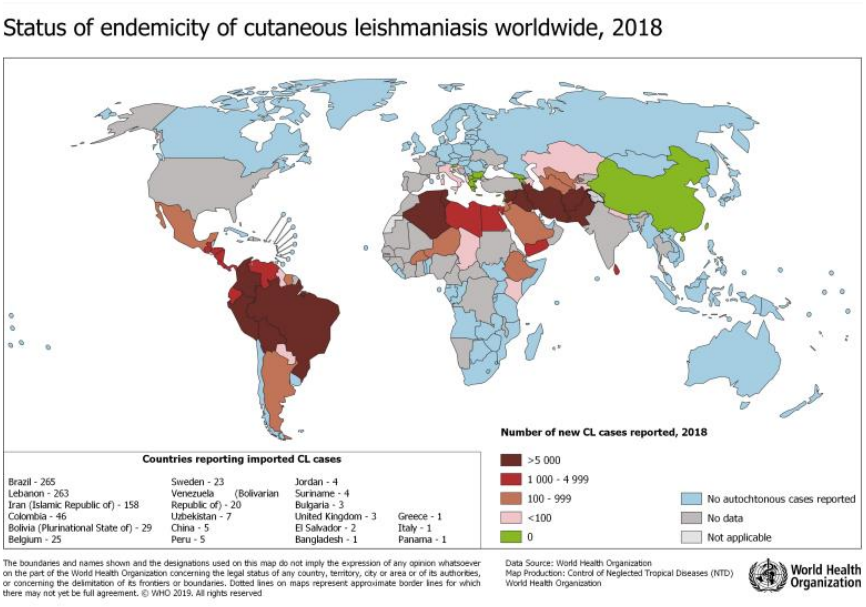


Figure 4: Geographical distribution of Cutaneous Leishmaniasis (Source: World Health Organization, WHO, 2018)

1.8 Cutaneous leishmaniasis in Nepal

Nepal is an endemic zone for visceral leishmaniasis usually the terai region (Joshi et al., 2008) 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 case of cutaneous leishmaniasis was reported 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 from Nepal which was suspected to be basal cell carcinoma was confirmed for CL caused by *L. major* by Safdarjung Hospital, New Delhi, India (Kumar et al., 2007). Recently, Ghimire et al. reported largest collection, 33 cases of CL over six-year period from January 2012 to January 2017, emphasizing the increasing trend of CL in Nepal. Cutaneous leishmaniasis is in increasing trend in Nepal (Ghimire et al., 2018). There is 21 new cases of Cl in 2019 (Pandey et al., 2021) There is no experience about MCL and there are no reports published in the literature from Nepal.

1.9 Molecular Characterization

Leishmaniasis is diagnosed by detecting *Leishmania* parasites (or DNA) in tissue specimens such as from skin lesions, for cutaneous leishmaniasis, or from bone marrow, for visceral leishmaniasis via light-microscopic examination of stained slides, molecular methods, and specialized culture techniques. Identification of the *Leishmania* species can be important, particularly if more than one species is found where the patient lived or traveled and if they can have different clinical and prognostic implications. The species can be identified by various approaches, such as molecular methods and biochemical techniques isoenzyme analysis of cultured parasites (CDC, 2021). The molecular tools such as amplification and subsequent RFLP or DNA sequence analysis of multicopy targets or multigene families, including coding and non-coding regions, and PCR-fingerprinting techniques recently developed.(Constantina et al., 2016) The digestion of ITS1 PCR product with the restriction enzyme HaeIII can distinguish all medically relevant *Leishmania* species. However, almost identical RFLP patterns arise for the representatives of the *L. donovani complex (L. donovani* and *L.infantum) or L. braziliensis complex (L. braziliensis, L. guyanensis, L. panamensis, L. peruviana* etc.) with a great variety of restriction enzymes (Scho¨nian et al., 2003).

1.9 Research hypothesis

Null Hypothesis

Only *Leishmania major* is the causative agent of CL in Nepal.

The *Leishmania donovani* responsible for the VL is not causing CL.

Alternative Hypothesis

More than one species of *Leishmania spp.* are the causative agent of CL in Nepal.

The *Leishmania donovani* responsible for the VL is also causing CL.

1.10 Objectives

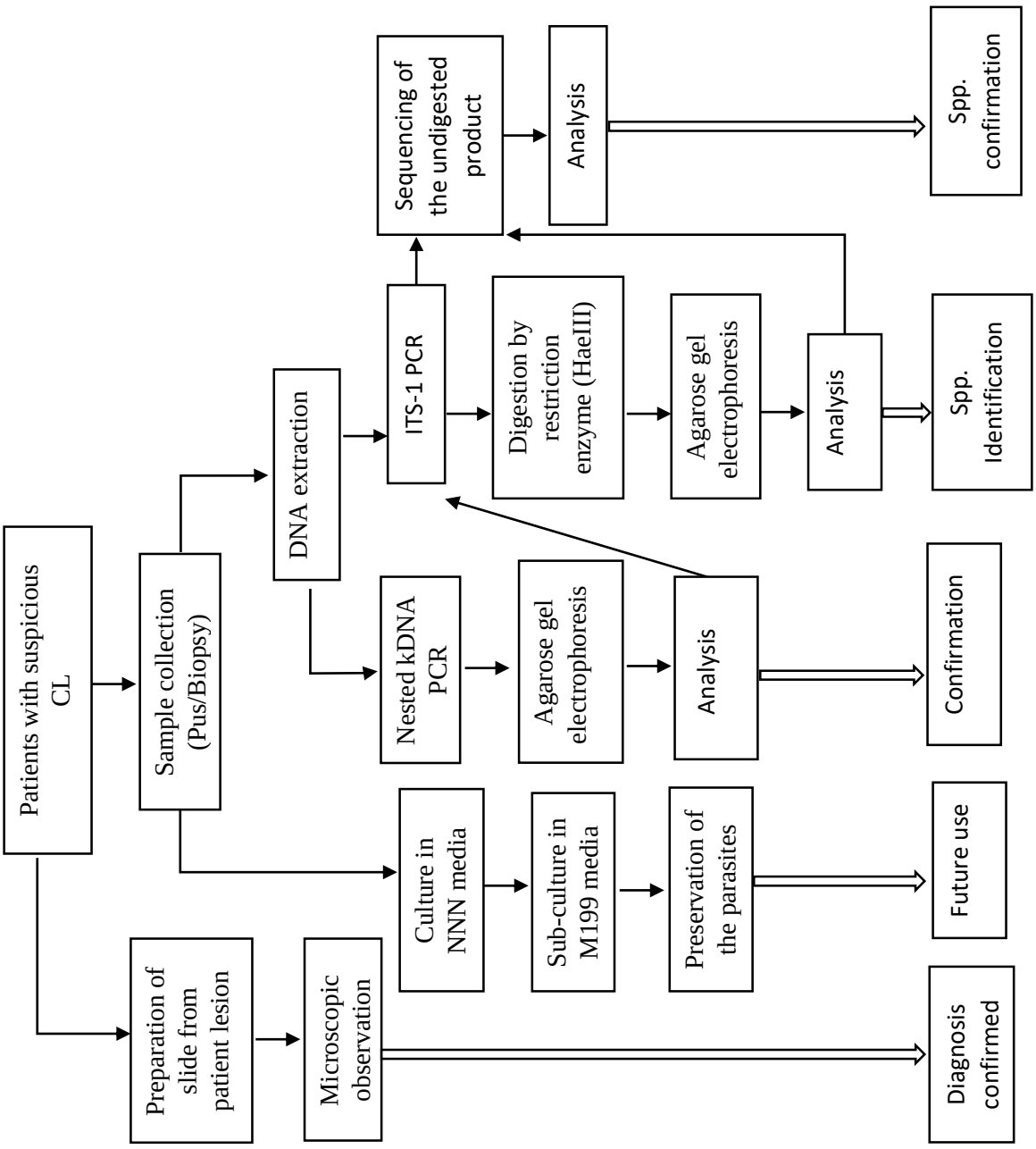
General objectives:

Molecular characterization of the *Leishmania* parasites that cause CL.

Specific objectives:

1. Identification of the *Leishmania* parasite causing cutaneous leishmaniasis by microscopy.
2. Culture of the parasite.
3. Confirmation of *Leishmania* parasite by kDNA PCR.
4. Species identification by ITS1-PCR-RFLP.
5. Species confirmation by sequencing of ITS-1 region.

1.11 Research plan



1.12 Rationale

Visceral Leishmaniasis VL (Kala-azar) is an endemic disease of Nepal that is common in Terai region. Although, CL is rare in Nepal, cases have been reported. CL can be diagnosed by Giemsa staining of the smear from the skin lesion by direct microscopy, culture in the NNN medium, M199 medium and demonstration of leshmanial DNA by PCR. In the endemic region, the clinical diagnosis is easier but there are difficulties in non-endemic countries like Nepal where CL is not common, it can easily be missed and treated as cutaneous tuberculosis, especially Lupus vulgaris. As well as simulate other disease conditions such as tumor-like lesions and, when ulcerated, may resemble basal cell carcinomas, especially in older individuals, making diagnosis difficult. The scar is noticeable for life also after the successful treatment. CL is emerging in Nepal but no data are available about the species of parasite in Nepal thus characterization is needed to know the circulating species in Nepal. In addition, the study will create awareness about the new emerging CL cases for prevention and help the nation not to list as CL endemic country.

The identification of the parasite and the analysis of genetic diversity are important for diagnosis, for epidemiological studies, and for taxonomic and population genetics investigations. Parasite species identification guides physicians in choosing and assessing a specific chemotherapeutic regimen. Since *Leishmania* species cannot be distinguished morphologically (Weiss, 1995), alternate methods of discrimination are essential

CHAPTER 2

LITERATURE REVIEW

It is an emerging and uncontrolled disease and is considered a severely neglected disease having serious and increasing threat (de Vries et al., 2015). People affected by poverty, malnutrition, displacement, and poor housing conditions are most at risk. The skin lesions that result from the disease form on the face or other exposed areas, leaving disfiguring, life-long scars that bring severe social stigma, particularly for women and children. (Bennis et al., 2017). So the early diagnosis of the disease is essential for the prevention of the complications arises. PCR-based methods in combination with restriction fragment length polymorphism analysis or sequencing enable correct species discrimination (de Vries et al., 2015). A recent study with large series of cases from Mid-western region of Nepal have demonstrated that cutaneous leishmaniasis is an under recognized medical condition posing health challenges (Ghimire PG et al., 2018). Recently, the causal species *Leishmania donovani* complex has been reported in a case study of CL in Nepal (Bastola et al., 2019). The outcome of infection will depend on whether the host mounts primarily a T-helper Th-1 or Th-2 response. Further, the interplay between the host determined delayed type hypersensitivity, antigen-specific T cell reactivity, and cytokine secretion, and the type and virulence of the particular infecting *Leishmania* species determine what type of disease expression develops in the host (Dereure et al., 2003). Here the review of most important diagnostic methods, species identification method and the Host immune response has been presented.

2.1 Disease diagnosis

The diagnosis of CL is based on clinical features 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/or indirect testing with serology and molecular diagnostics (de Vries et al., 2015).

2.1.1 Direct microscopy

Microscopical diagnosis of CL is performed by the direct identification of amastigotes in Giemsa-stained lesion smears of biopsies, scrapings, or impression smears. There are various procedures and laboratory techniques used to diagnose leishmaniasis.

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.

Fine needle aspiration cytology (FNAC): However, 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 (Adhikari and Shah, 2017).

2.1.2 In vitro Culture

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 (. 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. Recently developed mini- and micro-culture technologies have the advantage of being less costly because of the smaller volume of culture medium required, easier to use, and more sensitive, even when the parasite burden is low (Boggild et al., 2008).

2.1.3 Imunological 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 (Kar K, 2016). Furthermore, most currently available serologic tests are preliminary based on either a total parasite lysate or whole promastigote yielding in aspecific reactions. Recent developments 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). More commonly used assays for serodiagnosis in leishmaniasis are the indirect immunofluorescence assay (IIFA) and ELISA. Serodiagnosis is not a routine procedure for the diagnosis of cutaneous leishmaniasis in the Old World due to the variable or low sensitivity of the tests and cross-reactivity with other infections (Sarkari et al., 2018).

2.1.4 *Leishmania* Skin Test

The *Leishmania* intradermal skin test (LST) or Montenegro skin test (MST) is a marker of cellular immune response and occasionally used in CL diagnosis because of its simple use and because of its high sensitivity of 86.4 % up to 100 % (Antonio LF et al., 2014). 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 (Reithinger R & Dujardin J, 2007).

2.1.5 Nucleic Acid Amplification Tests

2.1.5.1 Polymerase Chain Reaction (PCR)

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 (Reithinger R & Dujardin J, 2007). In particular, PCR either as a single test or in a nested format or as a quantitative assay, has been widely exploited. PCR allows a highly sensitive and specific (up to 100%) detection of the *Leishmania* parasite irrespective of species or genus. *Leishmania* species identification can be performed by a series of PCR-based assays (Murray et al., 2005). PCR-based protocols have increased the speed and sensitivity of species-specific leishmaniasis diagnosis compared to the conventional techniques such as microscopy and parasite culture. However, these PCR based methods are often hindered by low sensitivity results that fail to detect amastigotes in samples that proved to be positive by microscopy. This caveat in molecular typing is often due to a low parasite load in the original sample (Shahbazi et al., 2008). Numerous tests targeting many different gene sequences have been developed over the last decades, with the ribosomal DNA internal transcribed spacer 1 sequence (Odiwuor et al., 2011), or sequences within the kinetoplast DNA of *Leishmania* genus as the main targets (Satow et al., 2013). The ribosomal DNA internal transcribed spacer 1 (ITS1) sequence or sequences within the kinetoplast DNA of *Leishmania* genus as the main targets (Ghasemloo et al, 2016). 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)(Bensoussan et al., 2006). 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), or assays based on HSP70 or tryparedoxin peroxidase gene targets amongst many others, are under evaluation (Auwera & Dujardin J, 2015).

Nested kDNA PCR

Nested PCR was developed to amplify the variable region of the kinetoplast minicircles of all *Leishmania* species which infect mammals. Each *Leishmania* parasite contains approximately 10,000 kinetoplast DNA minicircles, which are unequally distributed among approximately 10 minicircle classes. The PCR primers were designed to bind within the 120-bp conserved region which is common to all minicircle classes; the remaining approximately 600 bp of each minicircle is highly conserved within each minicircle class but highly divergent between classes.

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). The size of the PCR product may vary according to the species due to the variable region (Noyes et al., 1998).

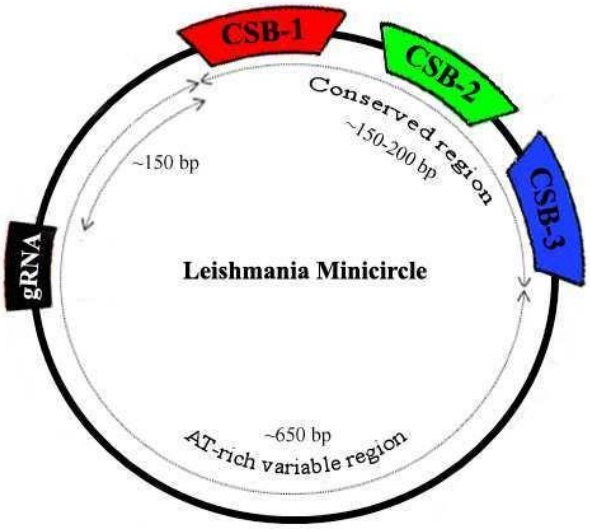


Figure 5: *Leishmania* Minicircle DNA Sciencedirect.com

PCR ITS-1 region

ITS1 is the sequence in between the 18S rRNA and 5.8S rRNA genes. It contains enough conservation to serve as a PCR target but sufficient polymorphisms to facilitate species typing and identification (Roelfsema et al, 2011). The samples were analyzed for ITS1 PCR using 400 nM primers: LITSR: 5-CTTG GATCATTTTCCGATG-3 and L5.8S 5-TGA TAC CAC TTA TCG CAT T-3(N.O.E El Tai et al., 2000). The major advantage of ITS1-PCR is that species identification can be achieved by digesting the PCR product by just one restriction enzyme (HaeIII) and this is sufficient to distinguish almost all medically important *Leishmania* species (Monray-Ostria et al., 2014). A universal PCR method targeting the internal transcribed spacer 1 (ITS1) region between the SSU and 5.8S rRNA genes were described for the direct diagnosis of different clinical manifestations of leishmaniasis and parasite

identification. The ITS1 sequence (300– 350 bp depending on the species). Ribosomal RNA genes are highly conserved and have proven value useful in phylogenetic studies of distantly related trypanosomatids (Tomasini N, 2017). The transcribed, noncoding regions of rRNA genes (Internal Transcribed Spacers or ITS) show extensive variability. Unlike the NTS, the ITS are relatively small (approx. 1 kb in *Leishmania*) and flanked by highly conserved segments to which PCR primers can be designed (Sogin et al., 1986). This method is applicable where several more than one parasite species are aetiologically relevant. It is highly specific and sensitive detecting approximately 0.2 parasites per sample (Schonian et al., 2003)

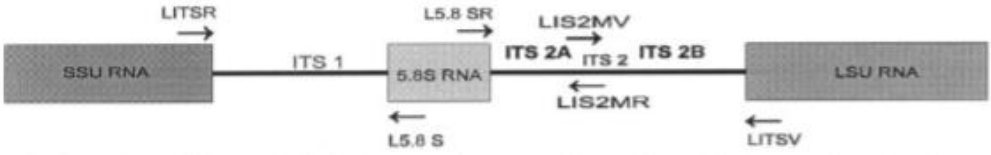


Figure 6: Ribosomal RNA genes and Internal Transcribed Spacers. (Sogin et al., 1986)

2.1.5.2 Isothermal Platforms

PCR requires adequate infrastructure and technically skilled operators, making tests based on this platform less suitable for resource-restricted laboratories in disease-endemic countries. In an attempt to partly circumvent these requirements, isothermal diagnostic platforms have been developed in recent years. Nucleic acid sequence-based amplification, an isothermal reaction targeting parasite RNA, has been developed for leishmaniasis (Beissner et al., 2015). A further development in isothermal molecular diagnostics is loop-mediated isothermal reaction (LAMP), which is performed at 60 and 65 C, uses only one enzyme (Bst DNA polymerase) for amplification, and is able to produce large amounts of DNA within 30–60 min. Importantly, the specificity of the reaction is high because it uses six primers and the end product can be visualized directly using simple detection methods. The initial LAMP test for CL was a generic reverse transcriptase (RT) LAMP, targeting the conserved region of the 18S ribosomal RNA gene. Amplification was visualized by the pre-amplification addition of fluorescent detection reagent and a simple ultraviolet lamp. By using a reverse-transcriptase step, the system detected infections between 10 and 100 parasites per mL and the sensitivity of RT-LAMP for CL patients was 98 % (Adams et al., 2010). Different research groups further developed this technology for various applications (Nzelu et al., 2014). The application of LAMP on boiled swab samples is an interesting advance to develop a simple and rapid diagnostic method for CL (Mikita et al., 2014). This approach has the potential advantages of using LAMP as a molecular diagnostic test in endemic regions where medical resources are limited.

2.2 Species determination

2.2.1 Multilocus Enzyme Electrophoresis (MLEE)

Multilocus Enzyme Electrophoresis (MLEE) is still considered to be the gold standard in parasite typing, even though the method is cumbersome, time-consuming, only applicable to cultured parasites, and exclusively applied in a few labs across the globe. Because it has been the reference test for so long, it is the only technique that has been evaluated for almost all currently identified *Leishmania* species. MLEE entails a biochemical characterization based on the pH dependent electrophoretic mobility of a predefined set of proteins (usually around 10 to 15) in a gel. The combined pattern of all these proteins constitutes a so-called zymodeme, which serves as the basis for species assignment as well as for classification below the species level (Tojal et al., 2006). As only a few labs nowadays persist in performing these analyses, and the method relies on parasite isolation and culture, MLEE is not suitable as a typing method for everyday use (Schönian et al., 2008).

2.2.2 Culture-Dependent Methods

The use of monoclonal antibodies to identify *Leishmania* species of the *Leishmania* (*Viannia*) (Saravia et al., 2002) or *Leishmania* (*Leishmania*) subgenus, or both (McMahon et al., 1985). These antibodies to some extent specifically recognize cultured promastigotes of different species, or they are genus specific. As such, all assays require parasite isolation and are unfit for analyzing clinical or environmental samples (van der Auwera & Dujardina, 2015).

2.2.3 PCR-Based Methods

The most widely accessible and most used sequence-dependent technique is RFLP analysis, whereby the PCR product is digested with one or several restriction endonucleases. Depending on the presence or absence of the enzyme's recognition site, differently sized DNA fragments are generated. Gel-based analysis of the resulting DNA fragment mixture subsequently allows classification of the parasite at hand. As RFLP is a simple technique that requires minimal lab infrastructure, it is available in each lab where PCR can be done.

2.2.3.1 ITS1-PCR-RFLP

The PCR-RFLP method was found to be highly sensitive and specific in detecting samples that have *Leishmania* when compared to the Giemsa-stain microscopy method, which is congruent with previous reports and can be used for Diagnose and direct species

identification in patient tissues, blood or other samples without prior parasite culturing, microscopic analysis or other technique (Hijjawi et al., 2016). The ITS1 PCR-RFLP assay is a multipurpose tool for diagnosis of *Leishmania* from clinical samples and enables determination of the infecting species of New World *Leishmania* in the field in relatively short time and low cost (Monroy-ostria, et al., 2014). The major advantage of the ITS1-PCR is that species identification can be achieved by digesting the PCR product with the restriction enzyme HaeIII. Thus, all clinically important species groups can be distinguished by their RFLP patterns (Schonian et al., 2003). The restriction endonuclease HaeIII, recognises and cleaves the double-stranded sequence GG^CC (https://www.ebi.ac.uk/) and is able to show different restriction patterns according to the species in the clinical sample. High levels of intra- and inter-specific variation were observed in 'New World' *Leishmania* species of the subgenus *Vianna* by amplifying and restricting the rapidly evolving internal transcribed spacers (ITS) in the ribosomal operons. Using this approach it has been possible to distinguish species and strains of 'New World' *Leishmania* isolates & based on their characteristic restriction patterns (Cupolillo et al., 1995).

2.2.3.2 High-resolution melting analysis

Talmi-Frank et al. (Talmi-Frank et al., 2010) developed a melting assay based on a 265- to 288-bp fragment within ITS1. After PCR, the temperature at which both DNA strands separate is recorded with high resolution, and this was found to be species specific. As with RFLP analysis, the Old World species could all be distinguished, with the exception of *L. infantum* from *L. donovani*. The assay was tested on a large panel of isolates from different geographic origins but so far has not been used outside the lab where it was optimized.

2.2.3.3 ITS2 typing

In contrast to the above-described ITS1-based methods, Cupolillo et al. (Cupolillo et al., 1995) could discriminate all tested species of the *Leishmania* (*Viannia*) subgenus by PCR-RFLP analysis of a fragment covering both ITS1 and ITS2. However, 10 enzymes were needed, while sequencing of this fragment was found to be complicated because of microsatellite regions in ITS2 (Parvizi & Ready, 2008). Davila and Momen separated most *Leishmania* (*Leishmania*) species on the basis of ITS1-5.8S-ITS2 rDNA sequences, but this showed no added value compared to the use of the LITSR-L5,8S fragment (Momen & Cupolillo, 2000).

2.2.3.4 SSU rDNA

The 18S or SSU rDNA has limited use for discriminating *Leishmania* species due to its conserved nature withinthe genus. Single-nucleotide polymorphisms have been exploited to identify the *L. mexicana* and *L. donovani* complexes and the *Leishmania* (*Viannia*) subgenus. Methods included probe hybridization, specific PCRs, RFLP analysis, and sequencing (Ulinia et al., 1994).

2.2.3.5 kDNA Minicircles

These PCR assays amplify the variable part of the *Leishmania* minicircles, but many species have minicircles of the same size. In all studies, however, *L. major* can be separated from *L. tropica, L. infantum* and *L. donovani*. Whether *L. aethiopica* can be distinguished based on size alone remains to be established. Aransay et al. complemented size analysis with sequencing of the obtained PCR products, but given the variability of minicircles in a single parasite, and even more so within all strains of the same species, sequence analysis of the variable region is difficult (Aransay et al., 2000).

Species-specific PCRs

Sequence polymorphisms in the minicircle kDNA have been exploited to design PCR primers that specifically amplify a species or group of species (Rocha et al., 2010). Only the specific primers discriminated 4 Old World species, but that assay was validated on one strain of each species only. Several authors have observed cross-reactions between different species (Weirather et al., 2011), which is probably caused by the use of slightly different reaction conditions across labs and the variability of the minicircle population in each species. De Bruijn and Barker (de Bruijn & Barker, 1992) reported a widely used *Leishmania* (*Viannia*)-specific PCR, validated on one strain each of 10 species, but cross-reaction of one primer with human and mouse DNAs was found (Vergel et al., 2005), which could lead to false-positive results. Also, Lopez et al. reported a PCR that amplifies several *Leishmania* (*Viannia*) species but which was evaluated on only 5 strains (Lopez et al., 1993).

RFLP analysis.

Several reports have described RFLP analysis of the variable minicircle region as an aid in species typing. The obtained fragment patterns form the basis of so-called schizodeme identification (Degrave et al., 1994). (Volpini et al., 2004) described an RFLP-based species discrimination test based on the conserved region of the minicircles, but only to discriminate *L. braziliensis* from *L. amazonensis*. Rocha et al. further evaluated this RFLP method and demonstrated that *L. lainsoni* and *L. infantum* could also be identified (Rocha et al., 2010).

LAMP.

Even though it is not a PCR procedure, loop-mediated isothermal amplification (LAMP) also constitutes an amplification technology (Notomi et al., 2000). Unlike PCR, it does not require a thermo cycler, and the amplified product is visualized in the reaction tube and seen by the naked eye, not requiring gels or fluorescence detection. Even though the technique has been used primarily in first-line diagnostics for parasite detection (Adams et al., 2010), an *L. donovani*- specific assay based on kDNA minicircles was developed (Takagi et al., 2009). It was tested on only a few type strains for some species, and hence it currently has limited validity.

Melting curve analysis.

Nicolas et al. used differences in melting temperature of PCR-amplified variable and conserved minicircle regions to differentiate some species in the Old World. Their assay was validated on only one or a few type strains from four species and could not separate *L. tropica* from *L. donovani* (Tomasini, 2017). The conserved region allowed Pita-Pereira et al. to differentiate a few type strains of *Leishmania* (*Leishmania*) from those of *Leishmania* (*Viannia*), but species identification was not possible (Pita-Pereira et al., 2012). Weirather et al. (Weirather et al., 2011) evaluated several primer sets that could discriminate groups of species.

2.2.3.6 Cytochrome *b* (maxicircles).

The *Leishmania* cytochrome *b* gene (*cytB*) is encoded on the kDNA maxicircles, of which an estimated 25 to 50 copies are present in each cell, making it a sensitive target for analysis of clinical samples without the need for culturing. Sequencing of the *cytB* coding region has successfully separated most tested species (Luyo-Acero et al., 2004) and PCRs were applied directly to clinical and sand fly samples (Kato et al., 2007) *L. donovani* and *L. infantum* cannot be separated, nor can *L. braziliensis* and *L. peruviana. L. guyanensis* can be separated from *L. shawi*. Sequence analysis of the gene is straightforward, as no size differences were observed, which facilitates sequence alignment. Some *cytB* realtime PCRs were evaluated by Weirather et al.(Weirather et al., 2011), but they could identify only *L. tropica*. In all, *cytB* is a good typing target, rendering a good resolution for all tested species across the globe, is sensitive enough for use on clinical and environmental samples, and is easily analyzed. Sequence validation on a more extended strain panel would further increase its reliability.

2.2.3.7 Antigen Genes

GP63. The metalloprotease glycoprotein 63 (GP63) is the major surface glycoprotein of *Leishmania* and is considered an important virulence factor and a strong immunogen (Rivier et al., 1999). The gene is arranged as a tandemly repeated unit, and both the intra-

and intergenic regions have been used for typing by RFLP analysis. Because of its multicopy nature, assays based on this gene array have been found to be sensitive enough for application directly on clinical samples, without the need for culturing. One report describes a sensitivity of 85% compared with that of kDNA minicircle amplification (Victoir et al., 2003).

CPB

Cysteine proteinase B (CPB) is another antigenic protein, and several copies of the gene are present in the *Leishmania* genome, arranged in a tandem array (Tintaya et al., 2004). The copies are not all identical and are classified into various subgroups (Kuru et al., 2007). Like GP63, it is an important factor in the host-parasite relationship and is therefore especially fit for looking at population structure in a clinical context. Both the coding sequence and the intergenic regions between the gene copies have been used for typing purposes, primarily using PCR-RFLP approaches to distinguish strains below the species level (Garcia et al., 2005). Quispe-Tintaya et al. included in a study of the *L. donovani* species complex a few outgroups that resulted in discriminant RFLP patterns, but *L. tropica* was not tested, nor was the intraspecies variability in *L. aethiopica* and *L. major* (Tintaya et al., 2004). PCR-RFLP analysis was also used in the New World, but not all species could be amplified, and only *L. braziliensis*, *L. peruviana*, and the *L. guyanensis* complex were identified (Garcia et al., 2005) . These PCR-RFLP assays have been used on clinical samples, without culturing (Veland et al., 2012). In summary, however, none of the *cpb* PCR-RFLP methods have been well validated for species typing. Because the *cpb* array is made up of non-identical isogenes, RFLP patterns are often complicated and vary within the same species (Tintaya et al., 2004).

Some authors have used species-specific *cpb* PCRs that amplifyone or a few. A more general assay, based on specific PCR amplification of certain groups followed by RFLP analysis to discriminate species within each group, but the assay was developed *in silico* and was not validated on type strains or compared with other methods. Given the complexity of the *cpb* locus, this is a major flaw. Because all these specific assays target only one particular copy of the *cpb* gene array, their sensitivity is reduced, and the PCRs have been applied only for typing of cultured parasites, not directly on environmental or clinical material (Lopez et al., 2012). A more sensitive LAMP assay for the detection of the *L. donovani* complex, but its specificity was validated on a limited set of type strains (Chaouch et al., 2018).

HSP70.

Heat shock protein 70 (HSP70) is also a *Leishmania* antigen, playing a role as a molecular chaperone in protein folding and transport (Hartl & Hayer-Hartl, 2002). Between 5 and 10 copies of the gene are present in the *Leishmania* genome , and minor differences between

them may exist in a particular strain (Folgueira et al., 2007). Fraga et al.(Fraga et al., 2010) studied *Leishmania* evolution based on HSP70. Because many studies have illustrated the value of the heat shock protein 70 gene for species discrimination, it has been used by various Researchers.

2.2.3.8 Miniexon

The miniexon or spliced leader is tandemly repeated in the *Leishmania* genome. The exon encodes an RNA that is added to the 5' end of each protein-encoding RNA during maturation. Even though the exon and intron are conserved in all *Leishmania* species, non-transcribed spacers are variable in both size and sequence (Fernandes et al., 1994). Around 100 to 200 copies are present in each genome, which makes the target sufficiently sensitive for analyzing tissue samples (Mosimann et al., 2013).

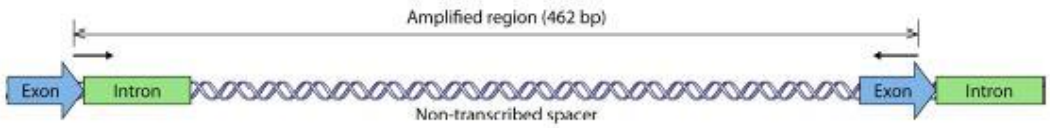


Figure 7: Miniexon segments (van der Auwera & Dujardina, 2015)

2.2.3.9 7 Spliced Leader RNA (7SL-RNA)

The 7SL-RNA is an RNA molecule of 250 to 300 nucleotides that plays a role in the translocation process of proteins across the endoplasmic reticulum. Because of its variability and abundance, Zelazny et al. proposed sequencing of a 140-bp PCR-amplified product for typing purposes (Zelazny et al., 2005). They tested the approach on 30 strains, and their analysis was complemented by Van der Auwera et al. (van der Auwera & Dujardina, 2015), using a larger panel of 71 isolates. These sequences allowed separation of species complexes rather than individual species, except for *L. major* and *L. lainsoni*, which could be typed individually.

2.2.3.10 Carbohydrate Metabolism Enzymes

Glucose-6-phosphate dehydrogenase (G6PDH or G6PD) has been exploited to discriminate between the subgenera *Leishmania* (*Viannia*) and *Leishmania* (*Leishmania*), and within *Leishmania* (*Viannia*) to distinguish the *L. braziliensis* complex, including *L. peruviana* (Castilho et al., 2003). These conventional and real-time specific PCR assays were validated mainly on Brazilian strains, and intraspecies variability was only moderately taken into account. They therefore have limited general use. Since they are based on single-copy genes, their sensitivity is lower than that of other methods based on multicopy targets, such as ITS1, HSP70, and kDNA minicircles (da Graça et al., 2012).

2.2.3.11 Multilocus Typing

Combining different genomic targets has an apparent advantage: one uses information from various loci to gather evidence for a certain species. Such an approach can use either a targeted strategy, whereby several predefined loci are characterized, or a nontargeted approach that randomly documents genome variation. These methods often have limited practical use for species typing, as they provide too much information, which is either redundant or better fit for population-based studies documenting intraspecies variation. When it comes to species typing, multilocus methods are primarily useful for discriminating species belonging to the same species complex, such as *L. infantum*-*L. donovani* (Rugna et al., 2018), *L. guyanensis*-*L. panamensis*, and *L. braziliensis*-*L. peruviana* (Bañuls et al., 1999).

2.2.4 Sequencing

Several DNA sequences have been used as targets for the specific identification of *Leishmania*, such as kinetoplast DNA (kDNA), ribosomal DNA (rDNA), and the g6pd gene, among other targets. kDNA makes up about 15% of the parasite's DNA and is formed by a network of concatenated circular molecules (maxicircles and minicircles). Glucose-6-phosphate dehydrogenase (G6PD) is one of the main enzymes used in the identification of *Leishmania* by zymodeme analysis. Other DNA targets described in the literature, such as the internal transcribed spacers (ITS) present in the rDNA cistron, the heat shock protein (HSP70) gene, the cysteine proteinase (CPB) gene, among others, have been studied in *Leishmania* identification tests, based on the polymorphic characteristics of DNA. Amplicon sequencing in this gene segment has been used to identify *Leishmania*. Based on polymorphisms in the sequence of that same gene this could be used to identify different groups of *Leishmania* have been described (Veasey JV et al., 2020).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Study site

Sukraraj Tropical and Infectious Disease Hospital (STIDH) and Bir Hospital was chosen for the collection of sample.

3.2 Sample collection

The sample were collected from suspected cutaneous leishmaniasis patient visited to our study site. The suspected patients were clinically diagnosed by the medical officer in the Hospital. The questionnaire form and the signed informed consent were taken individually. Lesion aspirate, imprint smear and blood was taken. Sampling was done in the aseptic condition. First, the lesion and its adjacent skin were cleaned with antiseptics. The sterile hypodermic syringe needle was inserted into the nodule or around the ulcers and rotated gently several times. Some tissue aspirate and freed tissue were withdrawn. Some part of the withdrawed sample was the dispensed in the epppendroffs tube containing RPMI medium for DNA extraction and some part in the NNN tube maintaining sterile condition for culture. The imprint smear was prepared by pressing the clean glassslide on the wound/ulcer and let dry.

After the collection of sample, the sample was transported to the laboratory at Central Department of Biotechnology, Tribhuvan University under ambient condition for further process.

There was sample stored in the lab of Central Department of Biotechnology, from which 17 samples were used for the characterization of the parasite on those samples.

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)

3.3 Calculation of sample Size

The required sample sample size for the study was calculated using the formula: $n = \frac{Z^2P(1-P)}{d^2}$
Where, P represents the incidence of CL in Nepal as per the literature Pandey et al., 2021, 21 new cases of CL in 2019, which is 1 in 136238: $P = \frac{1}{136238}$

P=0.00000734

Z=1.96 (at 95% confidence interval)

d=0.001 (desired precision)

n=$\frac{(1.96)^2 \times 0.00000734 \times (1-0.00000734)}{0.001^2}$

n=28.19 =28

3.4 Microscopy

3.4.1 Preparation of Giemsa stained slide

During sample collection the imprint smear slide was prepared on the clean and grease free glass slides. And the smear was left to dry at room temperature. Then the slides were fixed in 100% methanol for 30 seconds and rinsed with water and the slide was stained with 10% giemsa stain for 30 minutes. The slide was rinsed off with tap water and dried thoughly at room temperature. The slides were then viewed under objective 10X, 40X and 100X.

3.4.2 Preparation of Novy-McNeal Nicolle medium (NNN Media)

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. Rabbit blood was drawn from the ear and transfered 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) supplemented with penicillin-streptomycin solution with final concentration of 100 I.U. /ml penicillin and 100 µg/ml streptomycin was overlaid to each tubes in laminar hood and seal with parafilm.

3.4.3 Culture of the parasite

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 26^0C for 9 days. The tubes were checked for growth of parasites in inverted microscope taking a drop of media in a glass slide. As the promastigotes reaches about 100 promastigotes per field then 1-2 drops from the culture was sub-cultured in M199 in a 25 cm^2 culture flask.

3.4.4 Cryopreservation of *Leishmania* promastigotes

Promastigotes in the logarithmic phase were transferred to the 15 ml Polypropylene tube and centrifuged at 1200 rpm for 7 minutes at room temperature. The pellet was washed three times with sterile 1X PBS. The pellet was resuspended in Heat inactivated FBS until the parasite count was 1.2×10^7 per ml. In a cryovial 0.5 ml parasite suspension and 0.5 ml 20% DMSO (prepared in FBS) was added. The content was mixed by invertion and revertion 3 to 4 times. Immediately the vials were transferred to Mr. frosty containing Isopropanol and then Mr. frosty was kept at -70 ^{o}C. After 48 hours the cryovials were transfered to -70 ^{o}C freezer. 1 vial of the preserved vials was taken and cultured to test the stabilate.

3.5 Molecular diagnosis

3.5.1 DNA extraction

The DNA from the clinical specimen was extracted by using DNA extraction Kit (Quick-DNA^{TM} 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^{TM} 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.

3.5.2 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.

3.5.3 kDNA 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 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 2: 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. 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.

3.5.4 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.

3.6 Species Characterization

3.6.1 ITS-1 PCR

From the kDNA PCR positive samples, PCR of ITS-1 was performed using primer:

Forward primer: CTGGATCATTTTCCGATG

Reverse primer: TGATACCACTTATCGCAC

Table 3: PCR compositions

ITS-1 PCR	
Reagents	**Vol. Per reaction**
Master mix (5x)	3µl
LITSR	0.6µl
L5.8S	0.6µl
Template DNA	2µl
DNA grade water	8.8µl
Total	**15µl**

Table 4 Thermal cycling conditions

Steps			
	Cycles	**Temp.**	**Time**
Initial denaturation	1	94ºC	3 min
Denaturation		94ºC	0.4min
Annealing	35	52ºC	0.5min
Extension		72ºC	1min
Final extension	1	72ºC	10 min
Hold		4ºC	infinite

3.6.2 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.

3.6.3 Restriction Fragment Length Polymorphism

The product of the PCR positive for the ITS-1 was digested by enzyme HaeIII.

Table 5: Reaction composition for Restriction digestion:

Restriction digestion	
Reagents	**Vol. Per reaction**
Cut smart buffer	2.5µl
HaeIII enzyme	0.5µl
Template DNA	10µl
DNA grade water	12µl
Total	**25µl**

The reaction mixture was then incubated at 37^0C for 1 hour.

3.6.4 Agarose gel electrophoresis

For this 3% of agarose gel was prepared in 1X TAE (Tris base, acetic acid and EDTA) buffer and casted of gel electrophoresis tank. Ten microliters of digested product with 2 µl of loading dye were loaded along with suitable molecular weight DNA ladder (25bp,). Then the gel was run at 80 volts for 1 hour. Finally, the digested products on agarose gel, stained with ethidium bromide was visualized under UV Trans Illuminator and gel doc (MS major science UVDI) to observe the banding of the digested product and photographed to keep the record.

3.6.5 Sequencing

The Samples positive for kDNA were tested for ITS -1 region. The samples which were ITS-1 region positive were further subjected to ITS-1 PCR in final volume 25 µl. The amplification was performed by Agarose gel electrophoresis. The PCR products giving clear high intensity band without noise were transported to National academy of Science and Technology, Khumaltar for Sequencing. Sanger Sequencing was performed.

3.6.6 Sequence alignment and Phylogenetic analysis

Sequence alignments were performed using ClustalW. The sequence similarity and closest relationship among the selected sequences were analyzed by MEGA 7 software.

CHAPTER 4

RESULT

4.1 Demographic study of suspected CL cases.

4.1.1 Geographical Distribution

Cutaneous leishmaniasis was found in 17 districts during the period of this study. It was found in all province having highest number of cases from province 7, and lowest number of cases from province 5 and 2. Most of the patient in this study were included from Bajura. Incidence of the CL were found in the Jhapa, Bhojpur, Okhaldhunga and Solukhumbu district of Province 1, Parsa district of Province 2, Dhading, Lalitpur, Banepa and Sindhuli of Province 3, Gorkha, Baglung and Parbat of Province 4, Rukum of Province 5, Kalikot, Jajarkot of Province 6, and Bajura and Baitadi of Province 7. Highest numbers of cases were found in Province 7 (n=6) and Kalikot District of Province 6 (n=3).

Figure 8: Incidence of CL cases in the Districts and Provinces of Nepal

Table 6: Distribution of Patients according to District and Provinces

SN	District	Frequency	Province	Percentage
1	Bajura	4	7	17.4
2	Dhading	1	3	4.3
3	Kalikot	3	6	13.04
4	Jajarkot	1	6	4.3
5	Gorkha	1	4	4.3
6	Parsa	1	2	4.3
7	Baitadi,	2	7	8.7
8	Sindhuli	1	3	4.3
9	Bhojpur	1	1	4.3
10	Okhaldhunga	1	1	4.3
11	Banepa	1	3	4.3
12	Solukhumbu	1	1	4.3
13	Rukum	1	5	4.3
14	Baglung	1	4	4.3
15	Lalitpur	1	3	4.3
16	Parbat	1	4	4.3
17	Jhapa	1	1	4.3

(where,total number of sample n=23, Percentage=Frequency/total sample×100)

4.1.2 Gender based Distribution

The study cohort consisted of 69.57 %(n=16) males and 30.43 %(n=7) females. The male population was found to be higher than the female population, but there is no significant difference between the suspects of CL in Male:female population. The lesions appeared in the body of the male and female showed that there is no correlation of the lesion occurance specific to the sex. The ratio was found to be 16:7.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.

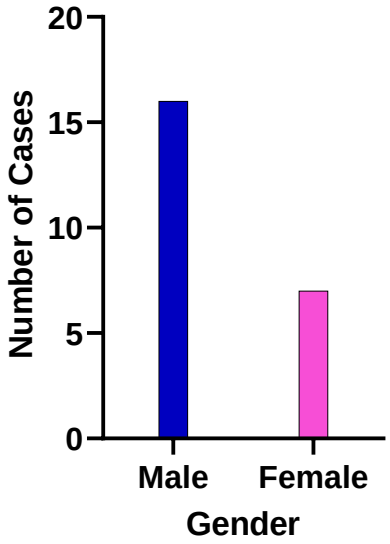


Figure 9: Graphical representation showing gender based distribution among the studied cases.

4.1.3 Age wise Distribution

A total of 23 patients ranging from 9 to 66 years involved in the study. Most of the patients infected were within the age group of 41-60 with the highest frequency 9 (39.13%) followed by Age group (0-20) having frequency 8 (34.78%) and (21-40) years old 5(21.75%). The lowest frequency was found in the Age group 61-80 with one case.

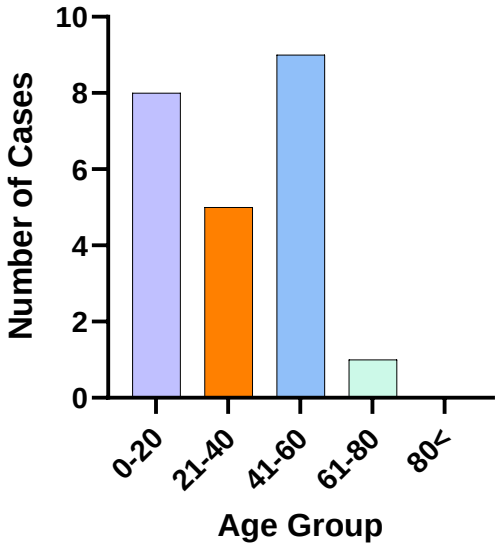


Figure 10: Distribution of research participants into different age groups

4.2 Epidemiology of CL cases in Nepal

4.2.1 Geographic Distribution

Cutaneous leishmaniasis cases were found in 10 districts during the period of this study. The highest number of cases i.e 4 from province 7, and only one case from province 5. No any case was found in Province 2 and 3.In province 1, 3 cases were found and 2 cases from Province 6. In province 4 2 cases are found to be positive.

Figure 11: CL positive cases in the different Provinces of Nepal

Table 7: CL positive cases in the different district and Provinces of Nepal

SN	District	Frequency	Province	Percentage
1	Bajura	3	7	25
2	Kalikot	1	6	8.3
3	Jajarkot	1	6	8.3
4	Gorkha	1	4	8.3
5	Baitadi,	1	7	8.3
6	Bhojpur	1	1	8.3
7	Okhaldhunga	1	1	8.3
8	Rukum	1	5	8.3
9	Baglung	1	4	8.3
10	Jhapa	1	1	8.3

4.2.2 Gender based CL positive cases

Out of 23 samples, 12 cases are positive for CL. Among which male were 48.14% (n=8) and those in female were 69.23% (n=4). There is no significant difference between positive and negative cases in bothe gender, at 95% confidence interval

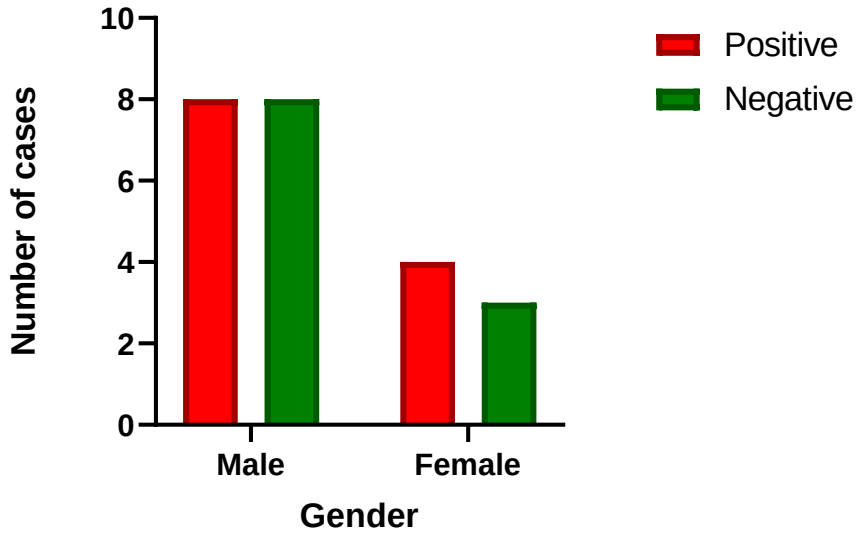


Figure 12: Gender based distribution of PCR positive cases.

4.2.3 Age based CL positive cases

The study subjects were divided based on the age gap of 20 years for evaluation. The data showed that highest (n=6) number fell into age group (41-60) years with 50 % positive cases and the lowest (n=1) number fell into age group (61-80). There was no any case in age group (>80). There is no correlation between the age and the number of individuals being infected.

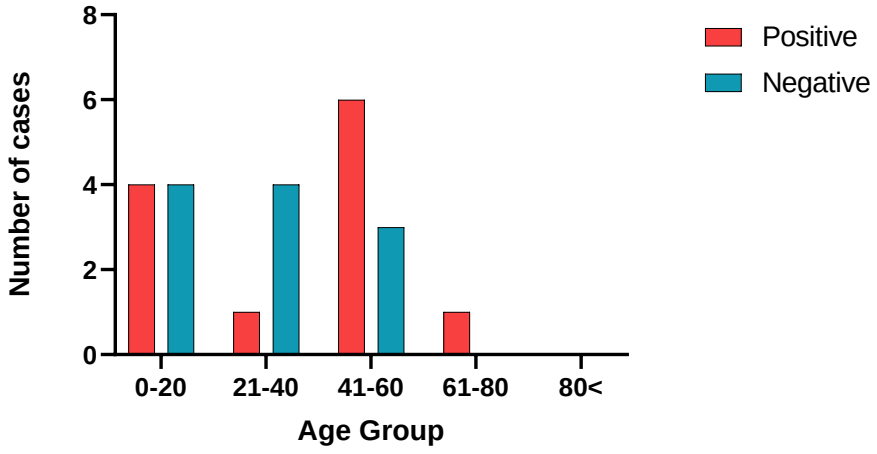


Figure 13: Age wise distribution of PCR positive case

4.2.4 Travel History of CL Positive cases

Out of 12 PCR positive cases, Among the PCR Positive cases most of them were from Hilly region. Only 3 patients had travel history outside Nepal of which all three had travel history in leishmaniasis endemic country. Rest of the patients hadn't travelled outside Nepal but two had travelled terai regions of Nepal.

Table 8: The travel history of the CL positive Cases.

SN	Sample code	Address	Travel history Outside country	Duration of stay	Travel history Inside country	Duration of stay
1	CL-18	Bajura	No		No	
2	CL-19	Bajura	No		No	
3	CL-22	Jagarkot	No		No	
4	CL-23	Gorkha	No		Yes, Chitwan	
5	CL-25	Baitadi,	No		No	
6	CL-27	Bhojpur	Yes, Dubai	7 years	No	
7	CL-29	Okhaldhunga	No		No	
8	CL-32	Kalikot	No		No	
9	CL-35	Rukum	No		Yes, Dang	7 Months
10	CL-36	Baglung	Yes (Saudi)	23 Months	No	
11	CL-39	Jhapa	Yes (Saudi)	4 and half year	No	
12	CL-40	Bajura	No		No	

Among 23 suspected samples 4 samples have traveled abroad. Among four patient 3 cases were diagnosed positive for the CL.

Table 9: Imported cases of CL among the studied cases.

sn	Sample ID	Travel History (Abroad)	PCR result
3	CL-27	Yes, Dubai(7yrs)	Positive
5	CL-36	Yes (Saudi, 23 months)	Positive
6	CL-39	Yes (Saudi, 4 year, 6months)	Positive

4.3 Comparative study of suspected verses Confirmed cases

Among the 4 suspects from the Province 1 3 were confirmed as CL and 1 was the disease other than CL. Similarly one case was confirmed as CL in province 2. In Province 3 none of the 4 cases were CL, one out of two was confirmed as CL in province 4 similarly, one from province 5, in province 6 2 out of 4 and in province 7 four cases were confirmed as CL. The remaining suspects which are negative for the diagnosis of CL are infection other than the CL.

Table 10: Comparisons of the suspects and confirmed cases in different province

S.N.	Province no.	suspected	Confirmed	Suspected but not CL
1	1	4	3	1
2	2	2	1	1
3	3	4	0	4
4	4	2	1	1
5	5	1	1	0
6	6	4	2	2
7	7	6	4	2

4.4 Clinical features

Clinically presenting 23 new cases cutaneous disease were diagnosed as cutaneous leishmaniasis according to the characteristic of the lesions.

4.4.1 Distribution/Location of lesions on the body of the patients

The lesions were mainly found to be in facial region, upper limb, lower limbs and the abdominal regions. Among them, most of the cases have lesion on the facial region. Most of the patients (47%) had lesions on their face followed by upper limb (17.39%) and abdominal region (13.04%). A patient had lesion covering the nose fig (27) and the mouth and one has disseminated lesion covered the entire face fig (38)

The patients complained about the pimple like eruption with slight itching and further developed the large lesion with no healing signs. Most of the patients had localized

lesions. While the patient had disseminated erythematous lesions fig (22, 23, 24, 27, 29, 30, 37, 38).

Some of the patients have multiple localized lesions in different parts of the body. A patient had lesion in the nose as well as in the upper limb fig (22 a and b), A patient had lesion in the lip as well as in the upper limb fig (25) another patient had lesion in upper limb as well as in the abdomen. Similarly, a patient had multiple lesions in the upper and lower limb fig (36 a and b). some had multiple lesions in a single part of the body (28: 3 lesions on the lip, 33: 3 lesions in the face ie., nose, lip, cheek, 39: 3 on leg, 40: cheek and lower lip).

Table 11: Distribution and frequency of lesions in CL suspected patients.

Site of Lesion	**Frequency of suspects**	**Frequency of CL Positives**
Face	11	5
Neck	1	1
Upper limb	4	2
Lower limb	1	1
Abdominal part or body	3	1
Face and upper limb	1	1
Upper Limb and lower limb	1	1
Upper limb and abdomen	1	0
Total	**23**	**12**

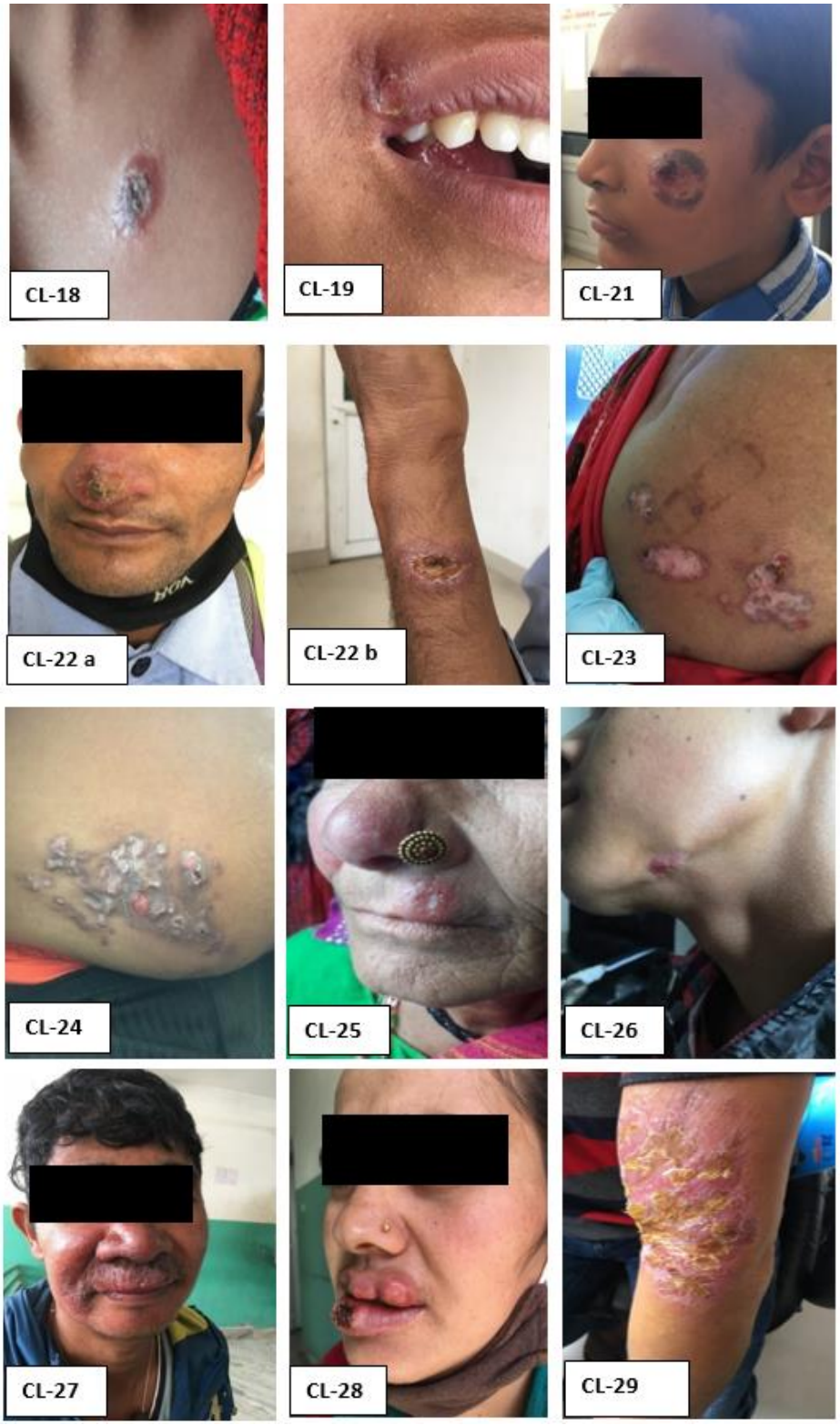


Figure 14: Clinical Feature and characteristics of lesions.

(CL 17) Ulcerated lesion in thigh. (CL 18) Dry crusted lesion in upper chest. (CL19) Dry crusted lesion in upper lip. (CL 20) Dry and dispersed. (CL 21) Raised nodulated lesion. (CL 22) Ulcerated, moist lesion in nose and arm. (CL 23) Dry and crusted lesion in waist. (CL 24) Dry desseminated lesion in abdomen. (CL 25) Moist invaginated lesions in upper lip and hand. (CL 26) Dry crusted lesion in chin. (CL 27) Dry and dispersed lesion in mouth and nose region. (CL 28) Dry nodulated crusted lesion in upper and lower lip. (CL 29) Dry erythematic plaque in arm.

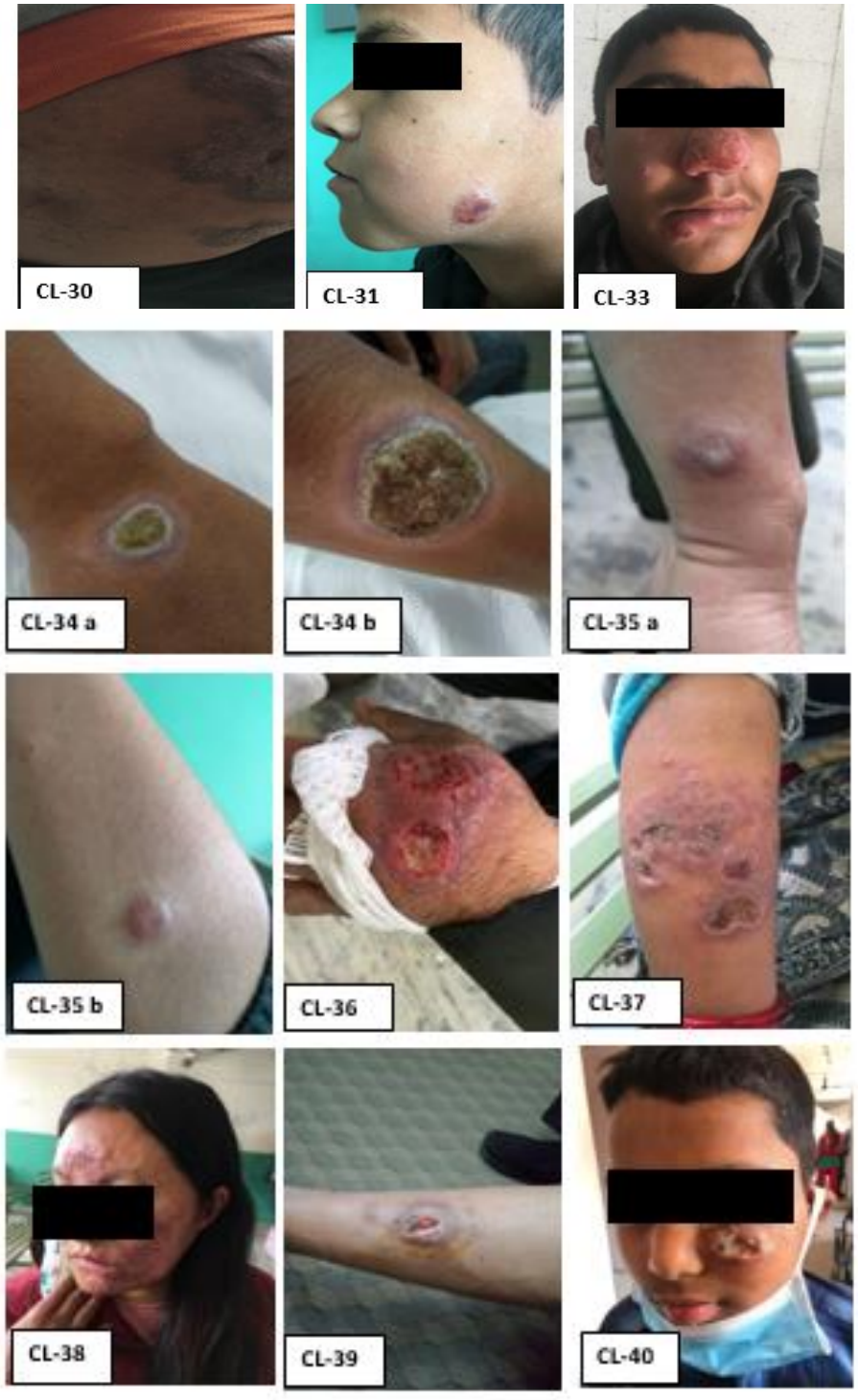


Figure 15: Clinical Feature of CL patients and characteristics of lesions

Characteristics of lesions. (CL30) erythematous plaque on buttock. (CL31) nodulated lesion on cheek. (CL33) Bulged, dry, nodulated lesion in nose upper lip and cheek. (CL34) Dry crusted lesion on both wrist. (CL35) Dry and raised lesion in both hands. (CL36) Central invaginated and moist lesion on both hand and leg. (CL37) Ulcerated lesion on right arm. (CL38) Erythematous plaque dispersed over the face. (CL39) Ulcerated lesion with Central invagination on left leg. (CL40) Raised crusted, dry, nodulated lesion on lower lip and left cheek.

4.4.2 Characteristics of Lesions

Out of 23 studied individual, most of the patient (n=17, 73.9%) have dry type of lesion without any ulcer and Exudate while, (n=6, 26.08 %) patient have wet type of lesion having ulcer and visible exudate. Among which 7 were found to be CL positive having dry type of lesion, and 5 cases were CL positive having moist type of lesion. At 95 % confidence interval, there was no significant difference between the Type of lesion and infection of Cl.

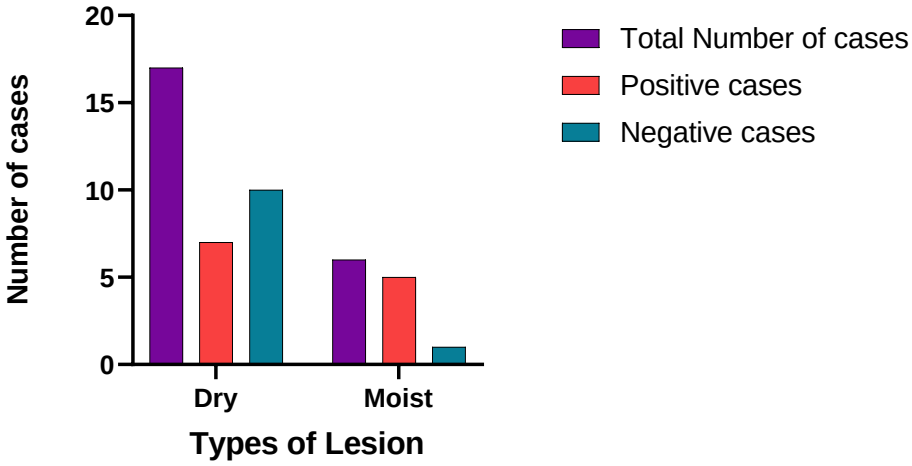


Figure 16: Graphical representations of lesion type with respect to number of cases

4.4.3 Mono and multilesions in the studied cases

Among the 23 suspected cases of cutaneous Leishmaniasis, most of the patients (n=10, 43.5%) have one lesion while n=5, 21.7%) have 2 lesions and n=8, 34.8%) have more than two lesions. Five patients having only one lesion was tested positive for CL. Simillarly, 2 patients were having two lesion and five were having more than two lesions on their body.

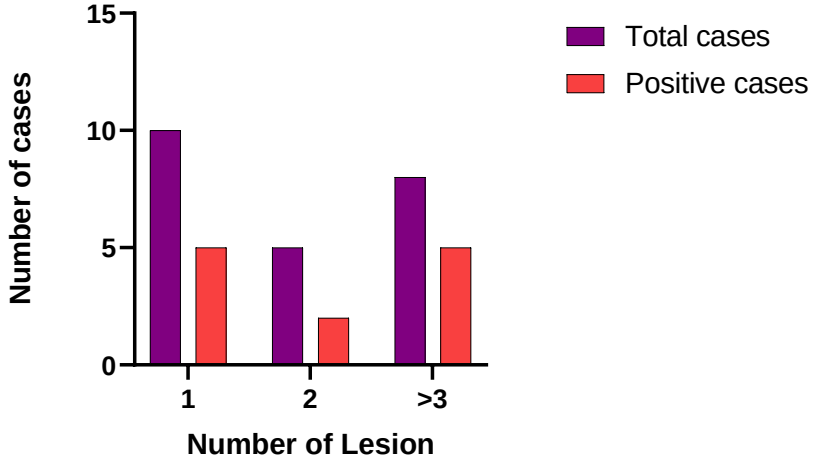


Figure 17: Graphical representations of number of lesions and number of cases.

4.4.4 Duration of Lesion at the time of Sample collection

Most of the patient visited hospital after 10 months of lesion eruption. In this study, 34.8% of patient visited hospital after 10 month of initial lesion eruption. Equal percentage, 26.08% patient visited after 8-10 months and 5-7 months of lesion eruption. No cases was found to be visited at earlier stage of the infection. Statistically it was found that with the increase in time duration of lesion visiting to the hospital increases as well the positive cases.

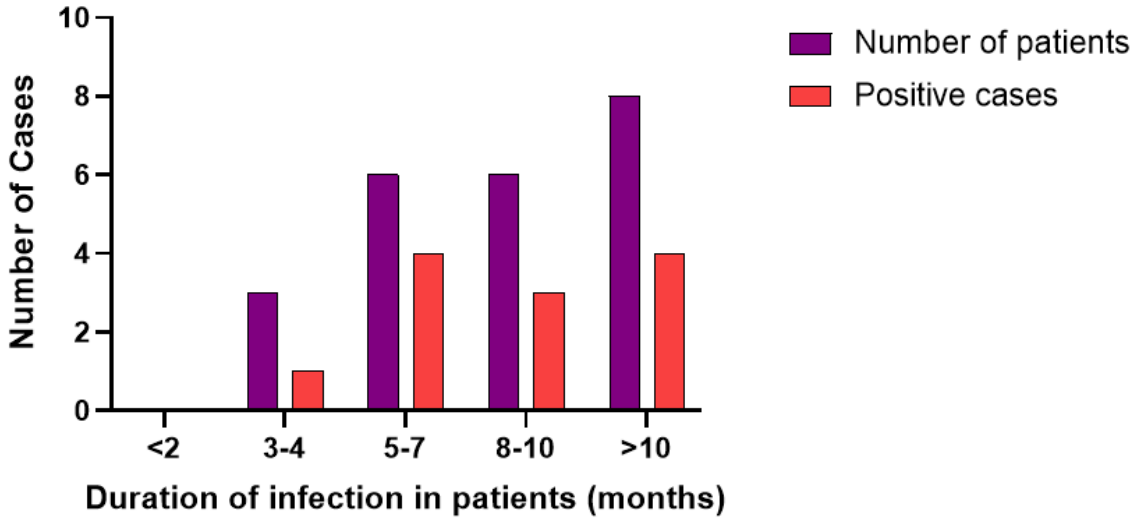


Figure 18: Graph showing the duration of infection in the studied patients

4.5 Diagnosis

4.5.1 Microscopy

Among 23 suspected CL cases LD bodies was observed in only one slide and on rest of the slides LD bodies were not observed. Two dotted structure was seen on the oval cell so it might be the amastigotes. The amastigotes were freely distributed outside the cell as well as inside the macrophages infecting the macrophages.

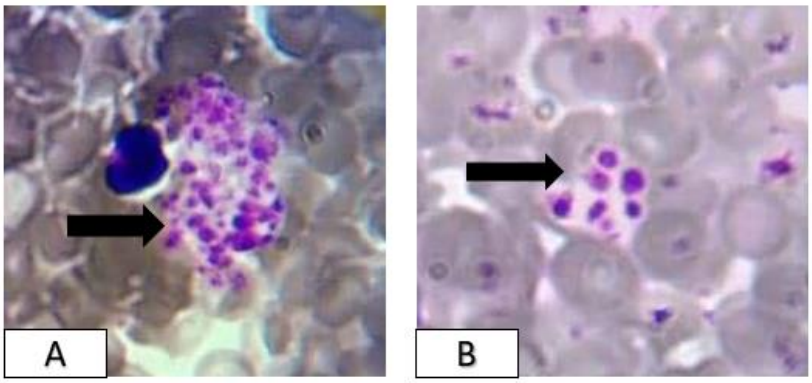


Figure 19: (A) Giemsa stained Smear showing macrophage infected by amastigotes. (B) Smear showing amastigotes under 100X as shown by arrows.

4.5.2 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 seen one of the culture tube. Further, subculture was done in liquid media, M199.

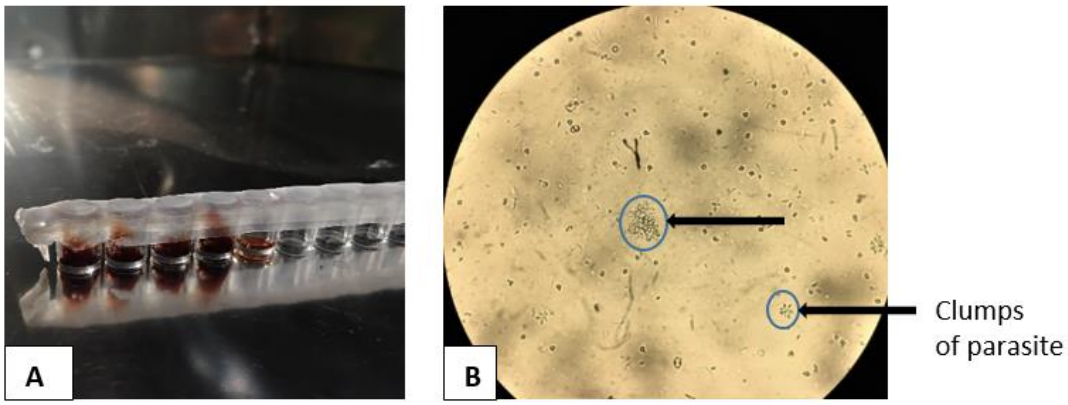


Figure 20: (A)Minicultures of NNN media (B)Microscopic Observation of parasites in NNN media

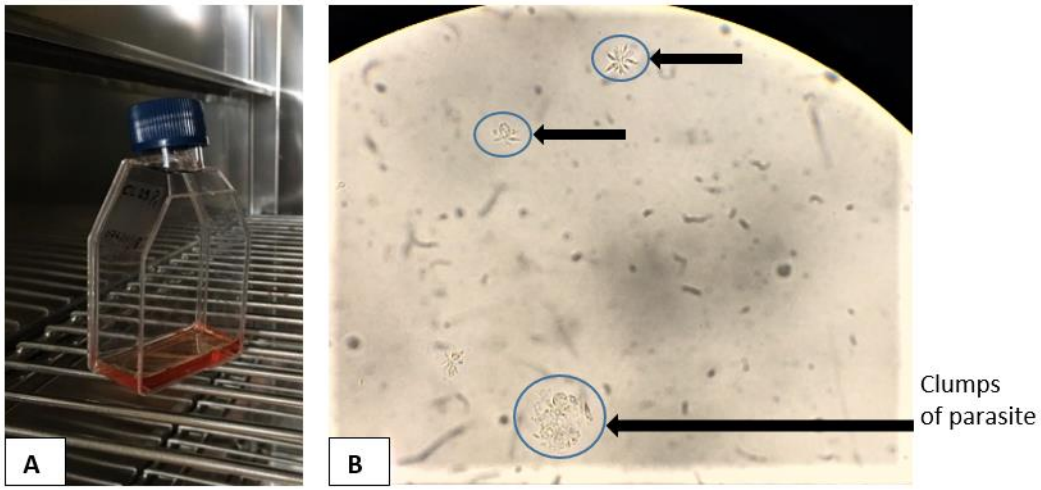


Figure 21: (A) Sub-culture in M199 media after 7 days (B) Growth of parasite after 10 days of subculture in M199

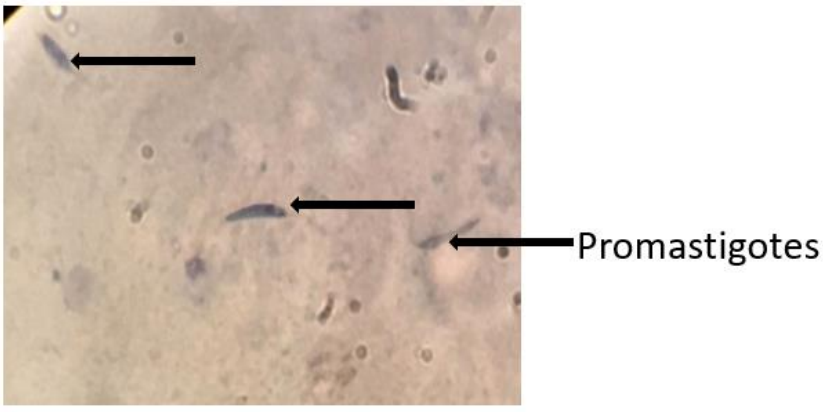


Figure 22: Giemsa stain of cultured parasite

4.5.3 Molecular diagnosis

4.5.3.1 DNA quantification

DNA extracted was measured for the quantification and its quality determination by Nanodrop.

Table 12: 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
18	CL18	1.965	0.963	23.42	2.04
19	CL19	0.765	0.421	41.25	1.81
20	CL20	0.852	0.465	25.80	1.83
21	CL21	0.963	0.540	59.02	1.78

S.N.	Sample name	Absorbance		Concn. (ng/µl)	Ratio(260/280)
		260nm	280nm		
22	CL22	1.231	0.689	78.13	1.78
23	CL23	1.365	0.785	85.63	1.27
24	CL24	0.982	0.625	89.98	1.57
25	CL25	0.452	0.236	63.15	1.91
26	CL26	0.258	0.165	96.36	1.56
27	CL27	1.067	0.851	120.52	1.25
28	CL28	0.753	0.451	53.12	1.66
29	CL29	1.896	1.112	41.30	1.70
30	CL30	0.598	0.315	74.13	1.89
31	CL31	0.529	0.326	12.30	1.62
32	CL32	1.684	0.987	73.12	1.70
33	CL33	1.023	0.651	36.21	1.57
34	CL34	1.365	0.810	74.41	1.68
35	CL35	0.852	0.504	13.12	1.69
36	CL36	1.325	0.763	85.12	1.73
37	CL37	0.592	0.321	17.23	1.84
38	CL38	0.733	0.421	47.01	1.74
39	CL39	1.832	0.892	96.12	2.05
40	CL40	0.574	0.312	20.12	1.83

4.5.3.2 PCR of kDNA

The DNA extracted from the samples collected from STIDH hospital were subjected to Nested PCR along with negative control. The First round PCR (PCR 1) electrophoresis showed bands of different sizes. Comparison was done with 100bp DNA ladder. However, in many of the samples did not show amplification. In the samples where PCR 1 gave products upon visualization, the amplicon sizes ranged from 700bp to 800bp. Some samples negative for PCR 1 was positive after Second round PCR (PCR 2). The amplicon

sizes of Second round PCR 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. Out of 40 CL suspected patients 22 were confirmed to be PCR positive CL cases.

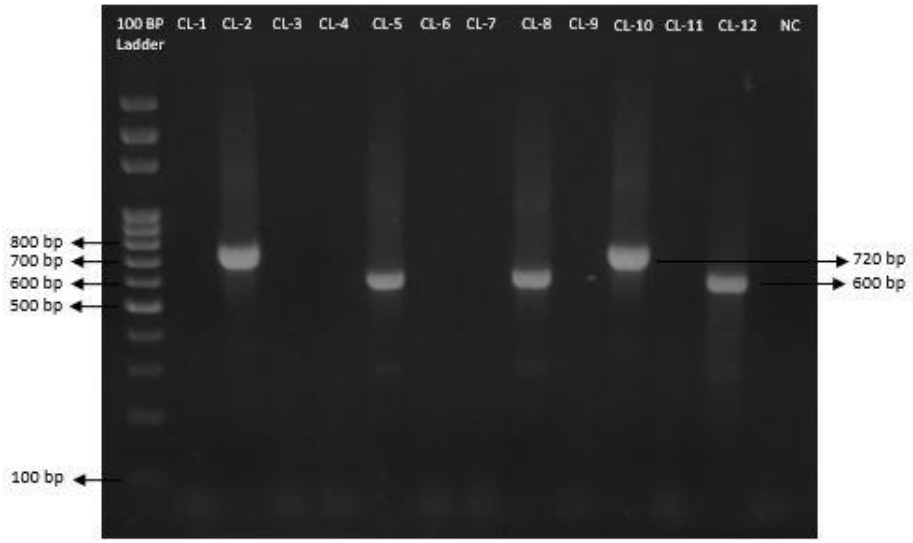


Figure 23: PCR 2 amplicon run for gel electrophoresis (a)

Lane1- 100 bp ladder, Lane 3 and 11 ~700bp lane 6,9 and 13 ~ 600 bp Lane 14- Negative control (NC) Lane 2,4,5,7,8,10,12 Not visualized or not amplified.

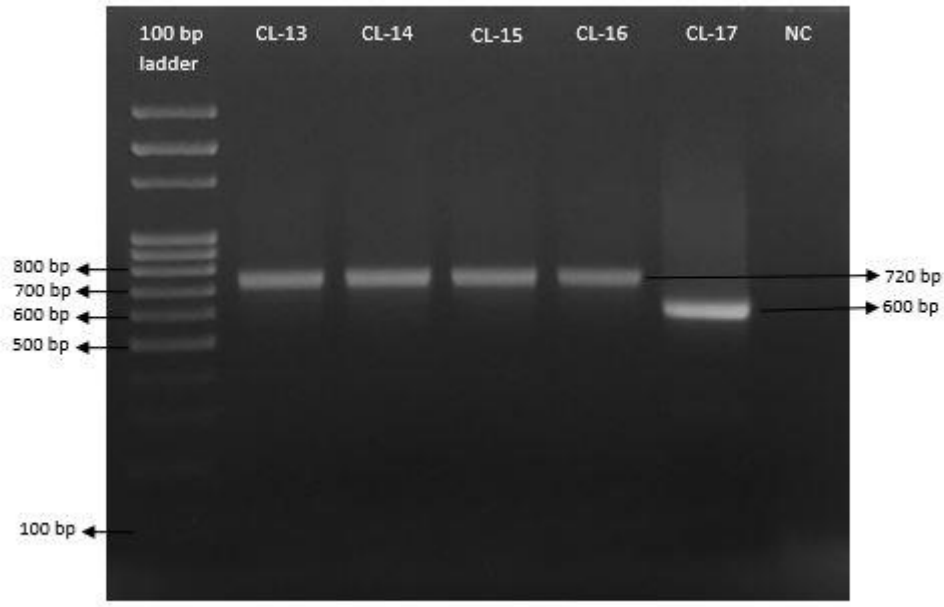


Figure 24: PCR 2 amplicon run for gel electrophoresis.(b)

Lane1- 100 bp ladder, Lane 1,2,3 and 4 ~700bp lane 5 ~ 600 bp Lane 6- Negative control (NC).

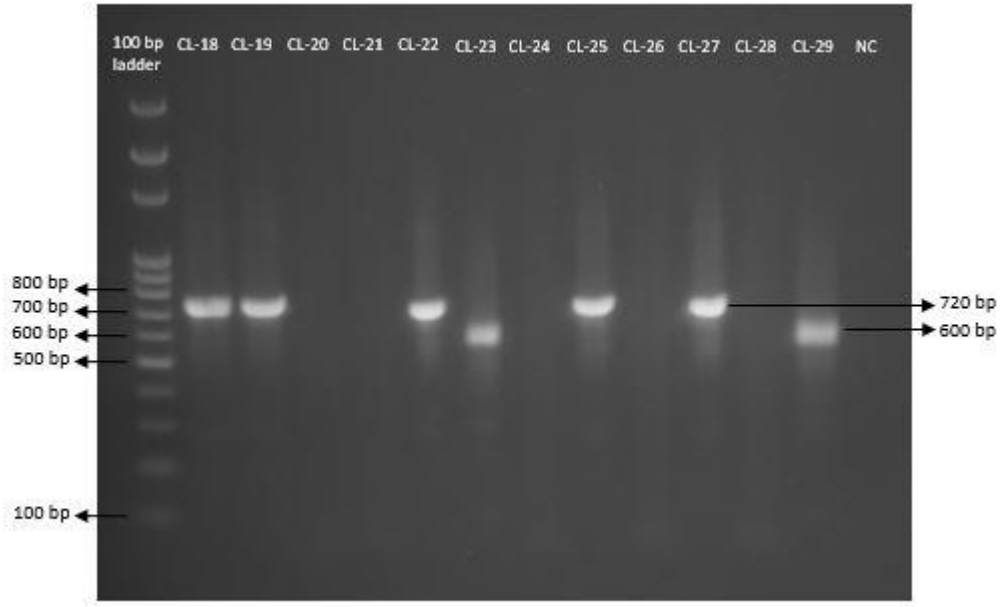


Figure 25: PCR 2 amplicon run for gel electrophoresis (c)

Lane1- 100 bp ladder, Lane 2,3,6,7,9 and 11 ~700bp lane 7and 13 ~ 600 bp Lane 14- Negative control (NC) Lane 4,5,8,10,12 Not visualized or not amplified.

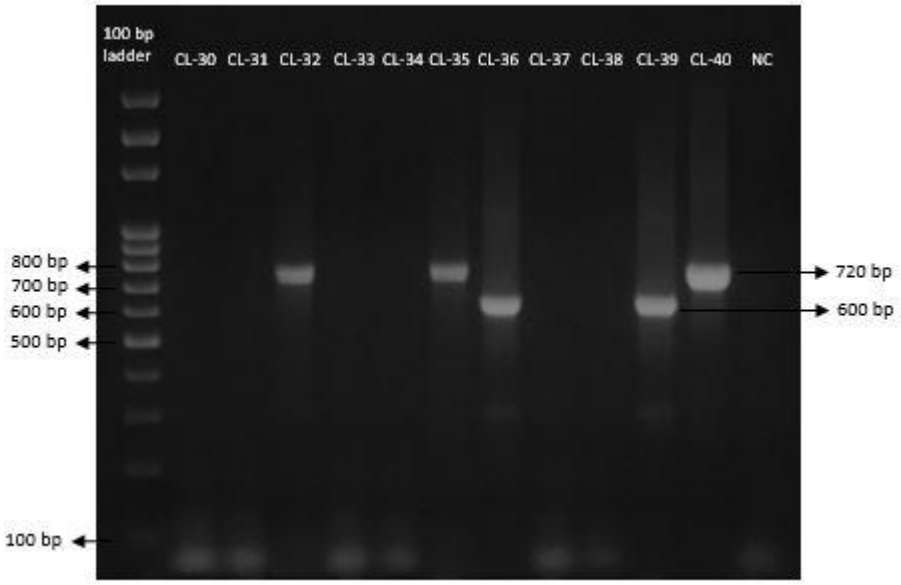


Figure 26: PCR 2 amplicon run for gel electrophoresis (d)

Lane1- 100 bp ladder, Lane 4,7 and 12 ~700bp lane 8 and 11 ~ 600 bp Lane 13- Negative control (NC) Lane 2,3,5,6,9 and 10 Not visualized or not amplified.

4.5.3 Comparison of diagnostic methods

Out of three diagnostic procedures used to validate the clinically confirmed cases, higher positivity results (n=12, 52.17 %) was found by nested PCR while it was 4.35% (n=1) by direct parasite examination in microscopy. And 4.35%, (n=1) by culture method. PCR positivity were found in all of the microscopy and culture positive cases, along with 11 additional cases which were negative by microscopy culture PCR. (Table 13).

Table 13: Results of the different diagnostic process

Microscopy	Culture	Nested PCR	Number (n=23)	Percentage	Diagnosis
-	-	+	10	43.47	Positive
+	-	+	1	4.35	Positive
-	+	+	1	4.35	Positive
-	-	-	11	47.83	Negative

4.6 Molecular Characterization

4.6.1 PCR of ITS-1 region

Among the samples tested positive for the kDNA were subjected to ITS-1 PCR amplification. Out of 22 samples tested positive for kDNA only 12 samples gave bands after agarose gel electrophoresis of ITS-1 region amplification. The bands size was of two types one is about 320 bp and another is around 340 bp.

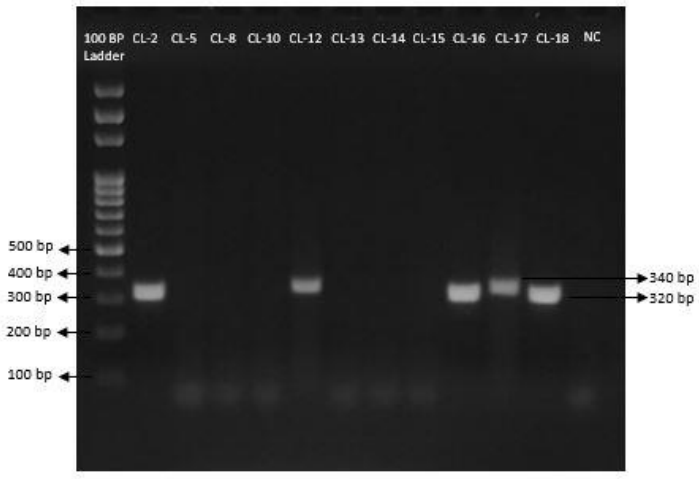


Figure 27: ITS-1 amplicon run for gel electrophoresis (a)

Lane1- 100 bp ladder, Lane 2,6,10,11,12 300-350bp, Lane 14- Negative control (NC)

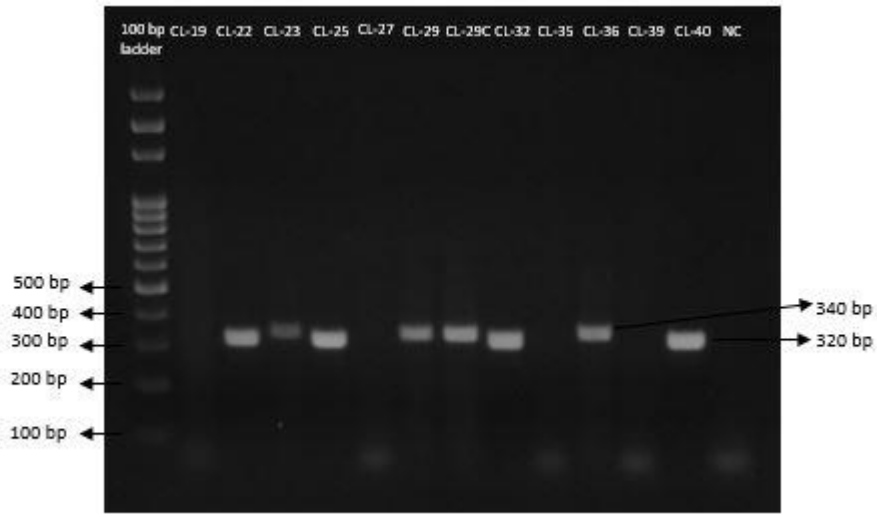


Figure 28: ITS-1 amplicon run for gel electrophoresis (b)

Lane1- 100 bp ladder, Lane 3,4,5,7,8,9,11,13 gives bands of around 300-350bp, lane 2,6,10 and 12 not visualized or not amplified, Lane 14- Negative control (NC)

4.6.2 Comparision of sensitivity of kDNA PCR and ITS-1 PCR

Out of 40 suspected cases, kDNA PCR gave 22 (55%) positive results while ITS-1 PCR gave only 12 (30%) positive result.

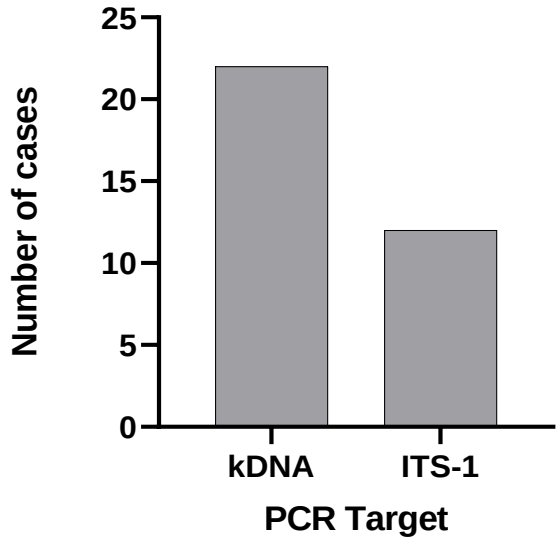


Figure 29: Graph showing the sensitivity of kDNA PCR and ITS-1 PCR

4.6.3 RFLP

After ITS-1 PCR, the PCR amplicon were subjected to restriction digestion using HaeIII enzyme. After gel electrophoresis two different pattern were observed, one is 210 bp, 140 bp another is 180 bp, 75bp, 50bp. The banding pattern 210bp, 140bp was seen in ITS-1 PCR product from five different samples. And another banding pattern 180 bp, 75bp, 50bp was seen in ITS-1 PCR product from seven different samples.

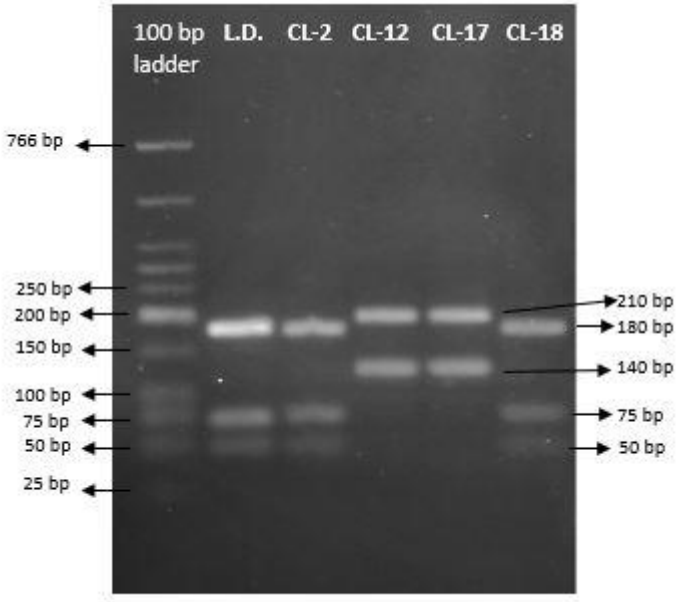


Figure 30: RFLP of ITS-1 region (a)

Lane-1 25bp ladder, Lane-2, 3 and 5 (50, 75, 180 bp), Lane 4 and 5 (140, 210bp).

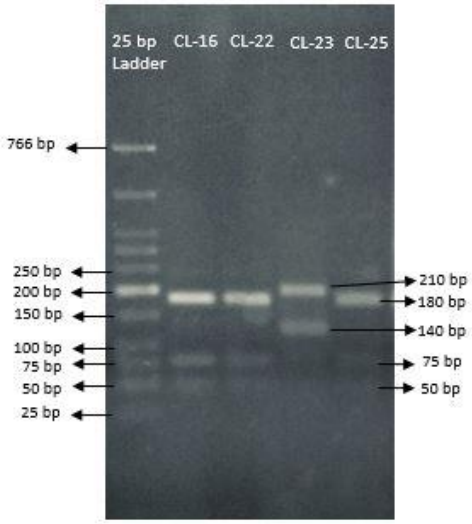


Figure 31: RFLP of ITS-1 region (b)

Lane-1 25bp ladder, Lane-2, 3 and 5 (50, 75, 180 bp), Lane 4 (140, 210bp).

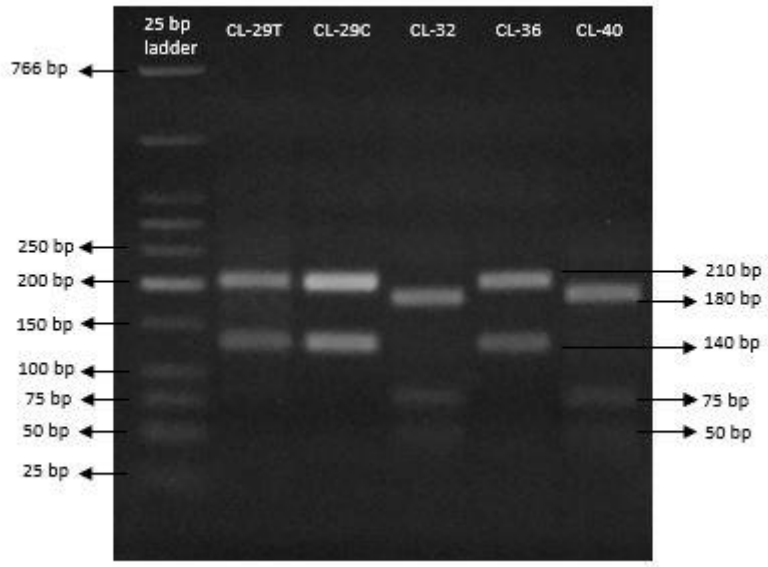


Figure 32: RFLP of ITS-1 region (c)

Lane-1 25bp ladder, Lane-2, 3 and 5(140, 210bp), Lane-4 and 6 (50, 75, 180 bp)

4.6.4 Sequencing and sequence analysis

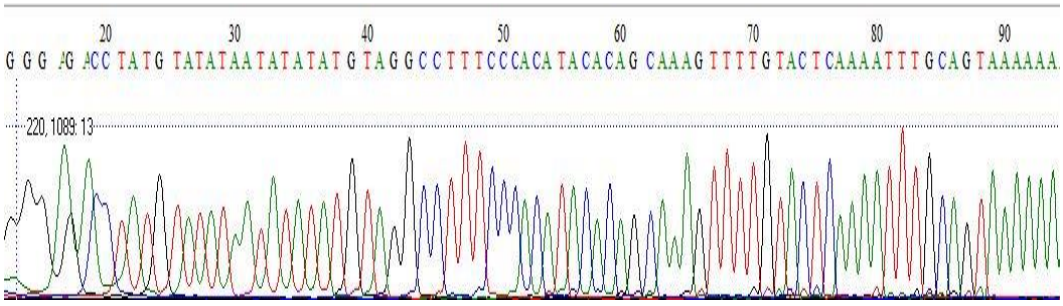


Figure 33: Chromatogram of the Sequence

The sequence of ITS-1 region of 7 tissue scraping and one from the culture was obtained as below. Among them, the size of the sequence from 4 samples including the Culture (CL-17, CL-23, CL-29 and CL-29C) was 337 base pair while rest of the 4 samples (CL-18, CL-22, CL-25 and CL-4) from the tissue scraping showed 321 base pairs. NCBI BLAST analysis revealed 99.12% and 99.47% homology with corresponding L. major and L. donovani sequence in the database (accession MT023531.1 and MW053328.1 respectively). The tool used for sequence alignment was CLUSTALW and the phylogenetic analysis was done by MEGA7 software. The analysis shows that the 3 samples (CL-17, CL-23 and CL-29) along with the cultured one (CL-29C), has close relation or lies with the *L. major* while remaining 4 samples (CL-18, CL-22, CL-25 and CL-4) has close association with *L. donovani*.

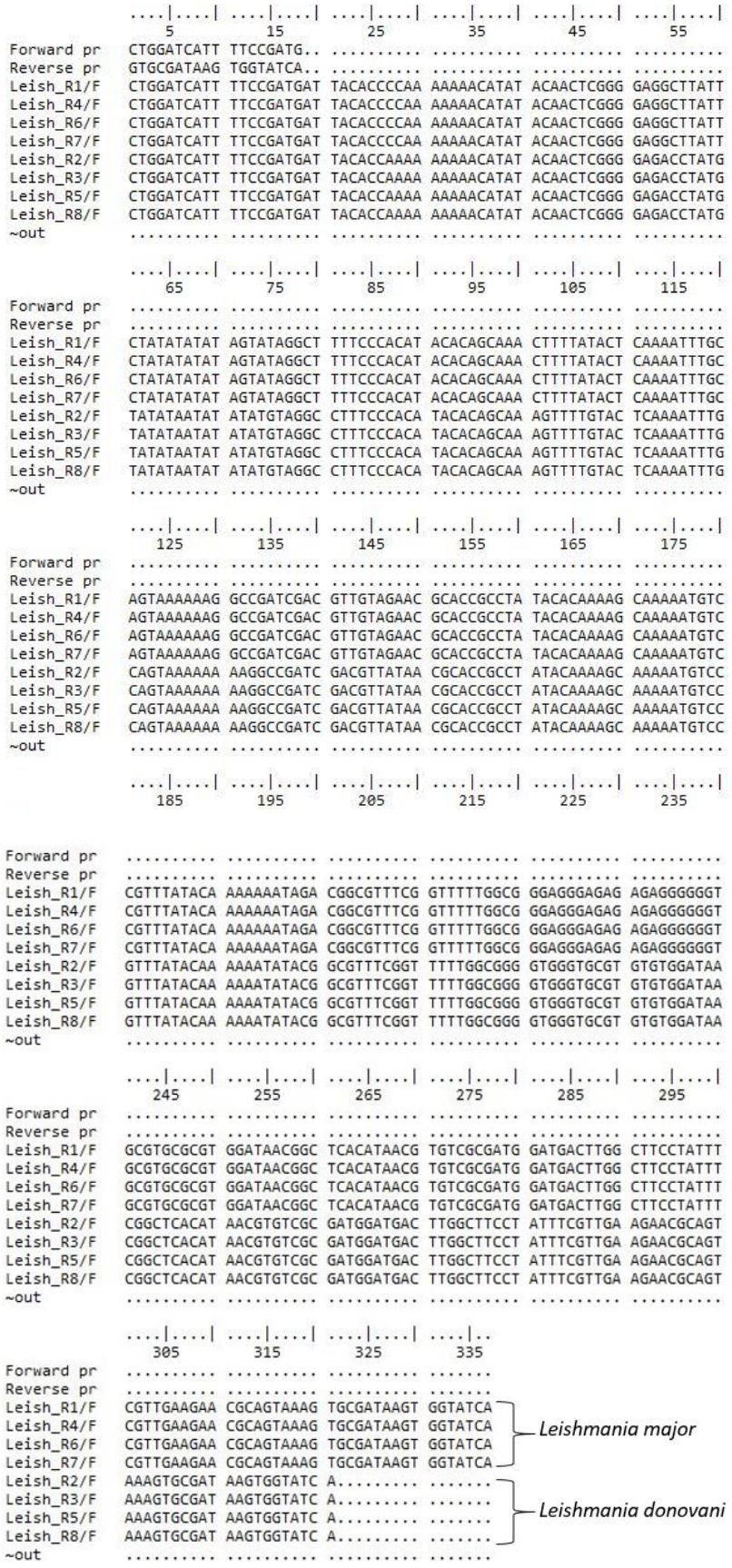


Figure 34: Sequence of ITS-1 region. (Leish_R1/F: CL 17, Leish_R4/F: CL 23, Leish_R6/F: CL 29 C, Leish_R7/F: CL 29 T, Leish_R2/F: CL 18, Leish_R3/F: CL 22, Leish_R5/F: CL 25, Leish_R8/F: CL 40)

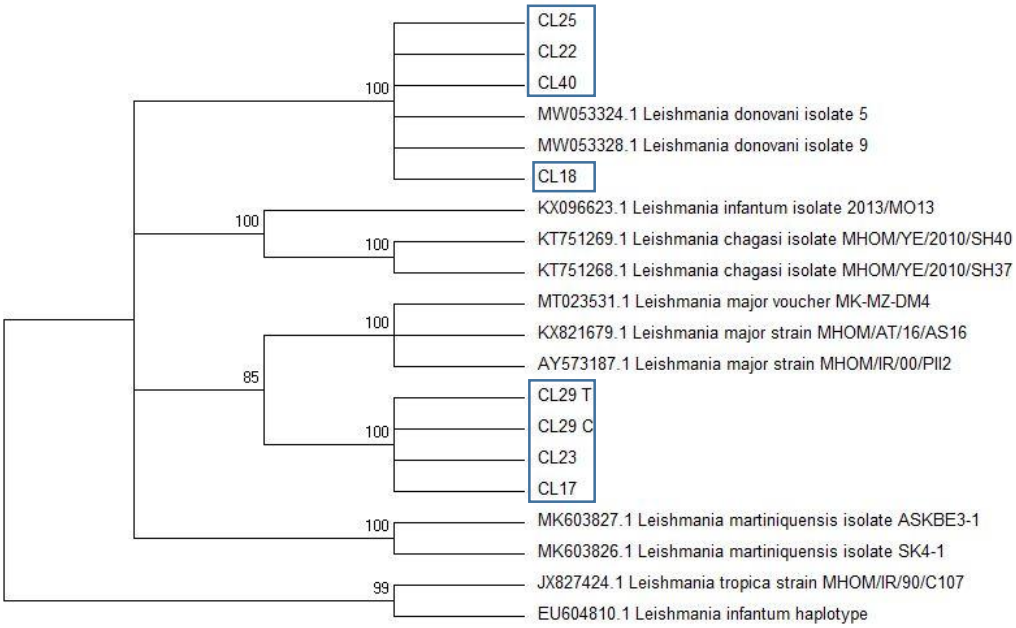


Figure 35: Phylogenetic tree of *Leishmania* species using MEGA7 using ClustalW for sequence alignment against NCBI Database. All the sequences in the tree are labelled as accession no with the isolate names. The label CL-17, CL-18, CL-22, CL-23, CL-25, CL-29C, CL29T and CL-40 are the isolates of the year 2018 sept to 2019 Dec.

Table 14: Causal *Leishmania* Species

SN	Samples	Band size kDNA PCR	ITS-1 PCR	RFLP Pattern	Species Identified
1	CL2	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani*
2	CL5	~600 bp	-		
3	CL8	~600 bp	-		
4	CL10	~720 bp	-		
5	CL12	~600 bp	~340 bp	210 bp, 180 bp	*Leishmania major*
6	CL13	~720 bp	-		
7	CL14	~720 bp	-		
8	CL15	~720 bp	-		
9	CL16	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani*
10	CL17	~600 bp	~340 bp	210 bp, 180 bp	*Leishmania major**

SN	Samples	Band size		RFLP Pattern	Species Identified
		kDNA PCR	ITS-1 PCR		
11	CL18	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani**
12	CL19	~720 bp	-		
13	CL22	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani**
14	CL23	~600 bp	~340 bp	210 bp, 180 bp	*Leishmania major**
15	CL25	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani**
16	CL27	~720 bp	-		
17	CL29	~600 bp	~340 bp	210 bp, 180 bp	*Leishmania major**
18	CL32	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani*
19	CL35	~720 bp	-		
20	CL36	~600 bp	~340 bp	210 bp, 180 bp	*Leishmania major*
21	CL39	~600 bp	-		
22	CL40	~720 bp	~320 bp	180 bp, 75 bp, 50 bp	*Leishmania donovani*

The banding pattern of the digests were correlated with the literature.(Kumar et al., 2007)

* In the above table indicates the species were validated by the result of sequencing.

CHAPTER 5

DISCUSSION

Diagnosis and characterization of *Leishmania* species in clinical infections in endemic areas are important for both clinical and epidemiologic reasons because of similar symptoms caused by other dermal manifestations (such as sarcoidosis and lupus vulgaris) and overlapping clinical presentation caused by different *Leishmania* species. Most commonly used methods for the direct detection of the parasite (e.g., microscopic examination of Giemsa-stained smears and in vitro cultivation) lack sensitivity because of the paucity of *Leishmania* parasites in some specimens or are hampered by the problem of contamination (Marfurt et al., 2003). Indirect methods (e.g., detection of anti-*Leishmania* antigen antibodies by serological methods) are also limited in sensitivity and are not able to discriminate between past and current infections (Salotra et al., 2003). Most of these assays are based on highly repetitive genomic gene loci or extrachromosomal kinetoplast DNA sequences, but their use is confined to the detection of the parasite at genus or complex level, and therefore, their application is limited to restricted geographical areas and diagnostic settings.

The clinical presentation of the lesion among the patients vary. Some are dry, scaly, nodulated whereas some are moist with invagination of the wound. Among them 17 (73.9%) lesions are dry type while 6 (26.1 %) are moist with central invaginations. Among the dry type 9 (39.13%) lesions have scales or crusted, 4 (17.39%) have bulged appearance or nodulated and remaining four (17.39%) have swollen erythematic appearance. Most of the lesions 13 (56.52%) were localized while 10 (43.47%) were dispersed lesions. Similarly, most of the lesions 10 (43.48%) have single lesion while 13 (56.52%) have multiple lesions. The lesions were in the exposed part of the body like face, hand, leg and neck. The highest frequency 11 (47.83%) was in the face, followed by hand 4 (17.39%), Neck 1 (4.3%), leg (4.3%), abdominal part 3 (13.04%) other having multiple lesion on these parts in the same patient includes 3 (13.04%). The result was found similar with the other study done by Yanik et al, CL lesions were on the exposed body parts such as face and hands (Yanik et al., 2004). Simillarly involvement of upper limbs were common in the studies conducted by other authors (Galwalama et al., 2017; Samaraj et al., 2009).

In this study, the suspected cases of cutaneous Leishmaniasis were found in 17 districts including all the seven province of Nepal. Among which the highest number of cases were found from the Province 7 whereas Bajura is the district having highest number of suspected cases and lowest number of cases were found from Province 2 and 5. Among the suspects 12 cases were tested positive .for CL. The highest number of positive CL cases

were found in Province 7 having four cases. And most of the suspected cases were from hilly region.

Cutaneous leishmaniasis cases were found in 10 districts during the period of this study The highest number of PCR positive CL cases were found in Province 7 having four cases, the two districts of province 7 i.e. Bajura and Baitadi in which Bajura has three cases and Baitadi has one positive case. Where two districts Gorkha, Baglung, of the province 4 and Kalikot, Jajarkot from province 6 are prone to CL. Among the PCR Positive cases most of them were from Hilly region. Most of the patients hadn't travelled outside Nepal but some had travelled terai regions of Nepal.

Most of the cases were from hilly region of the country, it may be due to the climate change which has the direct effect in the rise in temperature worldwide. The temperature of the hilly regions might be effective for the survival of the vector than that of the terai regions so the vectors might shift the habitat to the suitable environment that results in the distribution of vectors in the hilly regions.

In our study, the male population was found to be higher 16 (82.6%) than the female population (30.4%). The ratio of male:female was found to be 16:7. In Nepal, males are mostly involved on outdoor works and females are involved in the household chores. This may have predisposed the males to the bite of sandfly (Ghimire et al., 2018).

In this study, patients ranging from 9 to 66 years involved. The highest frequency 9 (39.13%) was within the age group of 41-60. Gurel et al found higher incidence (70%) found in patients of 5-9 years age group. Simillary, Higher incidence (27%) of Cutaneous Leishmaniasis was found in the age group 10-19 (Yemisen et al., 2012). However, Sharma et al., 2005 and Ghimire et al., 2018 found highest incidence from age group 21-40. Similarly Gunathilaka et al found the highest incidence (95%) from age group 26-35 years (Gunathilaka et al., 2020). The lowest frequency in this study was found in the Age group 61-80 with one case. The decreased number of patient with the increased age may be due to the Acquired immunity (Ghimire et al., 2018).

The gender based disribution of the positive cases in male were 69.23% (n=12) and those in female were 48.14% (n=8). The suspection of the disease cases were higher in case in male as well the Confirmed positive case for CL in Male population was higher. The difference in disease condition between males and females could be due to male-biased healthcare access (Ryan D. Lockard et al., 2019). It has been unclear whether this reflects a gender-related difference in the host response to the parasite or merely different intensities of exposure among men and women (Travi et al., 2002).

The study subjects were divided based on the age gap of 20 years for evaluation. The data showed that highest (n=6) fell into age group (41-60) years with (39.13) % positive cases

and the lowest (n=1) number fell into age group (61-80) yrs and (21-40), age group (<20) had four positive cases which is second highest infected age group. High prevalence of CL in Middle aged males it might be due to the practice that most of the males in this age group visits outside country for the labour in our study 3 males were infected in the CL endemic country before arriving Nepal. Childrens are generally considered to signal domestic transmission, which has expanded into the household settings of rural communities and periphery of urban centers. (Blanco et al., 2019).

Among 40 suspected samples 6 samples have traveled abroad. Among six patients 5 cases were diagnosed positive for the CL. out of 22 positive cases 5 were imported cases and 17 were indigenous to Nepal. The Banding pattern of the KDNA PCR were 700 bp and 600bp similar to the banding pattern of endogenous patients. Among 5 imported cases one sample CL36 was included in restriction digestion and the banding pattern was similar to the *L major* 210 bp, 180 bp.

From all the suspected patient touch skin smear or slit skin smear was prepared, and giemsa staining was performed. On microscopy the L.D bodies was unable to observe in all slides. It might be due to the low count of parasite during smear making.
Further, Cultivation of parasite among 40 samples, culture was successful once. Most of them were unsuccessful due to the bacterial and fungal contamination. Culture was done in NNN media prepared in 98 well strips to ensure the parasite load sufficient for the multiplication in less surface area. It was also done in M199 media directly from the samples. From NNN media the parasites were sub-cultured in M199 media. It was subjected to giemsa staining and DNA extraction. The patient was from Okhaldhunga District and no any travel history inside the country and outside the country as well. Further he was not subjected to any medications before. The factors that the culture was unsuccessful on other cases were might be patients were using some antifungal topical creams on the lesions, low parasite count on the aspirate, inappropriate conditions during the transport of samples from the collection site to the laboratory, further contamination was the mostly observed issue on the culture of the parasite. It is known that the positivity of parasitological diagnostic methods is directly associated with the duration of the presence of the lesion and parasitic load in the aspirate from the lesion (Ampuero et al., 2009). The sensitivity of the cultural diagnosis is increased by the microculture method than the conventional method (Boggild et al., 2007). Most of the culture in this study was done in 5 ml culture tubes, and modifying culture technique by microculture one of the culture was succeded.

This study was focused on diagnosis and species identification in an enough number of suspected CL patients in Nepal and it was confirmed that CL infections are caused by *L. major* and *L. donovani*. The Diagnosis of CL in clinical setting is based on Fine Needle

Aspiration Cytology (FNAC) (Paudel et al., 2020) (Abhimanyu & Dipendra, 2013). The kDNA PCR was the most sensitive diagnostic assay and was established as a valuable tool in the diagnosis (Noyes et al., 1998). kDNA PCR can be used as new diagnostic techniques in clinical settings for patients suspected of CL with negative microscopic examination and/or culture results, as former has more sensitivity than the later (Pezeshkpoor et al., 2013).

The diagnosis was done by kDNA Nested PCR, on which two sets of primer were used. The first set includes Forward Primer (CSB2XF), Reverse Primer (CSB1XR) which targets the conserved region of Kinetoplast minicircle DNA and amplifies the variable region of length of about 800 base pairs. The second round PCR was performed by second set of primer 13Z and LiR which targets the conserved region within the first round PCR product length. And amplifies about 700 base pairs. In this study, out of 40 suspected samples only 22(55%) were tested positive by kDNA Nested PCR.

In Nepal, extensive study about CL has not been done yet. The causal species *Leishmania donovani* complex has been reported in a recent case study of CL in Nepal (Bastola et al., 2020). The presence of multiple species of *Leishmania* in a region, with overlapping clinical pictures, demands the development of sensitive laboratory tests with simultaneous species identification.

The ITS1 PCR-RFLP assay is recommended for reliable characterization of *Leishmania* species. Species identification is a vital part of the diagnostic procedure, especially in areas where more than one species of *Leishmania* occurs (Monroy-ostria et al., 2014). In this study, 22 (55%) out of 40 samples were tested positive on kDNA PCR while, 12 (30%) on ITS-1 PCR as reported that the sensitivity of kDNA is higher than that of ITS-1 PCR (Bensoussan et al., 2006).

Molecular characterization of *Leishmania* species in clinical isolates of patients with CL was carried out by ITS1 PCR-RFLP analysis. There are reports which revealed an 0.9- to 1.2-kb non-coding spacer region present between Ssu and Lsu rRNA lying between the genes coding for 18S and 5.8S rRNA, which are highly repetitive variable regions that distinguish old world and new world *Leishmania* species by amplifying the internal spacer region and digesting with specific restriction enzymes such as HaeIII (Cupolillo et al., 1995). To appraise ITS1 PCR-RFLP genotyping, the amplicon was subjected to restricted digestion with HaeIII, which produced two different patterns that unambiguously differentiated *L. donovani* and *L. major*. The ITS1 PCR products amplified were subjected to sequencing before digestion, and confirmed the finding of RFLP. The finding that *L. donovani*, one of the causative agent of CL in Nepal shows similar in some parts of India and srilanka (Sharma et al., 2005), (Nawaratna et al., 2007). It is possible that new genetic strains of *L donovani* evolved due to mutation which causes the differences in the

pathogenesis and clinical outcomes from Visceral Leishmaniasis. Occasional reports on atypical cutaneous leishmaniasis reported in literature, indicate that more than one strain of *L. donovani* is likely to cause cutaneous disease. A study in srilanka affirms that there is sub-genetic diversity in *L. donovani* in the Indian subcontinent which are known to result in different clinical outcomes (Kariyawasam et al., 2017).

Molecular test from the cultured parasite was done by kDNA PCR as well as ITS-PCR-RFLP. The PCR product of ITS-1 region was sequenced along with the PCR product from the tissue scrapings. The sequence shows similarity between the cultured parasite and the tissue isolated parasitic DNA. It confirmed that the cultured parasite was same from the tissue scraping i.e. the origin of the parasite was not from other samples or any cross-contamination. The result of sequence BLAST and Phylogenetic analysis revealed that the cultured parasite was *L. major*.

Out of three diagnostic procedures used to validate the clinically confirmed cases, higher positivity results n=10, 47.47 %) was found by only nested PCR while it was 4.35% (n=1) by direct parasite examination in microscopy and nested PCR. And 4.35%, (n=1) by culture method and nested PCR. PCR positivity were found in all of the microscopy and culture positive cases, along with 11 additional cases which were negative by microscopy culture PCR.

The kDNA-PCR method detected *Leishmania* DNA in 87.5% (112/128) of the clinically suspected CL patients, while the traditional methods demonstrated the following percentages of positivity: 62.8% (49/78) for the Montenegro skin test, 61.8% (47/76) for direct investigation, and 19.3% (22/114) for in vitro culture (Satow et al., 2013).

CHAPTER 6

SUMMARY

Leishmaniasis is a vector-borne disease, caused by obligate intracellular parasite belongs to the order of Kinetoplastida, genus *Leishmania.* Sandflies of the species *Lutzomyia* serve as the vector in the New World, while the *Phlebotomus* species transmit infection in the Old World (Markle, 2004). Leishmaniasis is a severe health problem, endemic tropical and subtropical regions of 98 countries in Africa, Asia, Europe, and the Americas and 350 million people are at risk (Mcgwire & Satoskar, 2014). In the context of Nepal, the terai region is endemic to the Visceral Leishmaniasis and Cutaneous leishmaniasis is in the increasing trend (Ghimire et al., 2018). In this study, the Causative species of the *Leishmania* was identified. This also focused for the study if the same species was responsible for the visceral form and cutaneous form of the disease.

The kDNA PCR was the most sensitive diagnostic assay and was established as a valuable tool in the diagnosis (Noyes et al., 1998). The ITS1 PCR-RFLP assay is recommended for reliable characterization of *Leishmania* species (Monroy-ostria et al., 2014). Molecular characterization of *Leishmania* species in clinical isolates of patients with CL was carried out by ITS1 PCR-RFLP analysis and Sequencing. Statistical analysis was done using Graph Pad prism.

In this study, out of 40 suspected samples only 22(55%) were tested positive by kDNA Nested PCR. Most of the suspects of CL and Cases of CL were from the hilly regions of the country. Province no. 7 had the highest number of infection (n=4) followed by Province no. 1. (n=3). Male have the higher rate of infection than that of Female. Age group (41-60) years are highly affected, among which 3 cases were imported cases from the CL endemic country. The lesions were on the exposed parts, mostly affected part was face. Culture of one sample was succeeded and cryopreserved for the future use. PCR while, 12 (30%) on ITS-1 PCR as reported that the sensitivity of kDNA is higher than that of ITS-1 PCR (Bensoussan et al., 2006). ITS1 PCR-RFLP genotyping, the amplicon was subjected to restricted digestion with HaeIII, which produced two different patterns that unambiguously differentiated *L. donovani* and *L. major*. Further, sequencing of the amplified ITS1 PCR products validated the RFLP result. The discovery that *L. donovani*, a causal agent of CL in Nepal.

CHAPTER 7

CONCLUSION

Cutaneuos Leishmaniasis is imported, undervalued, emerging tropical disease of Nepal which is in increasing number. In this study 23 clinically suspected CL Cases were found within a year. Among which 12 are diagnosed that they have Cutaneous Leishmaniasis. This Study shows highest (n=6) number of infected persons fell into age group 41-60 years. Also, the disease was more common in males who are mostly doing outdoor works in comparison to females doing household works.

As the disease is recently occurring in Nepal it seems that many of the health workers are unknown about this disease and had misdiagnosed as the Lupus, Cutaneous Tuberculosis and Basal cell carcinoma. Similarly, most of the people are unaware about this disease and gone through the psychological trauma due to the non-healing ulcers as the disease if untreated with respective medications results in disfiguring, life-long scars, irreversible damage to the affected body parts. So, concerned stakeholders and organization need to bring a special awareness program and efforts should be made to make PCR platforms more user-friendly and cost-effective, especially in remote areas where leishmaniasis is endemic.

As known Nepal was an endemic for Visceral Leishmaniasis and its cutaneous form is emerging rapidly in the Hilly regions of the country. This study finds out that the species *L. donovani* which causes the visceral form of the disease is now causing the cutaneous form too. And the species of *Leishmania* circulating in Nepal which are responsible for the cutaneous Leishmaniasis is found to be *L. major* and the *L. donovani*.

LIMITATIONS OF STUDY

- The work is carried out only in limited number of samples who is visiting STIDH and Bir hospital.
- Due to the prior use of antifungal agent culture of all the samples are not succeeded.
- Due to Low quantity of samples the Immunological status of the lesion cannot be studied.
- Due to lack of resources issues not all the PCR positive samples were sequenced.
- Not all the possible Circulating Species were characterized by this study.

RECOMMENDATION

This research work supports the increasing trend of Cutaneous Leishmaniasis in Nepal and revealed the species causing Visceral Leishmaniasis is also equally causing cutaneous form of the disease. Once the country endemic for Visceral Leishmaniasis could be the Endemic for the Cutaneous Leishmaniasis if the disease spread is continued and Vector control programs is not performed. So, we should step forward to

- Screen Cases of the CL in every district should be done to minimize risk of Endemic situation of the country.
- Conduct public awareness programs in the CL reported Regions.
- Bring the vector control Programs effectively.
- Facilitate the diagnostic tools like PCR to the representative health centres.
- Extensive Research programs need to be conducted on the CL disease.

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APPENDICES

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

a. Acceptance Letter for Sample collection by STIDH.

Government of Nepal
Ministry of Health & Population
Department of Health Services
Sukraraj Tropical & Infectious Disease Hospital
Development Board
Teku, Kathmandu, Nepal
Tel.: 4253395
4253396

Date: 15th March 2019

To,

The Member Secretary

Nepal Health Research Council

Kathmandu, Nepal

Dear Sir/Madam,

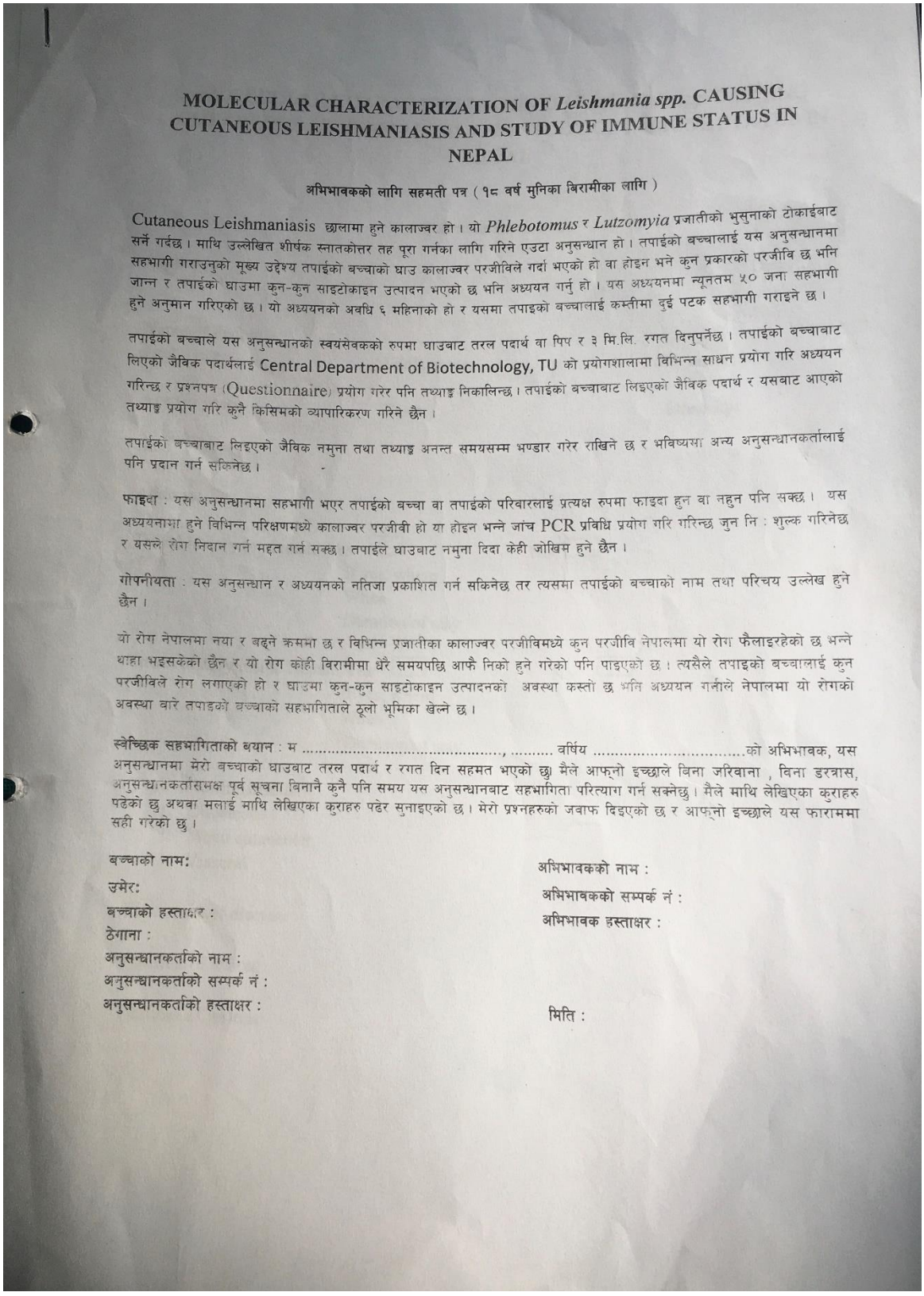
I along with Sukra Raj Tropical and infectious disease hospital, teku, Kathmandu, Nepal feel privileged to be one of the cooperating hands in connection with research proposed entitled "**Molecular characterization of leishmania spp. causing cutaneous leishmaniasis and study of immune status in Nepal**" by Ms. Tinmaya Rai, Central Department of Biotechnology, Tribhuvan University, Kathmandu, Nepal.

In reference to the research, blood sample and biopsy/needling from the lesions of the cutaneous leishmaniasis suspected patient coming in this hospital will be collected under my supervision with all required cautions. I will make sure that the written informed consent form will be read/detailed to the patient and signature will be taken from the patients who wish to give blood and biopsy sample for the proposed research.

Thanks

Dr. Anup Bastola, MD, MCTM
Head Academic Unit
Sukraraj Tropical and Infectious Disease Hospital
Teku, Kathmandu, Nepal.

b. Assent form



MOLECULAR CHARACTERIZATION OF *Leishmania spp.* CAUSING CUTANEOUS LEISHMANIASIS AND STUDY OF IMMUNE STATUS IN NEPAL

अभिभावकको लागि सहमती पत्र (१८ वर्ष मुनिका बिरामीका लागि)

Cutaneous Leishmaniasis छालामा हुने कालाज्वर हो । यो *Phlebotomus* र *Lutzomyia* प्रजातीको भुसुनाको टोकाईबाट सर्ने गर्दछ । माथि उल्लेखित शीर्षक स्नातकोत्तर तह पूरा गर्नका लागि गरिने एउटा अनुसन्धान हो । तपाईको बच्चालाई यस अनुसन्धानमा सहभागी गराउनुको मूख्य उद्देश्य तपाईको बच्चाको घाउ कालाज्वर परजीविले गर्दा भएको हो वा होइन भने कुन प्रकारको परजीवि छ भनि जान्न र तपाईको घाउमा कुन-कुन साइटोकाइन उत्पादन भएको छ भनि अध्ययन गर्नु हो । यस अध्ययनमा न्यूनतम ५० जना सहभागी हुने अनुमान गरिएको छ । यो अध्ययनको अवधि ६ महिनाको हो र यसमा तपाइको बच्चालाई कम्तीमा दुई पटक सहभागी गराइने छ ।

तपाईको बच्चाले यस अनुसन्धानको स्वयंसेवकको रुपमा घाउबाट तरल पदार्थ वा पिप र ३ मि.लि. रगत दिनुपर्नेछ । तपाईको बच्चाबाट लिएको जैविक पदार्थलाई Central Department of Biotechnology, TU को प्रयोगशालामा विभिन्न साधन प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र (Questionnaire) प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ । तपाईको बच्चाबाट लिइएको जैविक पदार्थ र यसबाट आएको तथ्याङ्क प्रयोग गरि कुनै किसिमको व्यापारिकरण गरिने छैन ।

तपाईको बच्चाबाट लिइएको जैविक नमुना तथा तथ्याङ्क अनन्त समयसम्म भण्डार गरेर राखिने छ र भविष्यमा अन्य अनुसन्धानकर्तालाई पनि प्रदान गर्न सकिनेछ ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाईको बच्चा वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनमा हुने विभिन्न परिक्षणमध्ये कालाज्वर परजीवी हो या होइन भन्ने जांच PCR प्रविधि प्रयोग गरि गरिन्छ जुन नि : शुल्क गरिनेछ र यसले रोग निदान गर्न महत गर्न सक्छ । तपाईले घाउबाट नमुना दिदा केही जोखिम हुने छैन ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको बच्चाको नाम तथा परिचय उल्लेख हुने छैन ।

यो रोग नेपालमा नया र बढ्ने क्रममा छ र विभिन्न प्रजातीका कालाज्वर परजीविमध्ये कुन परजीवि नेपालमा यो रोग फैलाइरहेको छ भन्ने थाहा भइसकेको छैन र यो रोग कोही बिरामीमा धेरै समयपछि आफै निको हुने गरेको पनि पाइएको छ । त्यसैले तपाइको बच्चालाई कुन परजीविले रोग लगाएको हो र घाउमा कुन-कुन साइटोकाइन उत्पादनको अवस्था कस्तो छ भनि अध्ययन गर्नाले नेपालमा यो रोगको अवस्था बारे तपाइको बच्चाको सहभागिताले ठूलो भूमिका खेल्ने छ ।

स्वेच्छिक सहभागिताको बयान : म ....., ... वर्षिय .....को अभिभावक, यस अनुसन्धानमा मेरो बच्चाको घाउबाट तरल पदार्थ र रगत दिन सहमत भएको छु। मैले आफ्नो इच्छाले बिना जरिवाना , बिना डरत्रास, अनुसन्धानकर्तासमक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

बच्चाको नाम:
उमेर:
बच्चाको हस्ताक्षर :
ठेगाना :
अनुसन्धानकर्ताको नाम :
अनुसन्धानकर्ताको सम्पर्क नं :
अनुसन्धानकर्ताको हस्ताक्षर :

अभिभावकको नाम :
अभिभावकको सम्पर्क नं :
अभिभावक हस्ताक्षर :

मिति :

c. Consent form

MOLECULAR CHARACTERIZATION OF *Leishmania spp.* CAUSING CUTANEOUS LEISHMANIASIS AND STUDY OF IMMUNE STATUS

सम्बन्धि अध्ययनमा सहभागी मञ्जुरीनामा फाराम

Cutaneous Leishmaniasis छालामा हुने कालाज्वर हो । यो *Phlebotomus* र *Lutzomyia* प्रजातीको भुसुनाको टोकाईबाट सर्ने गर्दछ । माथि उल्लेखित शीर्षक स्नातकोत्तर तह पूरा गर्नका लागि गरिने एउटा अनुसन्धान हो । तपाईलाई यस अनुसन्धानमा सहभागी गराउनुको मूख्य उद्देश्य तपाईको घाउ कालाज्वर परजीविले गर्दा भएको हो वा होइन भने कुन प्रकारको परजीवि छ भनि जान्न र तपाईको घाउमा कुन-कुन साइटोकाइन उत्पादन भएको छ भनि अध्ययन गर्नु हो । यस अध्ययनमा न्यूनतम ५० जना सहभागी हुने अनुमान गरिएको छ । यो अध्ययनको अवधि ६ महिनाको हो र यसमा तपाइलाई कम्तीमा दुई पटक सहभागी गराइने छ ।

तपाई यस अनुसन्धानको स्वयंसेवकको रुपमा आफ्नो घाउबाट तरल पदार्थ वा पिप र ५ मि.लि. रगत दिनुपर्नेछ । तपाइबाट लिइएको जैविक पदार्थलाई Central Department of Biotechnology, TU को प्रयोगशालामा विभिन्न साधन प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र (Questionnaire) प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ । तपाईबाट लिइएको जैविक पदार्थ र यसबाट आएको तथ्याङ्क प्रयोग गरि कुनै किसिमको व्यापारिकरण गरिने छैन ।

तपाईबाट लिइएको जैविक नमुना तथा तथ्याङ्क अनन्त समयसम्म भण्डार गरेर राखिनेछ र भविष्यमा अन्य अनुसन्धानकर्तालाई पनि प्रदान गर्न सकिनेछ ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ । यस अध्ययनमा हुने विभिन्न परिक्षणमध्ये कालाज्वर परजीवी हो या होइन भन्ने जांच PCR प्रविधि प्रयोग गरि गरिन्छ जुन नि : शुल्क गरिनेछ र यसले रोग निदान गर्न मद्दत गर्न सक्छ । तपाईले घाउबाट नमुना दिदा केही जोखिम हुने छैन ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन ।

यो रोग नेपालमा नयां तर बढ्ने क्रममा छ र विभिन्न प्रजातीका कालाज्वर परजीविमध्ये कुन परजीवि नेपालमा यो रोग फैलाइरहेको छ भन्ने थाहा भइसकेको छैन र यो रोग कोही विरामीमा धेरै समयपछि आफै निको हुने गरेको पनि पाइएको छ । त्यसैले तपाइलाई कुन परजीविले रोग लगाएको हो र घाउमा कुन-कुन साइटोकाइन उत्पादन अवस्था कस्तो छ भनि अध्ययन गर्नले नेपालमा यो रोगको अवस्था थाहा पाउन तपाइको सहभागिले ठूलो भूमिका खेल्ने छ ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो । मैल आफ्नो इच्छाले बिना जरिवाना , बिना डरत्रास, अनुसन्धानकर्तासमक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु । मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ । मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु ।

सहभागीको हस्ताक्षर :

अथवा सहभागीको हकमा अनुमती प्रदान गर्ने व्यक्तिको हस्ताक्षर)

अन्य व्यक्तिको हस्ताक्षर भए नाम र सम्बन्ध खुलाउनु होस् :

सहभागिको नाम :

ठेगाना :

सम्पर्क नं :

अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर :

मिति :

d. Questionaire

MOLECULAR CHARACTERIZATION OF *Leishmania spp* CAUSING CUTANEOUS LEISHMANIASIS AND STUDY OF IMMUNE STATUS IN NEPAL

Questionnaire format

Code no.: Date:

Name of Patient:
First name: Middle name: Last name:

Age (in years Caste: Ethnicity:

Sex:

Address:

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

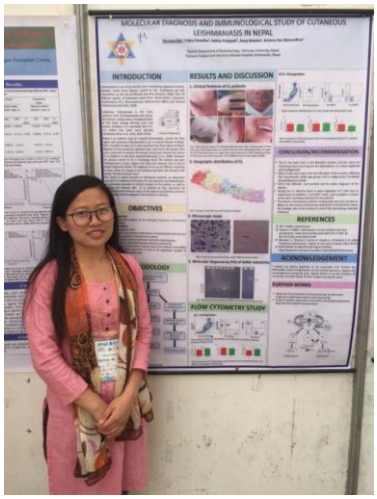
Type of leishmaniasis:
1. Cutaneous
2. Muco cutaneous
3. Visceral

First onset of disease:

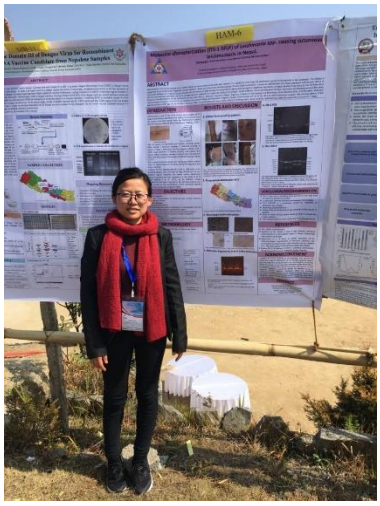
Position of lesion:

Number of lesions:

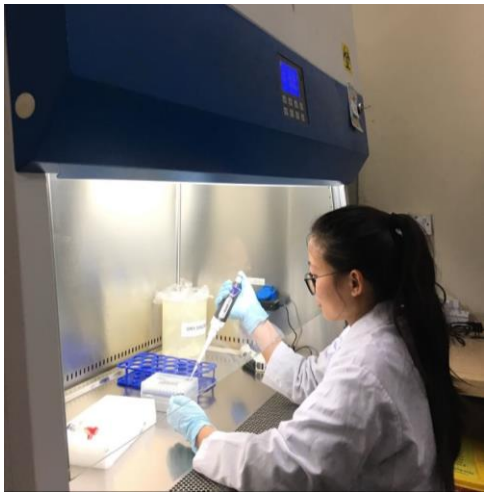
e. Photographs



Presenting poster on DNA day 2019.



Presenting poster on Southeast Asian Regional Symposium on Microbial Ecology (SARSME)-2020



Performing Parasite culture in NNN tubes Taking Nanodrop reading


During winter school 2020


Some memories with lab team

Memory with classmates and Teachers