UNDERSTANDING HOST-PARASITE COMPONENTS IN A PROTOZOAN DISEASE – LEISHMANIASIS

WITH SPECIFIC EMPHASIS ON MOLECULAR CHARACTERIZATION OF PARASITE SPECIES

Enrolment no - 111565

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May-2015

Project Report submitted in partial fulfilment of the requirement for the degree of

Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

Jaypee University of Information and Technology

Waknaghat

Certificate

This is to certify that project report entitled **"Understanding host-parasite components in a protozoan disease – Leishmaniasis with specific emphasis on molecular characterization of parasite species"**, submitted by **"Parul Sharma**" in partial fulfillment for the award of degree of **Bachelor of Technology** in Biotechnology to department of Bioinformatics and Biotechnology Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

Supervisor's Name – Dr. Manju Jain

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Acknowledgement

I would like to express my gratitude to my supervisor **Dr. Manju Jain** for the useful comments, remarks and engagement through the learning process in this project. Furthermore I would like to thank **Lovlesh Thakur** and other lab members for helping me in my endeavours and introducing me to the topic as well for the support on the way. I would also like to convey thanks to the department of Bioinformatics and Biotechology for providing the financial means and laboratory facilities.

I would like to thank my loved ones, who have supported me throughout entire process, both by keeping me harmonious and helping me putting pieces together. I will be grateful forever for your love.

Signature of the student :

Name of Student : Parul Sharma

Date :

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Summary

The project aims to characterize parasite species variants responsible for the development of atypical cutaneous leishmaniasis in the newly emerging endemic zone of the disease: Himachal Pradesh. For the project 20 cases of CL (CL26-CL45) were taken from Indira Gandhi Medical College, Shimla. PCR diagnosis using 3 primers sets LDS/LDK, LITSR/L5.8S and Uni21/Lmj4 were used to characterise the strains *L.major, L.donovani Dd8 and L.donovani Bob* and identify leishmania species in patient biopsy samples. Alongside, histopathological study of cutaneous leishmaniasis (CL) lesion tissue was also carried out.

List of figures and tables

1. **Introduction**

1.1 Epidemiology

Leishmaniasis is a vector borne disease caused by an obligate intracellular protozoan of the *Leishmania* species. It is a metazoonotic disease where the invertebrate host is the phlebotomine female sandflies of the genus *Phlebotomus* in the old world and *Lutzomyia* in the new world (Dawit G, Girma Z et.al, 2013). It is categorised as a neglected tropical disease with the disease burden of 58,000 VL cases and 2,20,000 CL cases annually (J.Alvar, Iva´n D et.al. 2012). Of all the VL cases 90% occur in four countries Bangladesh, India, Sudan, and Brazil whereas 90% of all the ML cases occur in Bolivia, Brazil and Peru. 90% of CL cases occur in Afghanistan, Brazil, Peru, Iran, Saudi Arabia and Syria (Desjeux P, World Health Organisation).

Of the seven continents, *Leishmania* exists in 5 of them except for Australia and Antarctica. There are more than 20 Leishmanial species transmitted by 30 phlebotomus species responsible for the infection of the disease (P. D Ready 2010; Centre for Disease Control and Prevention). *Leishmania* species complex comprising of *L.donovani* and *L.infantum* are the species responsible for causing visceral leishmaniasis (VL). *Leishmania donovani* is explicitly responsible for visceral leishmaniasis (Kala azar) in the Old World mostly affecting the north-eastern part of the Indian subcontinent and parts of Africa. *Leishmania infantum,* on the other hand, is associated with the spread of VL in the New World comprising of the Mediterranean region and parts of Latin America (P.D Ready 2014). *L. tropica, L. major and L.aethiopica* forms the "Major" complex and is responsible with the spread of cutaneous leishmaniasis (CL) in the Old and the New world (Richard Reithinger et.al, 2007).

The spread of the disease largely depends on environmental factors and the distribution of sandfies. Migration of individuals to an existing endemic zone, deforestation and settlement in areas near the forest are some the factors that increase the risk of exposure to vectors. Socio-econimic factors like poor housing and sanitation, improper waste management lead to the breeding of sandflies resulting in the spread of the disease. Malnutrition is another factor that leads to the pathogenesis of the disease due to lower immunity. Environmental factors like temperature, rainfall

and humidity greatly determine the distribution of sandflies and thus the spread of the disease (Dawit G, Girma Z et.al, 2013; (Richard Reithinger et.al, 2007).

1.2 Disease transmission and clinical manifestations

1.2.1 Life cycle

The transmission cycle can be both anthroponotic or zoonotic. The sandflies inject the parasite into the human host through the proboscis while taking the blood meal. This infective stage of the parasite present inside the sandflies is motile and is called as the promastigotes. The promastigotes then reach the human system where they are phagocytosed by the macrophages and other mononuclear phagocytic cells. The promastigotes are transformed into a non-motile form called as the amastigotes which then infect the surrounding cells. Sandflies become infected by ingesting infected cells during blood meals (Centre for disease control and prevention). In sandflies, amastigotes transform into promastigotes, develop in the gut, and migrate to the proboscis (as shown in Fig 1.1)

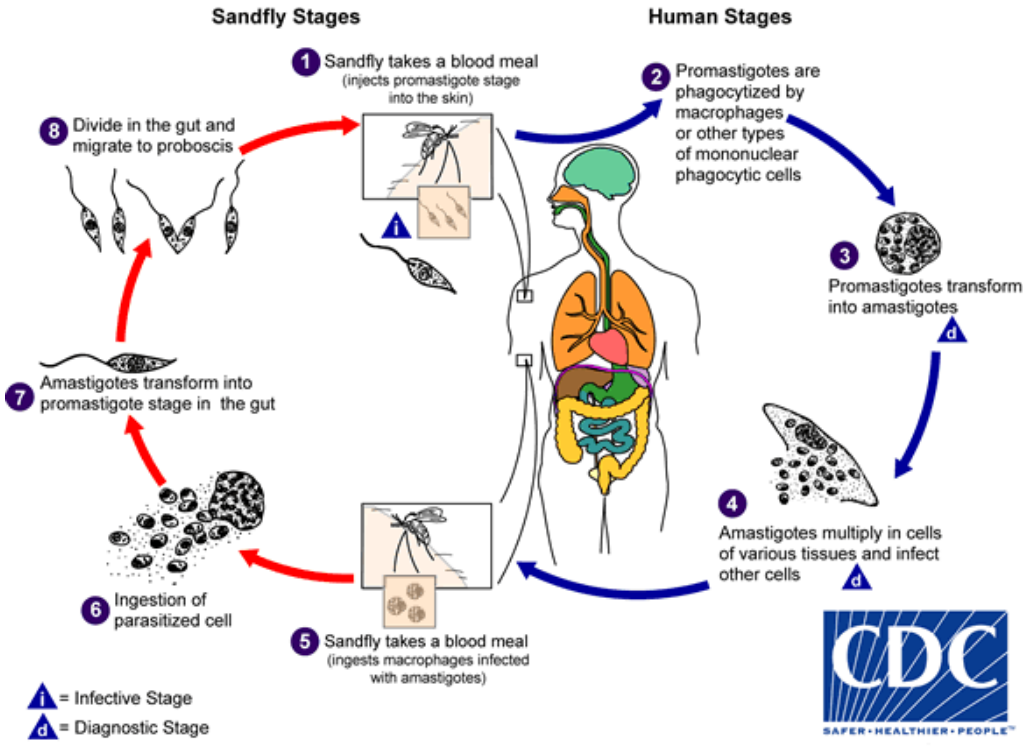


Fig1.1 Life cycle of Leishmania Source: CDC

1.2.2 Clinical manifestations of the disease

Leishmaniasis is associated with different clinical manifestations: self healing localized cutaneous leishmaniasis (LCL) (Fig1.2) marked by ulcerative lesions and the dispersed form of cutaneous leishmaniasis (DCL) marked by multiple non-ulcerative nodules; a severe form affecting the nasal, oral and pharyngeal mucosa producing a mutilating form called as mucosal leishmaniasis (Fig1.3); and the potentially fatal form called as visceral leishmaniasis (Fig1.4) (Richard Reithinger et.al, 2007). All the clinico-pathological manifestations are associated with different parasite species.

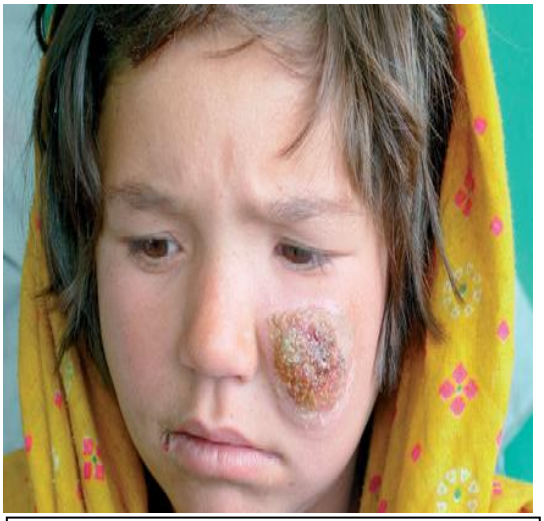


Fig1.2 A lesion caused by L tropica in Kabul,Afghanistan. (Richard Reithinger et.al, 2007).

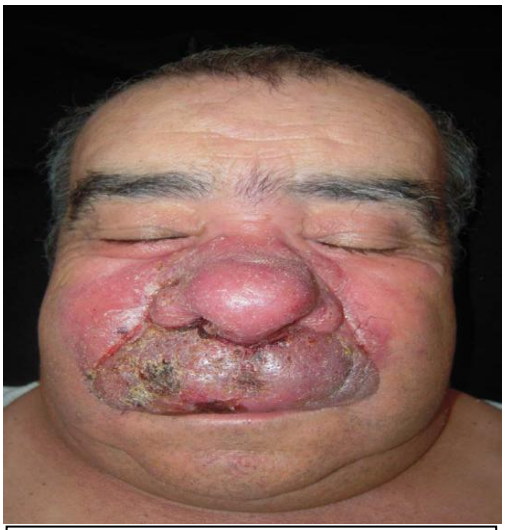


Fig1.3 Muco-cutaneous leishmaniasis in a patient in Portugal (L.Lopes et.al,2013)

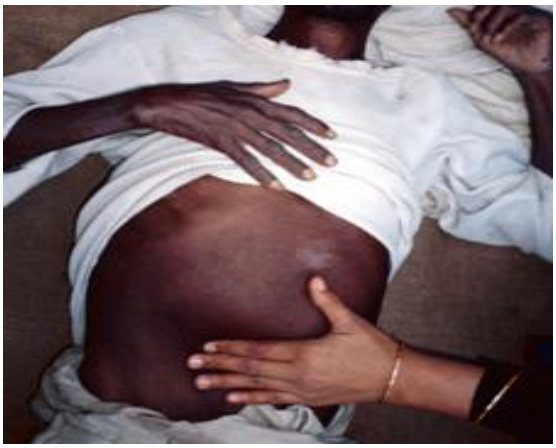


Fig1.4 Marked splenomegaly showing visceral leishmaniasis. (Credit: C. Bern, CDC)

1.3 Project rationale

While each clinical manifestation is associated with a different set of parasite species, a change in the pattern has been observed in the past few years. Reports of atypical leishmaniasis where cutaneous leishmaniasis is caused by visceral causing *L.donovani* have been reported in parts of Kenya, Sri Lanka, Yemen and India (Mebrahtu YB et.al, 1993; Pratlong F et.al, 1995; Karunaweera ND et.al, 2003). In India cutaneous leishmaniasis has been reported in states of Kerala, Assam and Haryana. Himachal Pradesh is the new endemic focus of leishmaniasis. The region is showing atypical pattern mainly the co-endemicity i.e. visceral and cutaneous forms co-exist and peculiar cutaneous form i.e. visceral causing *L.donovani* causing cutaneous leishmaniasis (NL Sharma et.al 2009; NL Sharma et.al 2005). The phenomenon points towards existence of parasite variants in this emerging focus of mixed infection.

Thus based on this information the project is designed to identify and characterize leishmanial species using PCR based analysis. Conventional methods like demonstration of leishmania amastigotes in skin smears using geimsa and H&E staining techniques will also be done. However the methods have lower sensitivity and specificity and thus PCR will give more conclusive results. Study of the histopathological and morphometric changes that occur in the skin lesions in cutaneous leishmaniasis will also be done.

1.4 Objective

Based on the project rationale two specific objectives are as follows:

Objective 1: Parasite species identification and characterisation using PCR based method and Geimsa staining.

Objective 2: Understanding and studying the histopathological and morphometric changes of the skin using paraffin embedding and H&E staining.

2. Review of literature

2.1 Immunology and pathogenesis of the disease

Leishmania is an intracellular parasite that resides and multiplies inside the macrophages. The outcome of the disease depends on the polarization of immune response and the balance between Th1 and Th2 response of the host. Macrophages and dendritic cells are the first and the most important cells that the leishmanial parasite interacts with (Dong Liu and Jude E. Uzonna, 2012). Uptake of the parasite by macrophages is a receptor mediated process and the neutrophils recruited at the site of initial infection act as intermediate host cells and enable the parasite to enter the macrophages silently thereby avoiding cell activation (Ifeoma Okwor et.al, 2012; Dong Liu and Jude E. Uzonna, 2012). Infected dendritic cells produce IL-12, which is critical for the development of IFN-ϒ-producing $CD4^{+}$Th1 cells. IFN-ϒ acts on infected macrophages leading to their activation (classical activation), upregulation of iNOS, and production of nitric oxide and other free radicals that are important for intracellular parasite killing. In contrast, the production of IL- 4 by other cell types supports $CD4^{+}$Th2 development. Th2 cells produce IL- 4 and IL-13, which leads to upregulation of arginase activity, alternative macrophage activation and the production polyamines that favor intracellular parasite proliferation. In addition, naturally occurring regulatory T cells (Treg) and infected macrophages also produce some immunoregulatory cytokines including IL-10 and TGF-β, which further deactivate infected cells leading to impaired parasite killing. (Dong Liu and Jude E. Uzonna, 2012).

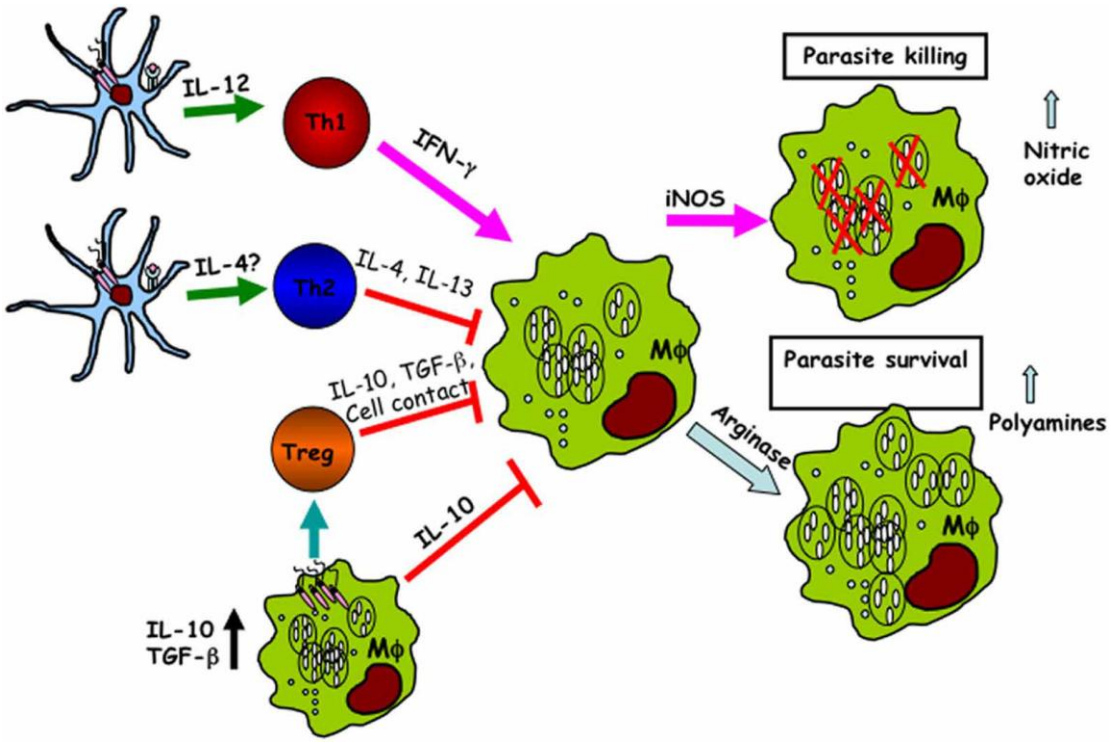


Fig2.1 Dendritic cells and macrophages regulate the outcome of *Leishmania* infection (Dong Liu and Jude E. Uzonna, 2012).

Cutaneous leishmaniasis is generally self healing leading to lifelong immunity and protection against re-infection. This state is achieved by specific INFϒ releasing $CD4^{+}$T cells. Furthermore, the resolution of the disease is characterized by increased secretion of interleukin (IL)-2 and IL-12 responses and the absence of classical Th2 cytokines or IL-10. Increased expression of IL-10 is a marker of disease progression (M. T. M. Roberts, 2006).

In case of visceral leishmaniasis, the infection ranges from asymptomatic to fatal. Individuals with subclinical or asymptomatic infection respond to stimulation with leishmanial antigen (LAg) and produce IL-2, IFNγ, and IL-12. The main disease-promoting activity of IL-10 in VL is probably conditioning host macrophages for enhanced survival and growth of the parasite. IL-10 can render macrophages unresponsive to activation signals and inhibit killing of amastigotes by down-regulating the production of TNFα and NO (Rajiv Kumar and Susanne Nylén, 2012).

2.2 Diagnostic method and treatment options

2.2.1 Diagnosis

Conventional methods of diagnosis of leishmanial parasite mainly include demonstration of parasite in tissue of relevance. For cutaneous leishmaniasis microscopic examination of Geimsa stained touch biopsy smear is the major diagnostic method. Leishmanial parasite is visualized as round or oval amastigotes in called as LD bodies mainly around the macrophages (Richard Reithinger et.al, 2007). Similar diagnostic method is used for visceral leishmaniasis where tissue aspirates from spleen and liver is obtained and parasite demonstration is done either through Geimsa staining or histopathological examination using H&E staining (Shyam Sundar and M. Rai, 2002). Although these methods are very frequently used, however these have very low sensitivity and specificity. Alternative methods include detection of DNA in tissue samples. Primers specific to conserved sequences of leishmania, like kDNA minicircle present in the kinetoplast, are used to amplify DNA in tissue samples. Species-level identification can also be done by analysis of amplified minicircle kinetoplast DNA (kDNA), by choosing primers from conserved regions of different leishmania species kDNA minicircles (Sacks, D. L et.al, 1995; Smyth, A. J et.al, 1992). Immunodiagnosis by detection of parasite antigen in tissue, blood, or urine samples, by detection of nonspecific or specific antileishmanial antibodies (immunoglobulin), or by assay for leishmania-specific cell-mediated immunity is another method. Two polypeptide fractions of 72-75 kDa and 123 kDa in the urine of kala-azar patients have been reported (De Colmenares et.al, 1995). ELISA has been used as a potential serodiagnostic tool for leishmaniasis. Several antigens have been tried. The commonly used antigen is a crude soluble antigen (CSA). A recombinant antigen, rK39, has been shown to be specific for antibodies in patients with VL caused by members of the L. donovani complex. This antigen, which is conserved in the kinesin region, is highly sensitive and predictive of the onset of acute disease (Shyam Sundar and M. Rai, 2002).

2.2.2 Current therapies

Visceral leishmaniasis	First line drugs	Sodium stibogluconate Meglumine antimoniate Amphotericin B Liposomal amphotericin B Pentamidine	Moderately toxic: cardiac effects, pancreatitis, nephrotoxicity, hepatotoxicity
	Clinical trials	Miltefosine Paromomycin Sitamaqine Other amphotericin B formulations	Gastrointestinal effects, nephrotoxicity, hepatotoxicity, possibly teratogenic
Cutaneous leishmaniasis	First line drugs	Sodium stibogluconate meglumine antimoniate Amphotericin B Pentamidine Paromomycin	Musculoskeletal pain, gastrointestinal disturbances, and mild to moderate headache.
	Clinical trials	Miltefosine Paromomycin Imiquimod Antifungal	Vomiting, nausea, kinetosis, headache, diarrhea, and a mild to moderate increase in aminotransferases and creatinine is associated with miltifosine

Table 2.1 – current therapies available for treatment (Johan van Griensven et.al, 2010; Simon L. Croft et.al 2006)

2.3 Disease prevalence in India

Until independence *L.donovani* was widespread in India. The number of cases of leishmaniasis went down after the start of the DDT spraying campaign during the national Malaria Eradicatio Programme. VL then returned in the form of large epidemic outbreaks (100,000 cases in 1977 and 40,000 in 1978) (Thakur CP, 1984). Currently, the endemic area covers the largest part of Bihar and extends to West

Bengal, Jharkhand and Uttar Pradesh. In addition, sporadic cases of VL occur in the foothills of the Himalayan mountain range in the northwestern sector of India. Recently, a few cases were reported from Gujarat and Kerala (Desjeux P, 1991; Simi

Bihar is currently the major endemic area for visceral leishmaniasis and extreme poverty, poor housing condition and sanitation are the major reasons for the spread of the disease. The number of cases in Bihar is a gross underestimation as most cases remain unreported due unaffordable treatment cost, unawareness about the disease and knowledge of control and prevention among the masses (Boelaert M et.al, 2009). Cutaneous leishmaniasis by *L. tropica* and *L. Major* occurs in the northwestern states of India (foci in Rajasthan and Punjab). The most affected area in Rajasthan is Bikaner district (Sharma MID et.al, 1973). Major areas affected by the disease are shown in Fig 2.2, Fig2.3

Fig2.2 areas affected by CL

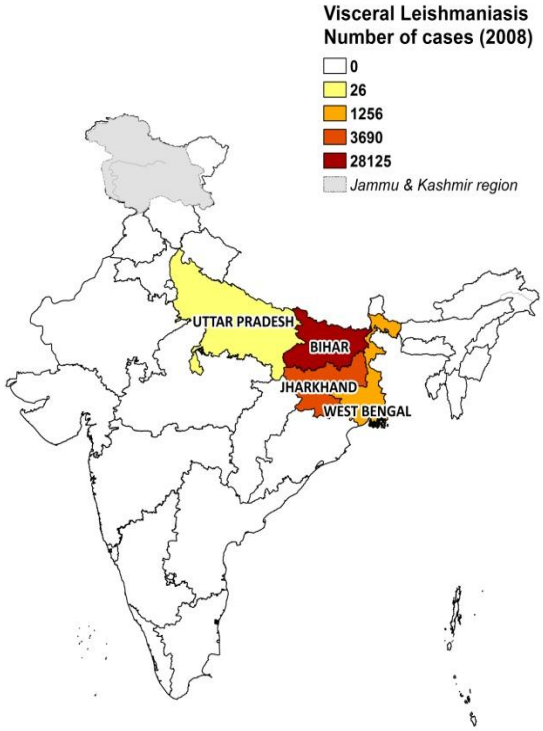


Fig2.3 areas affected by CL

CL was not known to occur in Himachal Pradesh before 1988. However Himachal Pradesh is recently identified as an endemic zone for CL. A study done by RC Sharma and colleagues showed 38 new cases of CL, acquired indigenously have been detected

from 1988 - 2000. Of these, 26 were from Kinnaur district and 12 from adjoining areas of bordering districts situated along the river Satluj (RC Sharma et.al, 2003). Satluj river belt (Fig2.4) has thus been identified as the major endemic zone. A study conducted by the Department of Medicine, Indira Gandhi Medical College, Shimla (Himachal Pradesh), India shows eighteen cases were diagnosed with VL over a five year period from January 2003 to December 2007. All the patients except one lived in a sub-alpine valley along the Satluj river that leads to the mountain deserts of the tribal district of Kinnaur and adjacent area of Shimla and Kullu districts (Sujeet Raina et.al, 2009). Sporadic cases of VL have been reported in the region.

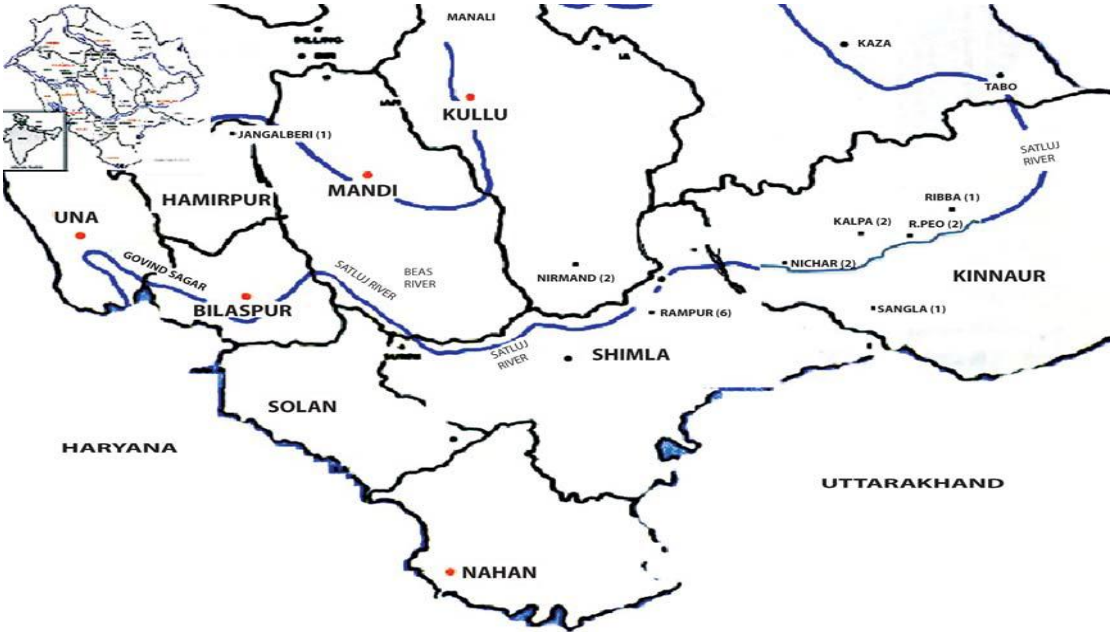


Fig2.4 Satluj river belt, the endemic zone in Himachal Pradesh (Sujeet Raina et.al, 2009)

Co-endemicity and peculiar CL have been the focus of attention in the area recently. *L. donovani* and *L. tropica* have been assigned as the species to the isolates from the CL patients of this new focus, preliminary studies showed that this being predominantly *L. donovani* (Nand Lal Sharma et.al, 2009). Another study was done by Nand Lal Sharma and colleagues where sandflies were collected during June 2003 to September 2007, relatively warmer months of the year from highly endemic pockets of cutaneous leishmaniasis which comprised five villages each of Kinnaur and Shimla districts. The study concluded that human leishmaniasis in endemic focus of Satluj river valley of Himachal Pradesh (India) has many features similar to those

of the disease present in Mediterranean countries; such as localized cutaneous leishmaniasis (CL) co-exists with visceral leishmaniasis (VL), and *Leishmania donovani* is predominant pathogen for CL whereas only a few cases have been due to *Leishmania tropica.*

3. Materials and methods

3.1 Collection of sample

20 patient samples (CL26-CL45) were collected from Indira Gandhi Medical College (IGMC) Shimla. The samples were collected with informed written consent from all patients and consent from the Ethical committee. Both blood and biopsy (from the affected area) of the patient was acquired. Touch biopsy smears were prepared for Geimsa staining. Biopsy was used for islolation of DNA for PCR analysis and paraffin embedding of the tissue. Paraffin embedded slides were prepared for H&E staining in order to analyze it further for histopathological and morphometric changes. The blood sample was stored at -80°C and the biopsy for paraffin was stored in 10% NBF.

3.2 DNA isolation

DNA of sample no CL26-CL45 as well as standard strains *L.major, L.d Dd8 (MHOM/IN/80/DD8), L.d Bob* was isolated using standard protocol of phenol-chloroform extraction. The biopsy sample was given a PBS wash followed by a short spin. The sample was then treated with NET buffer (150 mmol/L NaCl, 15 mmol/L, Tris-HCl, pH 8.3, 1mmol/L EDTA) (Rajesh Kumar et.al 2007). Proteinase K 200µg/ml for biopsy and 100µg/ml for pure culture was added to the sample and was incubated at 37°C overnight. Phenol-chloroform extraction followed by ethanol precipitation was done the next day. The DNA was dissolved in autoclaved distilled water. The isolated DNA was analyzed on 0.8% gel.

Nanodrop was performed for the quantitative and qualitative estimation of DNA.

3.3 PCR amplification

Three primer sets were used to characterize leishmania species namely *Leishmania major, Leishmania donovani Dd8, Leishmania donovani Bob.*

Sr no	Forward primer	Reverse primer	region	Band size
1.	LDS 5'GCGACGACAAGCC CATGATT 3'	LDK 5'GCGTCGGCTCGTT GATGATG3'	Cloned hsp70 cDNA	Expected band size – 243bp band for L.donovani Dd8. No bands for L.major

3.	LITSR 5'CTGGATCATTTTCC GATG 3'	L5.8S 5'TGATACCACTTATC GCACTT 3'	ITS1	300-350bp PCR product Digestion with HaeIII gives different RFLP pattern L.donovani: 3 bands-50bp, 80bp & 190 bp L.major: 2 bands-160bp and 210 bp
4.	Uni21 5'GGGGTTGGTGTAA AATAGGCC 3'	Lmj4 5'CTAGTTTCCCGCCT CCGAG 3'	kDNA minicircle	Differentiates L.major from L.donovani L. major: 650bp L.donovani: 850bp

Table 3.1 Primer sets used in study

Each PCR was optimized using Leishmania DNA prior to use for diagnosis of patient derived samples. The first PCR was carried out using the primer set LDS/LDK. PCR master mix from NEB(New England Biolabs) was used. 50ng of the DNA sample was used. PCR condition were used as described by S.K. Arora et al., 2008; N.L Sharma et al., 2009. Initial denaturation at 95°C for 5min, followed by 30 cycles of denaturation at 95°C for 1min, annealing at 60°C for 1min, extension 72°C for 1min and final extension at 72°C for 10min.The PCR product was analyzed on 1.5% gel. The fragments were visualized by UV light and the sizes of the restriction products determined.

The third PCR was carried out using the primer set LITSR/L5.8S. PCR master mix from NEB(New England Biolabs) was used. 50ng of the DNA sample was used. PCR conditions were used as described by G. Schonian et al., 2003; E. Bensoussan et al., 2006; R. Kumar et al., 2007; S.Khanra et al., 2012. Initial denaturation at 95°C for 2min, followed by 35 cycles of denaturation at 95°C for 20sec, annealing at 53°C for 30sec, extension 72°C for 1min and final extension 72°C for 6min. The PCR product was analyzed on 1.5% gel. . RFLP was done using restriction enzyme HaeIII from (New England Biolabs) according to the manufacturer's instructions, and the restriction fragments were analyzed by gel electrophoresis at 100 V in 2.0% agarose

gel. The fragments were visualized by UV light and the sizes of the restriction products determined.

The last PCR was carried out using the primer set Uni21/Lmj4. PCR master mix from NEB(New England Biolabs) was used. 50ng of the DNA sample was used. PCR condition were used as described by G. Anders et al., 2002 R.Kumar et al., 2007. Initial denaturation at 94°C for 6min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 55°C for 30sec, extension 72°C for 30sec and final extension at 72°C for 5min.The PCR product was analyzed on 1.5% gel. The fragments were visualized by UV light and the sizes of the restriction products determined.

Sequence analysis -To understand the basis of expected amplicons, the species specific *in-silico* analysis of Leishmania sequences was done using NCBI BLAST.

3.4 Tissue smears and paraffin embedding

3.4.1 Tissue smears

Touch biopsy smears were prepared by placing the biopsy between two slides and crushing the tissue between the two. Geimsa staining was then performed on this touch smear. It was done by fixing the slides in 100% methanol for 5min. The fixed slides were then subjected to geimsa stain solution for 25-30 minutes. The slide was washed and dried. It was given a small dip in xylene and the observed under microscope.

3.4.2 Paraffin embedding

Pre-processing - of the tissue biopsy was done by storing the biopsy samples transported in fixative (usually 10% NBF) (It remains in this preservative for a minimum of 24 hours prior to processing). Grossing was done in order to check sample size,shape etc. and tissue was packed in blotting sheets in cassettes with proper sample ID no.

Processing - The biopsies were then processed in the tissue processing machine for 24 hours. The machine was an automated system that processed the tissues in graded alcohols and xylene. The tissues was dehydrated in ascending grades of alcohol 50%,70%, 80%, 90% or absolute alcohol. Clearing was done using xylene (4 steps -

10 mins each)**.** The tissue was then impregnated in molten paraffin to remove xylene and was ready for embedding.

Embedding or block preparation - Skin biopsies were embedded in metal moulds filled with paraffin wax using ice to hold the tissue in place and were left to set on ice. The mould was removed whilst cold and the excess wax was trimmed manually using a dissecting blade. The wax blocks were cooled in ice and sectioned at a thickness of 4 μm using a microtome. The sections were floated on a 40ºC water bath and collected on adhesive slides to minimize section loss during heat-mediated retrieval They were then incubated at 37 ºC overnight for 24 hours on slide racks, and kept in special slides container at room temperature.

Haematoxylin & Eosin staining of Paraffin sections – To perform H&E the slides were first deparaffinised using by placing the slide on a hot plate. It was then emmersed into three sets of xylene for 10 minutes each and passed through descending grade of alcohols : 95%, 80%, 70%, 60% -2 mins each. The slides were rinsed with tap water (to remove the wax and dehydrate the sections). Slides were placed into **haematoxylin** for 20 minutes and then rinsed by tap water for 4-5 minutes. Excess of haematoxylin was removed by adding 1% acid alcohol (1% HCl in 70% (v/v) alcohol) for 5 seconds followed by a tap water wash. The pink haematoxylin stain was converted to blue by adding Scott's tap water, for approximately 10 seconds until the sections turned blue. It was rinsed in tap water and then stained in **eosin** (1% (w/v)) for 15 seconds with a subsequent wash in running tap water for 1-5 minutes. The sections were then dehydrated by ascending grades of alcohol followed by two washes of xylene for 10 minutes each. The slides were mounted in DPX mountant and covered with glass cover slip

4. Results and discussion

4.1 DNA isolation

DNA was successfully isolated from biopsy samples CL26-CL45. Biopsy and blood sample of patient 40 was not available.

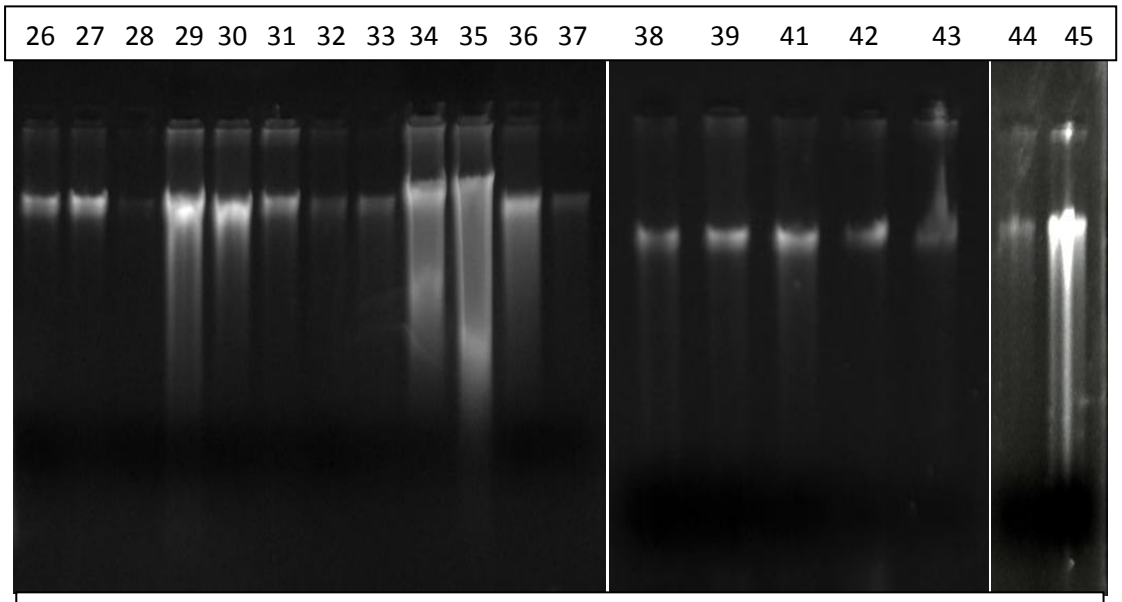


Fig4.1 Shown are the results of DNA isolation of biopsy samples CL26-CL45. Genomic DNA analyzed run on agarose gel as described in Materials and Methods.

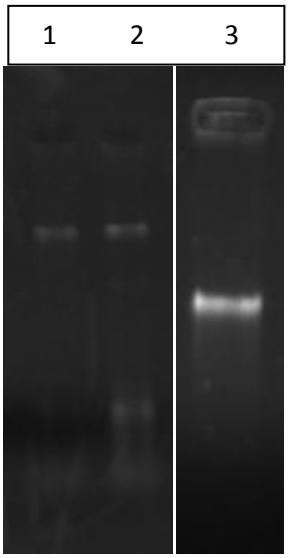


Fig4.2 Shown are the results of DNA isolation of pure cultures. lane 1, 50 ng of *L.major*; lane 2, 50 ng of *L.donovani Bob*; lane 3, 50 ng of *L.donovani Dd8.* DNA analyzed on agarose gel as described in Materials and Methods.

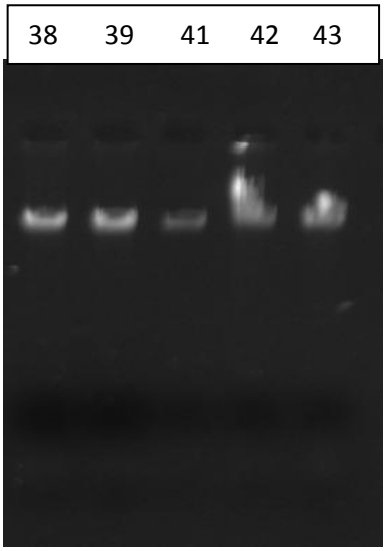


Fig4.3 Shown are the results of DNA isolation of patient blood samples CL38-CL43. DNA analyzed on agarose gel as described in Materials and Methods.

Patient No.	Concentration ng/µl
CL26	16
CL27	16.4
CL28	3.1
CL29	100
CL30	50.3
CL31	1.5
CL32	1.3
CL33	10.9
CL34	277
CL35	231
CL36	50.9
CL37	8.7
CL38	136
CL39	9.1
CL41	17.5
CL42	54.9
CL43	258
CL44	5
CL45	66.9

Table 4.1 nanodrop reading

4.2 PCR results

4.2.1 LDS/LDK primer set.

The primer set is *L.donovani* specific and amplifies clonedhsp70 cDNA region and gives a 243bp band. The basis of the said *L. donivani* specific amplication is depicted in Fig 4.4

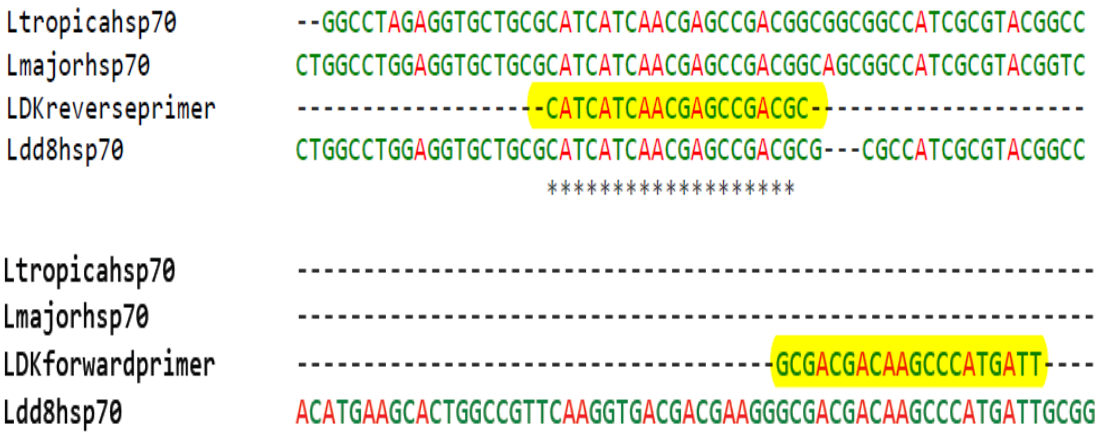


Fig4.4 Multiple sequence alignment done via Clustal omega gives a sequence similarity of the reverse primers for all the three leishmania species but the forward primer shows sequence similarity only for *L.donovani* but not for *L.major* or *L.tropica* .

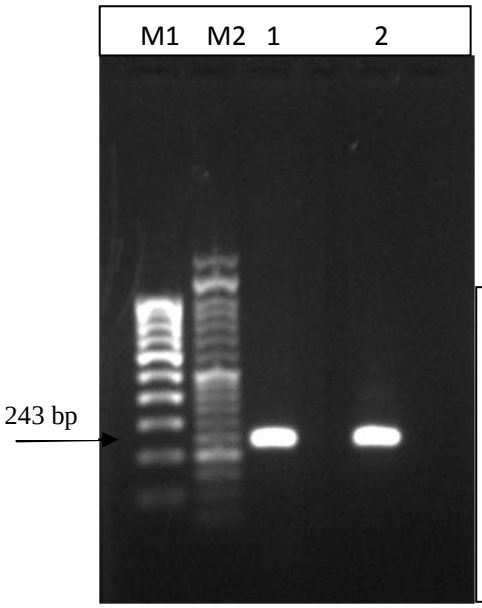


Fig4.5 Shown are the results of PCR amplification pure parasite cultures by LDS/LDK primer set analyzed on agarose gels. DNA was extracted from parasite cultures and amplified as described in Materials and Methods. Lane M1, 100bp Ladder (New England Biolabs); lane M2, 50 Ladder (New England Biolabs); lane 1 *L.donovani Dd8*, ; lane 2, *L.donovani Bob*.

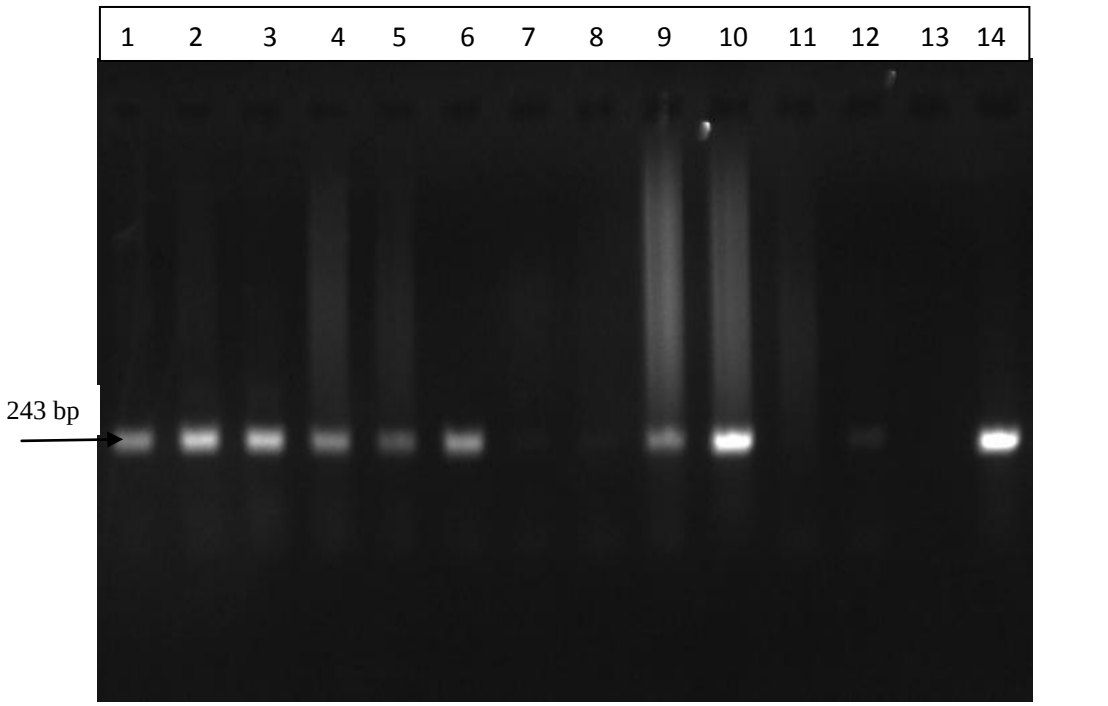


Fig4.6 Shown are the results of PCR amplification of patient biopsy samples LDS/LDK primer set analyzed on agarose gels. DNA was extracted from parasite cultures and amplified as described in Materials and Methods. Lane 1, 50 ng of CL26 DNA; lane 2, 50 ng of CL27 DNA; lane 3, 50ng of CL28 DNA; lane 4, 50ng of CL29 DNA; lane 5, 50ng of CL30 DNA; lane 6, 50ng CL31 of DNA, lane 7, 50 ng of CL32 DNA; lane 8, 50ng of CL33 DNA; lane 9, 50ng of CL34 DNA; lane 10, 50ng of CL35 DNA; lane 11, 50ng CL36 of DNA; lane 12, 50 ng of CL37 DNA; lane 13, nil; lane 14, 50 ng of *L.donovani Bob*.

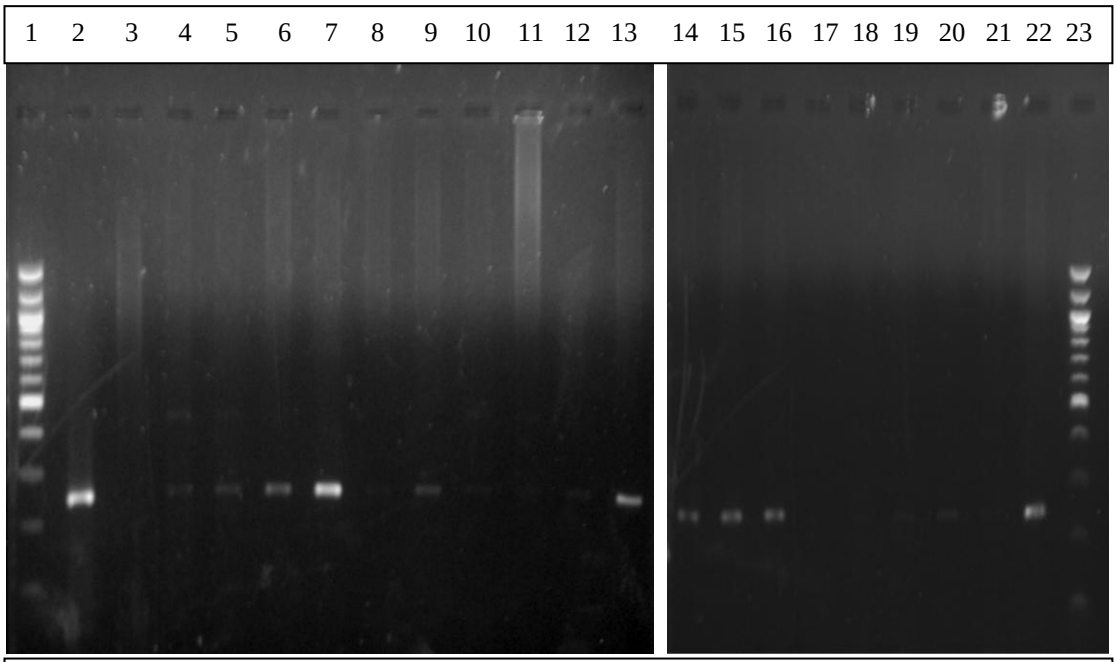


Fig4.7 Shown are the results of PCR amplification of patient biopsy samples LDS/LDK primer set analyzed on agarose gels. Lane 1, 100bp ladder; lane 2, *L.donovani Bob*; lane 3, *Mycobacterium (negative control)*; lane 4, 50ng of CL29 DNA; lane 5, 50ng of CL30 DNA; lane 6, 50ng CL34 of DNA, lane 7, 50 ng of CL35 DNA; lane 8, 50ng of CL36 DNA; lane 9, 50ng of CL38 DNA; lane 10, 50ng of CL42 DNA; lane 11, 50ng CL43 of DNA; lane 12, 50 ng of CL44 DNA; lane 13, 50 ng of CL45 DNA; lane 14, 50 ng of CL26 DNA; lane 15, 50 ng of CL27 DNA; lane 16, 50 ng of CL28 DNA; lane 17, 50 ng of CL31 DNA; lane 18, 50 ng of CL32 DNA; lane 19, 50 ng of CL33 DNA; lane 20, 50 ng of CL37 DNA; lane 21, 50 ng of CL39 DNA; lane 22, 50 ng of CL41 DNA; lane 23, 100bp ladder.

The expected 243bp band was amplified using LDS-LDK primer specific for *L.donovani* species *L.donovani Bob* and *L.donovani Dd8* (shown in Fig 4.5).The same primer set was used to amplify biopsy samples CL26-CL45. A 243bp band was observed indicating the presence of *L.donovani* DNA in cutaneous leishmaniasis samples. Very faint bands were observed in CL32, CL33, CL36 and CL37 as shown in (Fig 4.6). Biopsy sample CL26, CL27, CL28, CL29, CL30, CL31, CL34, CL35 show dark bands indicating the presence of leishmanial DNA (Fig4.6). Upon repeating the PCR, faint bands were obtained in the same biopsy samples (Fig 4.7). Faint bands were obtained in other biopsy samples CL31, CL32, CL42, CL43, CL44 and CL45 (Fig4.7).

4.2.2 Uni21/Lmj4 primer set

The primer set amplifies kDNA minicircle and differentiates between L.major (expected bad size -650bp) and L.donovani (expected band size -850). Unexpected band sizes were observed in this PCR. Thus another primer set was used.

4.2.3 LITSR/L5.8S primer set

The primer amplifies the ITS1 region and gives a 350bp band for all leishmania species. The PCR product is then digested with HaeIII restriction enzyme which yields different fragment for every species.

```
L5.8Sreverse      ----------------------------------ACTATGGTGAATA------------
LmajorITS1        CAAAAATGTCCGTTTATACAAAAAAATAGACGGCGTTTCGGTTTTTGGCGGGAGGGAGAG
LtropicaITS1      CAAAAATGTCCGTTTATACAA----ATATACGGCGTTTCGGTTTTG------------TT

LITSRforward      CTGGATCATTTTCCGATG-----------------------------------------
LmajorITS1        CTGGATCATTTTCCGATGATTACACCCCAAAAAA---CATAT--ACAACTCGGGGAGGCT
LtropicaITS1      -TGGCTCATTTTCCGAAGATTACCCCCCAAAAAAAAACATATACAAAACTCGGGGAGGCC
```

Fig4.8 Multiple sequence alignment done via Clustal omega gives a sequence similarity of both forward and the reverse primers for both *L.major* and *L.tropica*.

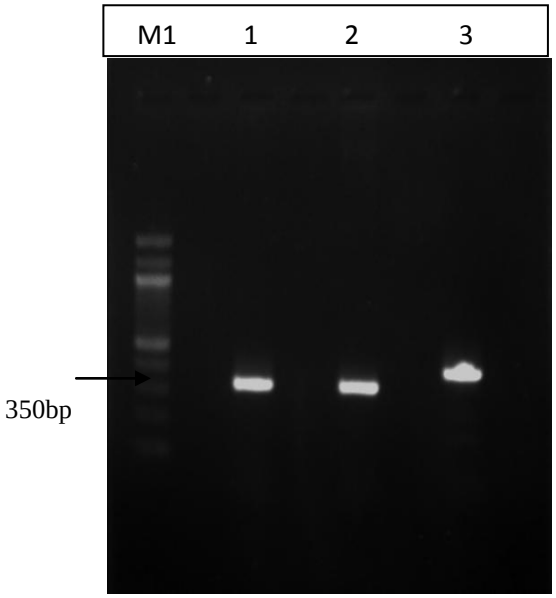


Fig4.9 Shown are the results of PCR amplification of pure parasite cultures by LITSR/5.8s primer set analyzed on agarose gels. DNA was extracted from parasite cultures and amplified as described in Materials and Methods. Lane M1, 100bp Ladder (New England Biolabs); lane 1, 50ng *L.major;* lane2, 50ng *L.donovani Bob;* lane 3, 50ng *L.donovani Dd8*;

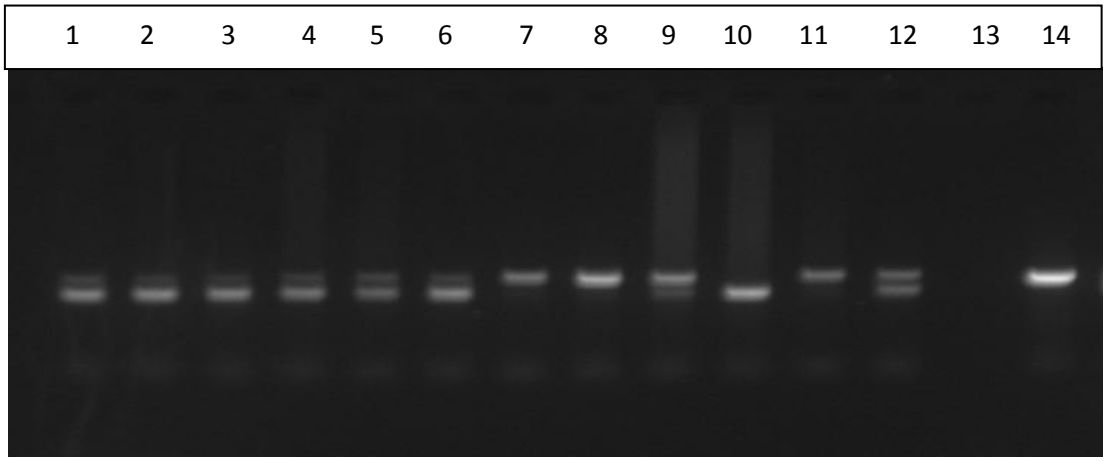


Fig4.10 Shown are the results of PCR amplification of patient biopsy samples LDS/L5.8S primer set analyzed on agarose gels. DNA was extracted from parasite cultures and amplified as described in Materials and Methods. Lane 1, 50 ng of CL26 DNA; lane 2, 50 ng of CL27 DNA; lane 3, 50ng of CL28 DNA; lane 4, 50ng of CL29 DNA; lane 5, 50ng of CL30 DNA; lane 6, 50ng CL31 of DNA, lane 7, 50 ng of CL32 DNA; lane 8, 50ng of CL33 DNA; lane 9, 50ng of CL34 DNA; lane 10, 50ng of CL35 DNA; lane 11, 50ng CL36 of DNA; lane 12, 50 ng of CL37 DNA; lane 13, nil; lane 14, 50 ng of *L.major*.

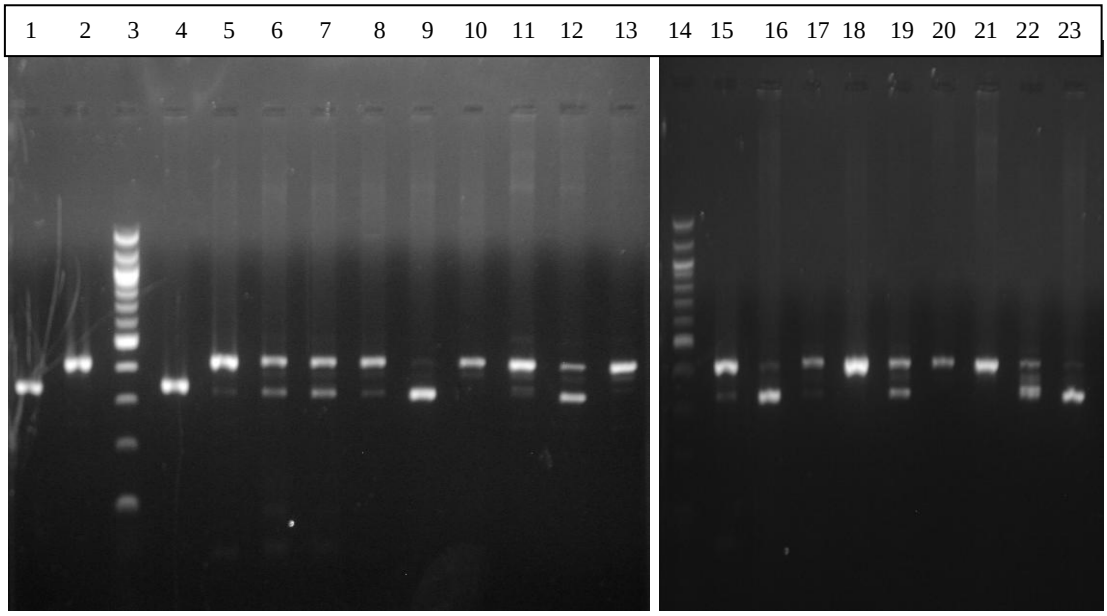


Fig4.11 Shown are the results of PCR amplification of patient biopsy samples LITSR/L5.8S primer set analyzed on agarose gels. Lane 1, 50ng *L.major*; lane 2, *L.donovani Dd8*; lane 3, 100bp ladder(NEB); lane 4, 50ng of *L.donovani Bob*; lane 5, 50ng of CL26 DNA; lane 6, 50ng CL29 of DNA, lane 7, 50 ng of CL30 DNA; lane 8, 50ng of CL34 DNA; lane 9, 50ng of CL35 DNA; lane 10, 50ng of CL36 DNA; lane 11, 50ng CL38 of DNA; lane 12, 50 ng of CL41 DNA; lane 13, 50 ng of CL42 DNA; lane 14, 100bp ladder (NEB); lane 15, 50 ng of CL27 DNA; lane 16, 50 ng of CL28 DNA; lane 17, 50 ng of CL31 DNA; lane 18, 50 ng of CL33 DNA; lane 19, 50 ng of CL37 DNA; lane 20, 50 ng of CL39 DNA; lane 21, 50 ng of CL43 DNA; lane 22, 50 ng of CL44 DNA; lane 23, 50 ng of CL45 DNA

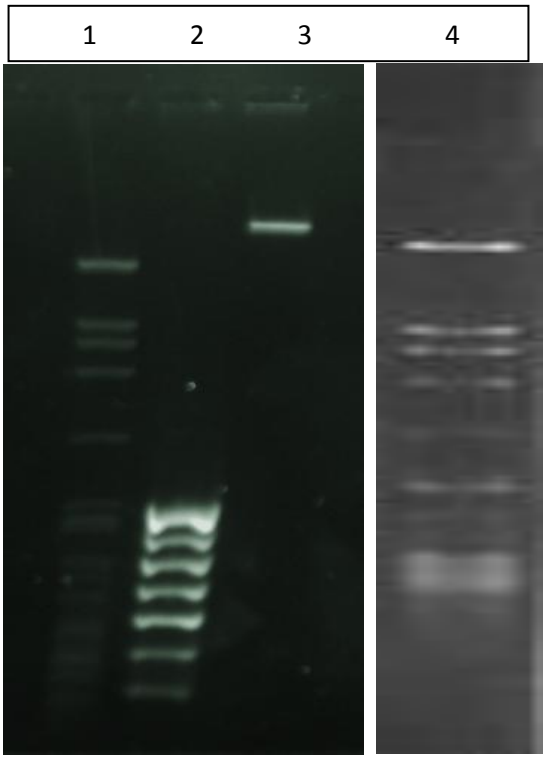


Fig4.12 Shown are the results of restriction digestion of λ DNA by HaeIII enzyme analyzed on agarose gels. Lane 1, λ DNA (HaeIII digested); lane2, 100bp ladder; lane 3, Control (Undigested λ DNA), lane 4, standard digestion pattern.

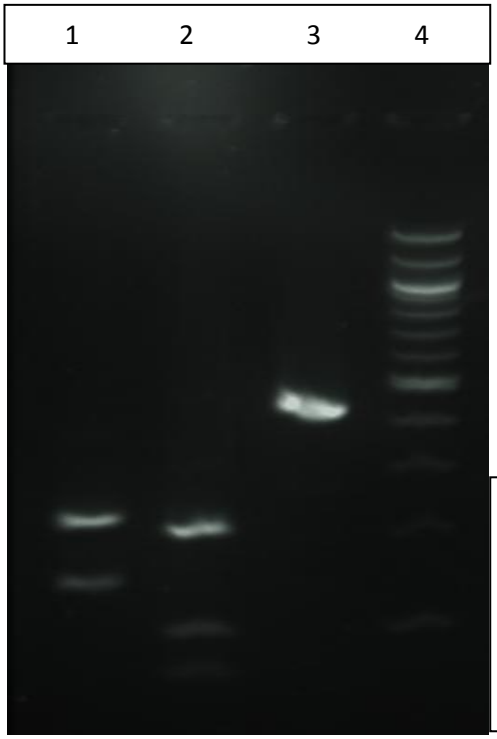


Fig4.13 Shown are the results of restriction digestion of PCR product of LITSR/L5.8S primer set analyzed on agarose gels. Lane 1, 50ng *L.major;* lane2, 50ng *L.donovani Bob;* lane 3, 50ng *L.donovani Dd8* . Lane 4, 100bp Ladder (New England Biolabs).

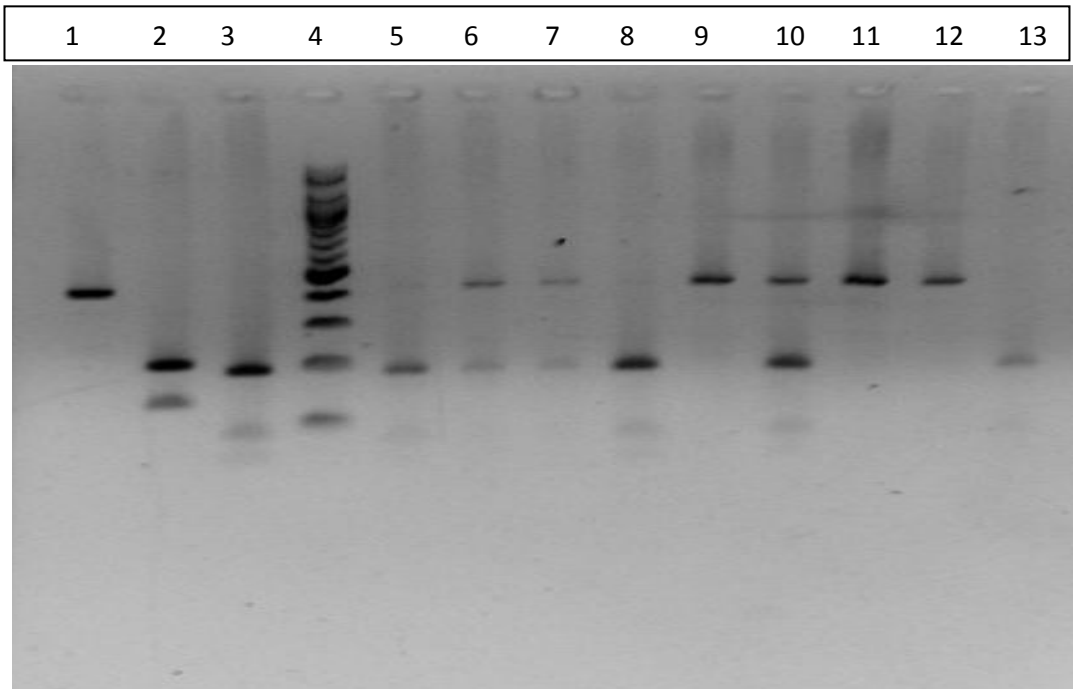


Fig4.14 Shown are the results of restriction digestion of PCR product of LITSR/L5.8S primer set analyzed on agarose gels. . Lane 1, *L.donovani Dd8*; lane 2, *L.major*; lane 3, *L.donovani Bob*; lane 4,100bp ladder (NEB); lane 5, CL28; lane 6,CL29; lane 7 CL30; lane 8, CL35; lane 9, CL36; lane 10, CL41; lane 11, CL42; lane 12, CL43 DNA; lane 13, CL45.

A 400bp band was observed in *L.donovani* Dd8 (Fig4.9). *L.major* and *L.donovani Bob* showed a 300bp band (Fig4.9). Upon PCR amplification of biopsy samples a pattern of single and double bands was observed.

Double bands were observed at 350bp and 400bp in biopsies CL26, CL27, CL28, CL29, CL30, CL31, CL34, CL37, CL38, CL41, and CL44 (Fig4.10,Fig4.11). Band corresponding to *L.donovani Dd8* amplicon at 400bp was seen in biopsies CL32, CL33, CL36, CL38, CL42, CL39, and CL43 (Fig4.10,Fig4.11). The position of band corresponded *L.major* and *L.donovani Bob* amplicons in biopsies CL35 and CL45 (Fig4.10, Fig4.11).

Restriction digestion was not successful on the PCR product initially. To check the activity of HaeIII enzyme, restriction digestion was performed on λ DNA and compared to the standard restriction pattern expected by the digestion. Similar results were obtained showing showing the enzyme working well (Fig4.12).

After restriction digestion of the PCR using HaeIII enzyme, *L.major* showed 2 bands-160bp and 210 bp. *L.donovani Bob* showed 3 bands -50bp, 80bp & 190 bp and *L.donovani Dd8* showed no restriction digestion (Fig4.13). Biopsy samples CL29, CL30, CL36, CL42, CL43 showed band corresponding to *L.donovani Dd8* indicating the presence of *L.donovani* in cutaneous leishmaniasis patient (Fig4.14). CL 41 showed two bands corresponding to *L.major* ITS1-PCR RFLP pattern (Fig4.14).

CL28 and CL35 showed bands corresponding to *L.major* and *L.donovani Bob PCR RFLP* pattern(Fig4.14)

4.3 H&E and Geimsa staining

4.3.1 Geimsa staining

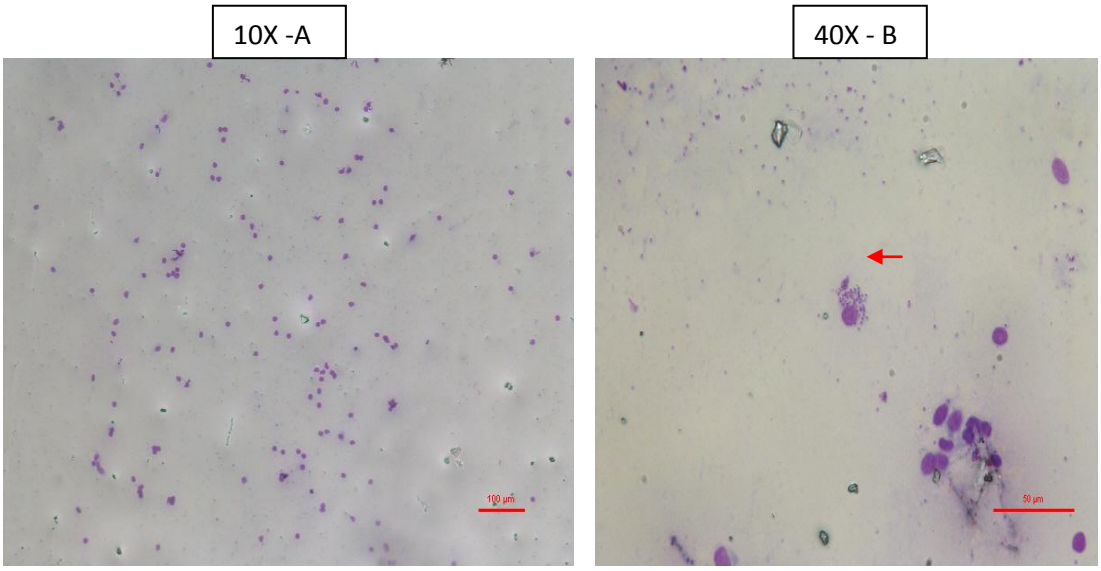


Fig 4.15 The figure A and B shows geimsa stained slide of patient CL26 at 10X and 40X resolution respectively. The (➔) shows an affected macrophage with LD bodies confirming the patient is positive for leishmaniasis.

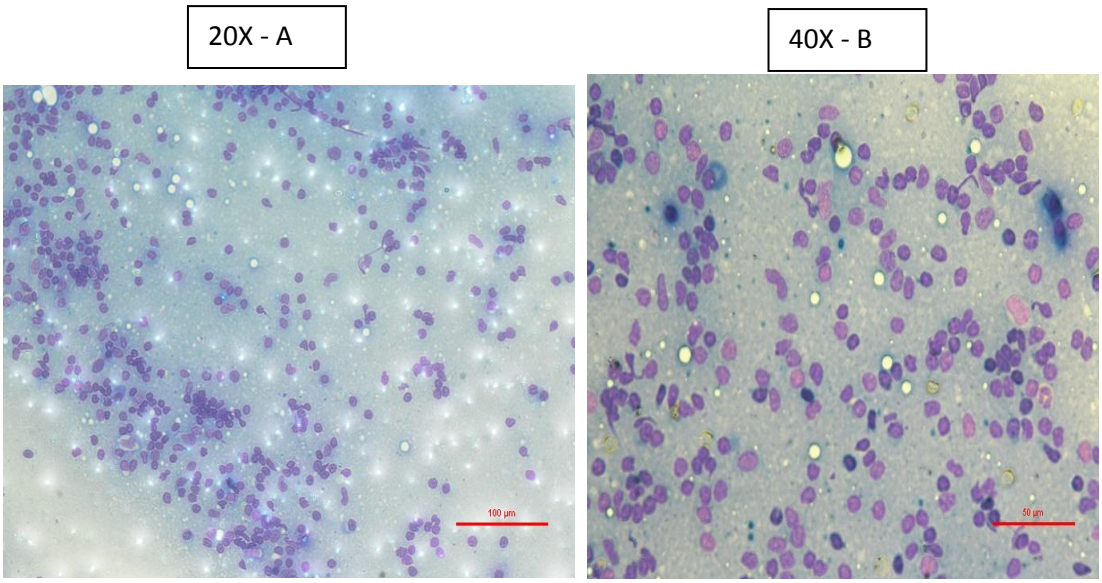


Fig 4.16 The figure A and B shows geimsa stained slide of patient CL34 at 20X and 40X resolution respectively. No LD bodies were seen in the slide.

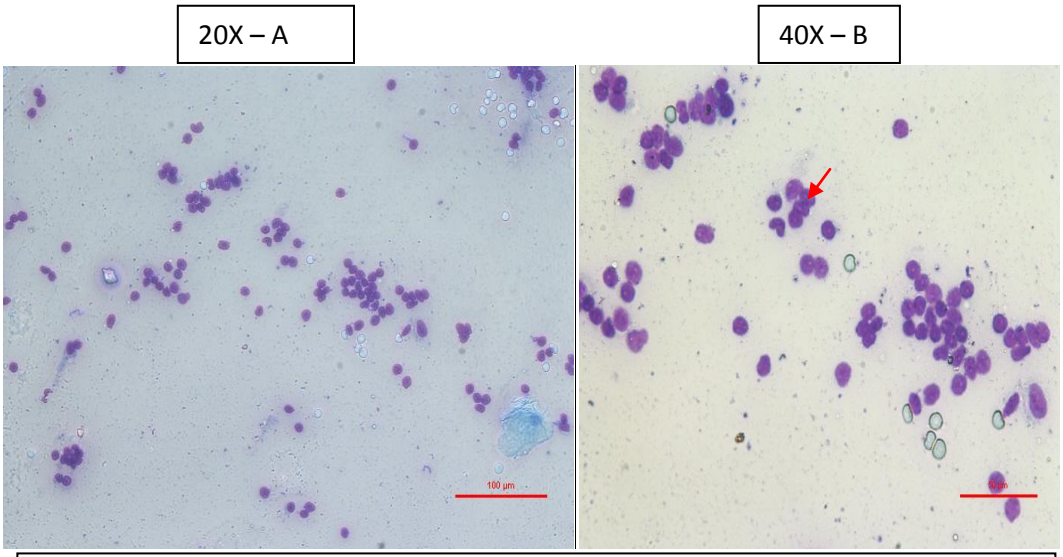


Fig 4.17 The figure A and B shows geimsa stained slide of patient CL35 at 20X and 40X resolution respectively. Intracellular LD bodies shown by (➔) were observed inside the macrophage confirming the patient is positive for leishmaniasis.

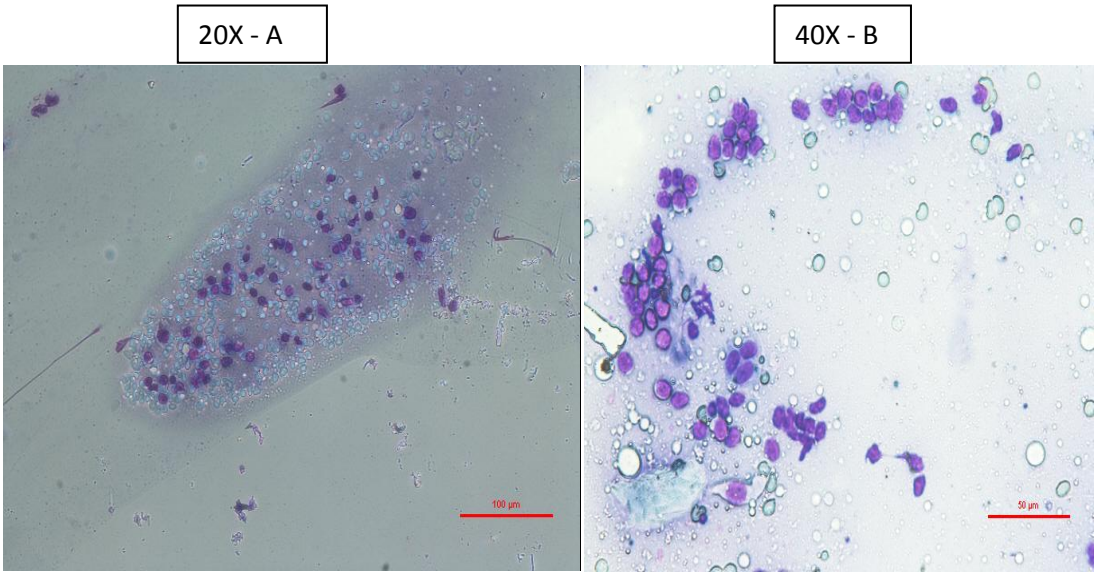


Fig 4.18 The figure A and B shows geimsa stained slide of patient CL34 at 20X and 40X resolution respectively. No LD bodies were seen in the slide.

4.3.2 H&E staining

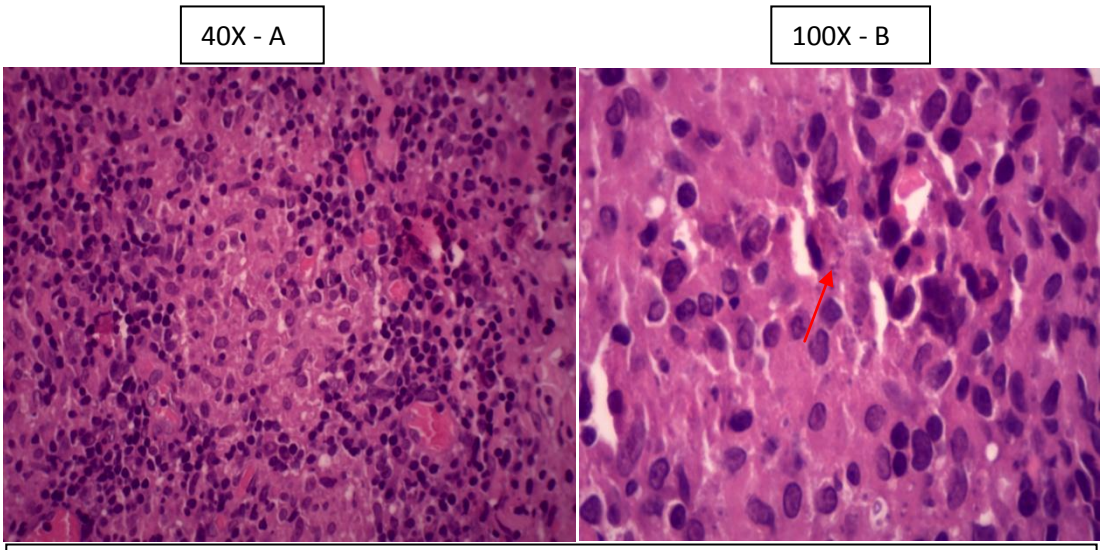


Fig4.19The figure A and B shows H&E stained slide of patient CL26 at 40X and 100X resolution respectively. The figure B shows presence of LD bodies shown by (→)

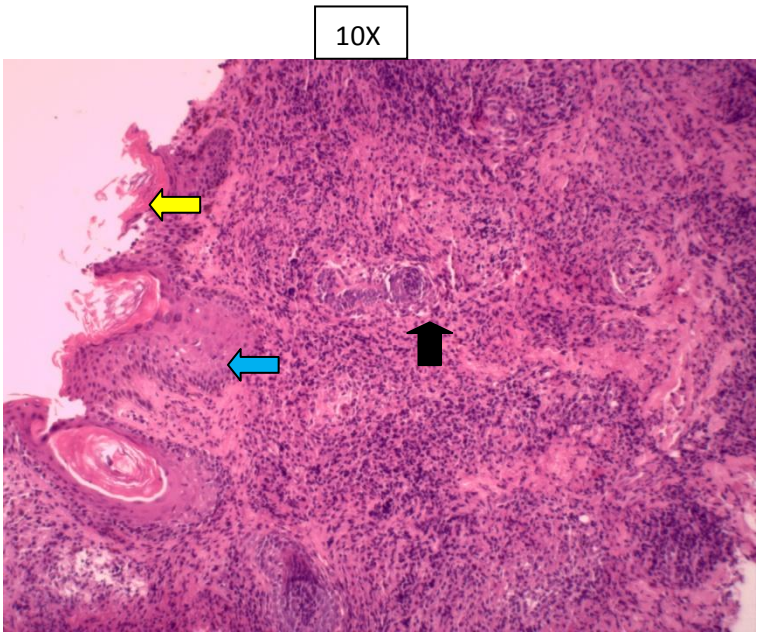


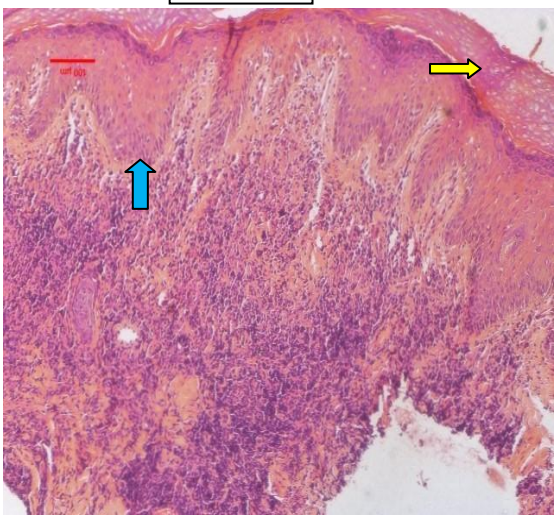
Fig4.20 The figure A and B shows H&E stained slide of patient CL30 at 10X.

Skin biopsy shows keratinised stratified squamous epithelium with parakeratosis (⇦) and mild acanthosis or slightly club shaped elongated rete ridges (⇦)

Chronic inflammatory cell infiltrate of lymphocytes, plasma, histiocytes, neutrophils (⬆)

LD bodies not seen.

10X – A



20X – B

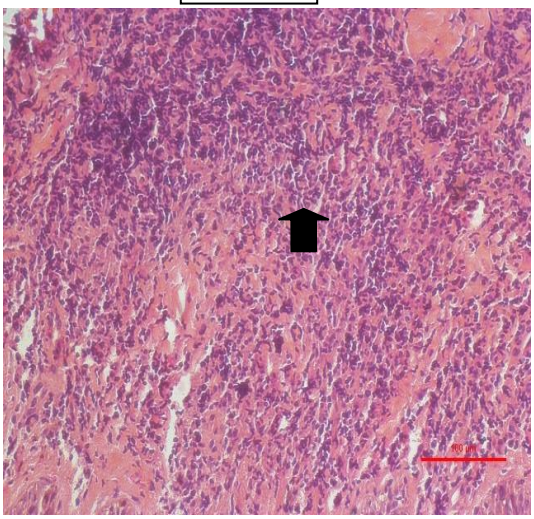


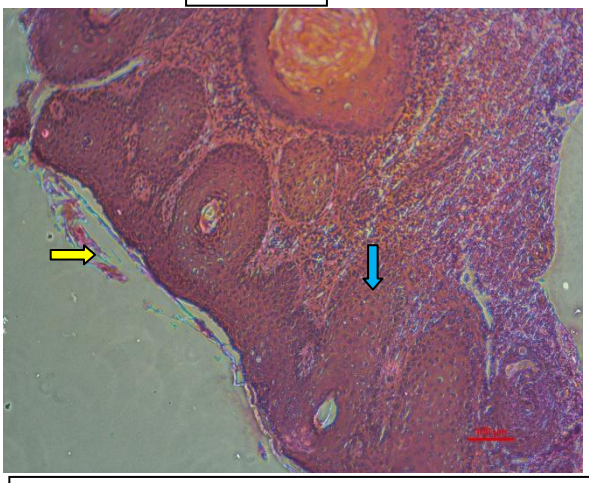
Fig4.21 The figure A and B shows H&E stained slide of patient CL27 at 10X and 20X

Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis (⇧)

Mild Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis (⇨)

Chronic inflammatory infiltrates of lymphocytes, plasma, histiocytes, neutrophils (⬆) . No LD bodies were observed

10X – A



40X - B

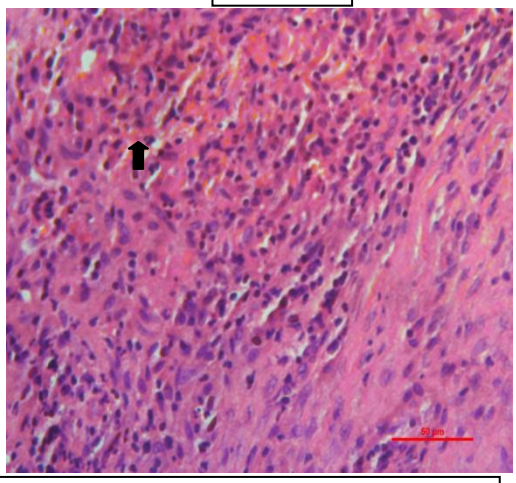


Fig4.22 The figure A and B shows H&E stained slide of patient CL27 at 10X and 20X

Regular acanthosis or slightly club shaped elongated rete ridges with thickened epidermis (⇧)

Mild Hyperkeratosis and Parakeratosis in the stratum corneum in the scales over epidermis (⇨)

Chronic inflammatory infiltrates of lymphocytes, plasma, histiocytes, neutrophils (⬆)

No LD bodies were observed

Patient no.	Histopathaological features			
	Acanthosis	Hyperkeratosis and parakeratosis	Immune infiltration	LD bodies
CL26	-	-	++	+
CL27	+	++	+	-
CL28	+	+	++	-
CL30	+	+	+	-
CL34	-	-	+	-
CL35	+	+	+	-
CL36	+	++	+	-
CL37	+	-	+	-
CL38	+	+	+	-
CL39	++	+	+	-
CL43	+	+	+	-

Table 4.2 Samples showing positive/negative CL histopathological features.

The histopathological changes that occur during leishmaniasis were analysed by H&E staining. A characteristic set of changes were seen in the morphology as shown in the representative images above (Fig4.19, 4.20, 4.21, 4.22). The major histopathological changes include acanthosis, hyperkeratosis, parakeratosis, shows perivascular infiltration of lymphocytes, plasma and neutrophils. The patients positive for these features are give in table 4.2. Patient samples CL29, CL31, CL32, CL33, CL40, CL41, and CL42 slides did not show characteristic features and/or gave low quality sections.

Conclusion

Leishmaniasis is a major tropical disease with a wide clinical spectrum of cutaneous, mucocutaneous and visceral involvement. Presentation is often varied and diagnosis can be challenging. The diagnostic methods like demonstration of a single amastigote upon microscopic examination of tissue smears or multiple promastigotes in cultures disease is considered sufficient but the sensitivity of the method is low. Besides, culturing parasites is expensive and time consuming and requires expertise and costly equipment, severely restricting its use in routine clinical practice. Due to these limitations PCR based diagnosis has gained importance. Primers designed to amplify conserved regions of leishmanial parasite like kDNA regions present in the minicircles are used for diagnosis. The method not only identifies parasite DNA but help in the characterization of various leishmania spp. Characterization of parasite species has recently gained importance due to reported cases of variation in parasite genome leading to atypical presentation of symptoms. Economic development, including widespread urbanization, deforestation, and development of newer settlements, besides migration from rural to urban areas, is responsible for the spread of the sandfly as well the reservoir system of leishmania. The resurgence of leishmaniasis, its emergence in newer geographical areas and in newer hosts, besides changing the clinical profile of infected patients, has put forward newer challenges in the areas of diagnosis, treatment, and disease control. The disease in endemic in five continents and part of the efficient control strategy is the development of sensitive diagnostic technique. PCR methods hold promise due to its high sensitivity and specificity.

Thus the project was aimed at characterizing various *Leishmania* species present in Himachal Pradesh as it is an emerging endemic zone the disease. Apart from endemicity, atypical presentation of the disease in which visceral causing donovani species has been documented to cause cutaneous leishmaniasis. The reason for this atypical disease prevalance could be because of the host immunobiology or variance in the genome of the parasite. The project deals with the parasite genome variance which can be identified by PCR. For further analysis at molecular level, genome variance techniques like MLMT, MLST can be utilised which characterise the parasite at the strain level.

Out of our experiments carried out with 20 CL patient samples, we re-affirm that CL cases in endemic belt of Himachal are infected with *L. donovani* variants that manifest cutaneous leishmaniasis as the disease form instead of VL form. This is apparent from the amplification products obtained in PCRs based on LDK-LDS primer sets and ITS1 PCR-RFLP pattern as discussed under result sections. We got digestion pattern specific to *L. donovani* species although a higher size band undigestable with the HaeIII enzyme was also obtained. Further molecular studies based on MLMT and MLST is required to pinpoint the variation at the strain level.

Immuno-histological studies of H & E stained slides demonstrated morphometric features specific to CL patients' lesion biopsy. Out of 20, 11 lesion sections (H & E stained), exhibited atleast 2 features seen in CL skin lesions viz acanthosis, parakeratosis, hyperkerakosis, perivascular infiltration.

More rigorous analysis of Geimsa stained slides is required.

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