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Epidemiological And Molecular Study Of Leishmania In Tribal Districts Of Khyber Pakhtunkhwa

Waseem Sajjad[1], Mudassir Ahmad[2], Sania[3], Sabahat Rasul[4], Adeel Ahmed[5], Ahsan Ali[6],

[1]Department: Department of Microbiology Institute: Abbottabad University of Science and Technology, Abbottabad.
Email ID: waseemsajjad303@gmail.com
[2]Institute: Poultry Research Institute, Rawalpindi, 46000, Pakistan
Email ID: mudassir.ahmad@uvas.edu.pk
[3]Department: Department of Microbiology Institute: Abbottabad University of Science and Technology, Abbottabad
Email ID: shahsania247@gmail.com
[4]Institute: Poultry Research Institute, Rawalpindi, 46000, Pakistan
Email id: sabahatrasul@gmail.com
[5]Department: Cardiology Department Institute: Punjab Institute of Cardiology Lahore, Pakistan
Email ID: adeelahmedmbbs@gmail.com
[6]Department: Department of Veterinary Surgery Institute: University of Veterinary and Animal Sciences, Lahore Pakistan
Email id : ahsankamboh046@gmail.com

*** Correspondence authore**
Mudassir Ahmad
Institute: Poultry Research Institute, Rawalpindi, 46000, Pakistan
Email ID: Mudassir.Ahmad@Uvas.Edu.Pk

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ABSTRACT
Leishmaniasis is a vector-borne disease that is caused by an obligate intra-macrophage protozoan named Leishmania. It is assessed that more than 12 million individuals are infected worldwide with approximately 7000,000 to 1 million new cases of different clinical structures and 20,000 to 30,000 passing every year. It ranges in severity with varied signs and symptoms ranging from pathologically asymptomatic to lethal severity, causing severe human suffering, death, and great economic loss. Current study was conducted to find out the outbreak of Leishmaniasis and to construct the phylogenetic relationship based upon the sequencing of Leishmaniacytochrome-B gene. Total 100 samples positive for CL were taken from different regions of KPK. They all were confirmed by PCR of cytochrome-B gene. Among 100 patients, 71% male were children and 29% were female children. 89% children were between the age of 1-12 years and 11% were teenagers. 25 samples were selected for Sanger sequencing after the amplification of cytochrome-B gene. The cytochrome-B based sequence analysis showed that two major strains were reported in the tribal districts of Khyber Pakhtunkhwa (23, Leishmania Tropica; 1, leishmania infantum). Six species of the L.tropica were reported closely related with the Tunisia and China L.tropica species, while the leishmania infantum specie was reported closely related with L.donovani and L.infantum stated from china. The SNPs changes were reported in the alignment of the sequences which makes 5 groups of the L.tropica specie. The tribal districts of Khyber Pakhtunkhwa are important in tracking possible ingress of leishmaniasis.

1. INTRODUCTION
Leishmaniasis is a vector-borne disease. It is caused by an obligate intra-macrophage protozoan named Leishmania. It is a highly widespread disease that popularly occurs in large tropic and subtropics areas across the world. 23 known species of protozoan potentially cause Leishmaniasis [1, 2].
The taxonomical classification of Leishmania [3, 4] is as follow:

Kingdom	Protista
Sub Kingdom	Sacro Mestigopora
Phylum	Protozoa
Sub Phylum	Mastigopora
Class	Kinetoplastea
Order	Trypanosomatida
Genus	Leishmania
Major Species	*L.donovani* , L. Tropica

WHO conducted a study in 2010 which concluded that Leishmania has affected 325 million people globally and the number of people falling prey to the disease each year is increasing by 2 million. It is assessed that excess of 12 million individual are infected worldwide with approximately 7000,000 to 1 million new cases of different clinical structures and 20,000 to 30,000 passing every year. All of them reportedly belong to the tropical and sub-tropical region [5].

Leishmaniasis is endemic in 98 countries The diseases caused by the Leishmania spp. range in severity with varied signs and symptoms ranging from pathologically asymptomatic to lethal severity, causing severe human suffering, death, and great economic loss [1, 2].

There are three main clinical forms of this disease:

Visceral Leishmaniasis (VL)

Mucocutaneous Leishmaniasis (ML)

Cutaneous Leishmaniasis (CL)

Local name of VL is kala-azar. This form of Leishmania is caused by *L.donovani* and *L.infantum* in most area. Individuals do not develop wounds however in several cases, small lumps may occur partially over the entire body of the patient. Common symptoms are irregular bouts of fever, loss of weight, spleen and liver become enlarge, and anaemia. Brazil Africa India Iraq are the worst effected regions. Annually 50,000 to 90,000 estimated cases of VL occur worldwide, while 25 to 45 % cases are reported.

95% of the cases are accounted for to happen in 10 countries.I.e. Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South, and Sudan.

ML prompts the fractional are absolute destruction of the bodily fluid film of the nose, mouth and throat. Over 90% of ML cases happen in Bolivia (the plurinational province of Brazil, Ethiopia, and Peru), South America, Paraguay, Colombia, and Venezuela. It is caused by L.barazilensis, *L.major*, *and L.tropica*and characterized by lesion near the mucosal membrane. The first site of infection is small red papule that ulcerates in a few weeks. The lesions are flat, no raised rim and offer oozing infection of the ear, nose and mouth area leading to a degeneration of the cartilage and soft tissue resulting disfigurements.

CL has different names such as oriental sore, al-mohtafirah, al-domal. It is the supreme common form of leishmaniasis and causes skin lesions mainly ulcers on exposed parts of the body leaving life-long scars and serious disability and stigma. 95%of CL cases occur in America and the Middle East and Central Asia. In 2018 over 85% of new CL cases occurred in 10 countries like Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, and Pakistan. It's estimated that 600,000 to 1 million new cases occur worldwide annually [6].

CL is majorly caused by many species of Leishmania including,

L.major

L.aethiopica

L.tropica

Approximately 90% of CL is mainly reported in Algeria, Afghanistan, Iran, Brazil,

Peru, Saudi Arabia and Sudan in accumulation to Syria. Syria, especially the region of Aleppo, has continuously been at the highest risk of CL and this can be traced back to centuries ago. Therefore, the disease is locally known as “Aleppo Boil”. 1985 is reported as the year with the highest and most severe cases of CL. By 2008, approximately 30,000 cases were reported. At first, *L.major* was considered responsible for the disease mainly, but now L. tropica represents almost 90% of all CL cases. It’s one of the most important public health problems in the Syrian Arab Republic [7].

The Leishmanial genome consists of 34 Mbps. It is dispersed among 36 chromosomes with size ranging from 0.3 to 2.8 Mb [8, 9]. The members of the sub-genus Viannia (L.V) are generally blamed for CL in the central and South America. Some of these members are as follow:

(L.V) panamensis

(L.V) braziliensis

(L.V) peruviana

(L.V) guyanensis

(L.V) lainsoni

(L.V) naiffi

The most widely known vector of the species is sandfly; popularly known as Phlebotomus in the old world and Lutzomiya in the new world. Phlebotomus papatasi or P.papatasi causes *Leishmania major* (*L.major*) and P.sergenti and P.alexandri cause *Leishmania tropica* (*L.tropica*).

Taxonomic classification of Sandfly

Kingdom Animalia

Phylum Arthropoda

Sub phylum Euathropoda

Super class Antenata

Class Insecta/Hexopoda

Order Diptera

Suborder Nematocera (considered the most ancient,

Dating to the Jurassic period)

Subfamily Phlebotominae

Genus old world Phlebotomis/Sergentomyia

Genus new world Lutzomyia (with six subgenera:

Dampomyia, Pintomyia, Myssomyia,Psychodop and

Peruensis/Brumotimyia/Warileya/Psychodopyg.

Species Omleca, Flaviscutellata, Trapidoi, Diab andLongipalpis

General characteristics:

It has a weak flight for a short distance and a short level.

Nocturnal habitat.

From an epidemiological point of sight, it is important to know which Leishmania species flow in a geographical area. However, from a health perspective, the comparison between Leishmania species causing human infection might be more relevant because the choice of treatment strategy is based on geographical location and mainly in the infecting species. The currently existing diagnostic protocols for Leishmania are in-effective due to its insensitive nature and is labour-intensive nonspecific lab work. Due to under-developed, un-approved vaccines and the high toxicity of the therapeutic agents the treatment of the disease, the cure is a difficult task [10, 11].

In Pakistan Hussain et al., reported the Leishmania identification by Microscopy on GSS and molecularly confirmed by the RITS1G amplification. In their study, L. tropica was confirmed in 351 samples while *L.major* specie was identified in 6 biopsy samples, which confirms the CL circulating in the Khyber Pakhtunkhwa Pakistan[12]. Qureshi et al., [13] reported the prevalence of CL in schoolboys. The CL was identified using the microscopic examination of Giemsa-stained smears of

lesion exudates and molecularly confirmed by minicircle DNA amplification. Accurate identification of *Leishmania* species is important for monitoring clinical outcome, adequately targeting treatment, and evaluation of epidemiological risk in tegumentary leishmaniasis. This is

especially the case in regions where several species coexist and for travel medicine where the geographical source of infection is not always obvious. Species identification presently depends on parasite isolation, which is not very sensitive and not necessarily representative of parasites present in human tissues. We evaluated a polymerase chain reaction.

The cytochrome b gene is used previously for the identification of all Leishmania species using the PCR and DNA sequencing techniques [14]. To identify all genotypes of Leishmania in Federally Administered Tribal Areas (FATA) Pakistan, sequencing of the cytochrome b gene will be used for the first time in the proposed study.

LITERATURE REVIEW

Leishmania remains eukaryotic protozoan parasites of vertebrates in the family Trypanomastidae (order Kinetoplastida) [15, 16]. Distinctive of members of the order Kinetoplastida is the occurrence of a visible Feulgen stain-positive (i.e., DNA-containing) kinetoplast. Altogether members of the family Trypanomastidae remain parasitic for vertebrates or invertebrates plus undergo morphological variations during transition amongst stages of their life progression [17]. Of the numerous genera in this family, single species of Leishmania in addition to Trypanosome are human pathogens. Binary subgenera of Leishmania, L. (Leishmania) and L. (Viannia) are documented. Conservatively, at minimum 14 Leishmania species remain pathogenic for mammals, of which nine are known parasites of humans [18]. Leishmania occurs as morphologically distinctive forms. In mammals, amastigotes are an obligate intracellular parasite of mononuclear phagocytes. The elongated motile promastigote procedure originates in female sandflies (genus Phlebotomus in the Old World then Lutzomyia and Psychodopygus in the New World), which remain the only known vector for Leishmania [19]. Promastigotes keep a single nucleus and are mutable in length (15–25 μm) and form (ellipsoid to slender) [20]. The maximum prominent structures of stained promastigotes are the nucleus, the kinetoplast, and the flagellum the kinetoplast and the origin of the flagellum define the anterior region of the parasite (see 'Flagellum') [21]. Amastigotes are round to oval shape with a 2–10 μm diameter. This phase is a flagella (i.e., the flagellum does not spread past the cell borderline), but the kinetoplast and nucleus remain observable in stained amastigotes [22-24]. According to Peacock, [25] Leishmania is a parasite which has a broad range of clinical infection. *L.infantum and* L. braziliensis are two species whom genome sequencing is to be reported here. Merely ∼ 200 genes with a distinctive sharing among the three species are revealed by comparing *L.major* genome with these two specie sequences with clear preservation of synteny.

Victoire [26] investigated the significance of precise identification of *Leishmania* is important for observing clinical consequence and assessment of the wide-ranging threat in *Leishmania*especially in areas where numerous species live together. Only if the parasite is strictly segregated or isolated, species can be identified which is not essentially the characteristic of parasites present in tissues of a human. PCR test in combination with amplification of the gene gp63, followed by RFLP (Restriction fragment length polymorphism) analysis is performed in 1999, to identify *Leishmania*assembled from Peruvian patients. Our PCR test revealed a sensitivity of detection by 85% in comparison with a PCR which is DNA-based. Some expected species were found in samples of patients like: *Leishmania (Viannia) peruviana*, *Leishmania (Viannia) braziliensis*, and *Leishmania* (Viannia) *guyanensis* having similarity with previous studies of *Leishmania*s in Peru. But still some unpredicted results were also observed which elevate questions concerning about (i) the occurrence of hybrid or assorted contaminations (ii) human movement to the occurrence of the novel focus of certain species and (iii) the ability of pathogen activity of some species.

Meredith [27] explored Leishmaniasis recognition and analysis might be challenging because patient samples to contain less number of parasites. The precise PCR assay with advanced sensitivity has been established for amplification of DNA of whole Leishmania species which is based on the SSU ribosomal RNA (rRNA) gene. 3`endprimers has been created via the exact point mutations existing in the SSU region of ribosomal RNA (rRNA) of the Leishmania. PCR testing allows the usage of blood spots, biopsy material and blood. The PCR analysis from blood spots was performed and related and compared with existing data. For the detection of active VL, PCR on blood spots is a sensitive and simple process as indicated by the first results.

The precision and accuracy of the probe were calculated by DNA amplification from twenty-one strains used as reference via RLB and biotinylated primers for PCR. The probes which were species-specific discriminated among all the Leishmaniasis of the previous world, whereas genus-specific probes identified all inspected species of *Leishmania*. The results exposed that the RLB is 10- 100 times much more precise and sensitive than ITS1 PCR. The comparison between reverse line blot hybridization test (RLB) with ITS1 PCR and kinetoplast DNA (kDNA) was performed in the West Bank and Israel, by means of 67 CL patients. The results were astonishing as RLB precisely detected 58/59 positive samples, which was comparable with the results of kDNA PCR which detect 59/59 and much improved than ITS1 PCR which showed 50/59 of the result. In short, for diagnosis and characterization of old-World CL, RLB can be used.

Iqbal [28] stated Leishmaniasis transmission is because of female sandfly having an infection, affecting humid and underdeveloped areas. The spreading of disease particularly in prevalent areas is presented by numerous Leishmania species so only clinical inquiries are not enough for representation and identification of the specific parasite. Suspected forty-one patients of CL samples including both genders and having variability in age groups and locality were registered for the examination. Samples were gathered from the lesions active edge followed by staining with Giemsa stain and approved by microscopic techniques. For Leishmania identification, PCR product examination was performed by 2% agarose gel electrophoresis. DNA extraction was performed consuming proteinase k followed by amplification through precise primers of kDNA. The fragment of *Leishmania Tropica* having length of 186bp was specified approving it as a widespread species in KPK, Pakistan. By comparing PCR assay with Giemsa stain and culture methods, PCR is a very sensitive and consistent technique to identify Leishmania DNA.

Rehman [29] studied CL is a lethal disorder caused by parasites. In the current study, 45 objects from the region of Hangu, who were infected by the Leishmania infection caused by L. tropica, were inspected through PCR. When observed under microscope, it revealed 87.61% (99/113), 53.98% (61/113) cases proving that PCR is far more accurate than microscopy. During the trials, PCR technique was used to identify 186bp L. tropica whereas the existence of L. tropica exposed the diseased person serum through SDS method analysis.

Abd El-Salam [30] investigated the CL patient's samples were detected for *Leishmania tropica* species presence collected from Kohat (KPK) and was recognized by PCR, culture methods and microscopic techniques. The results indicate that out of 113 patient samples, examined through PCR showed 87.61% of samples, whereas microscopic techniques and culture methods give 53.98% and 46.90% of specie dominance. PCR assay resulted into186bp *Leishmania tropica* gene because of its high sensitivity ratio in comparison with other detection techniques.

According to Wang [31], In Wenxian County, Gansu Province the study was performed to evaluate *L.infantum* contamination of a Kala-azar area. Two sets of primers, RV1-RV2 and K13A-K13B were designed for DNA based PCR of Leishmania species followed by rK39-dipstick and ELISA. The results clearly indicate positive ratios of ELISA, rK39-dipstick, and PCR with percentage of 24.2%, 0 and 30.9% respectively. PCR assay regulated based on RV1-RV2 and K13A-K13B pair of primers is a delicate and precise technique for detection of the contamination. Chaudhary [56] says that Intracellular protozoal parasites from Leishmania species is the major cause of the disease called CL whose unusual appearance and extensive development is due to suppression of Immune response triggered by HIV infection. HIV co-infection and scattered CL has been emerged as an exceptionally severe infection. The strange case is reported with prevalent cutaneous presentation concealed as lepromatous leprosy. A person with HIV +ve, age 38 years, having 1 year history of developed papule and nodular outbreaks on face and margins with intrusions of oral and nasal mucosa.

According to Bettini and Gradoni [32], Mediterranean Sea bordered countries revealed the circulation of canine leishmaniasis and frequency of contamination. The scientific profile and most figured laboratory calculations are studied. Canine and human forms of visceral leishmaniasis together are the mediators of *L.infantum.* Furthermore, the procedures of inquiry and regulation of canine leishmaniasis are debated.

Berman [58] examined the latest concern of Leishmanias with respect to transportable medicine, human distresses, operation of veterans and impurity with HIV is of significance to physicians. Over the last 10 years chemotherapy for the cure of this disease has considered. For VL various therapies including amphoteric in B and latest lipid formulas are active which are in comparison with pentavalent antimony. For the management of CL high dosage of antimony treatment is mostly operative.

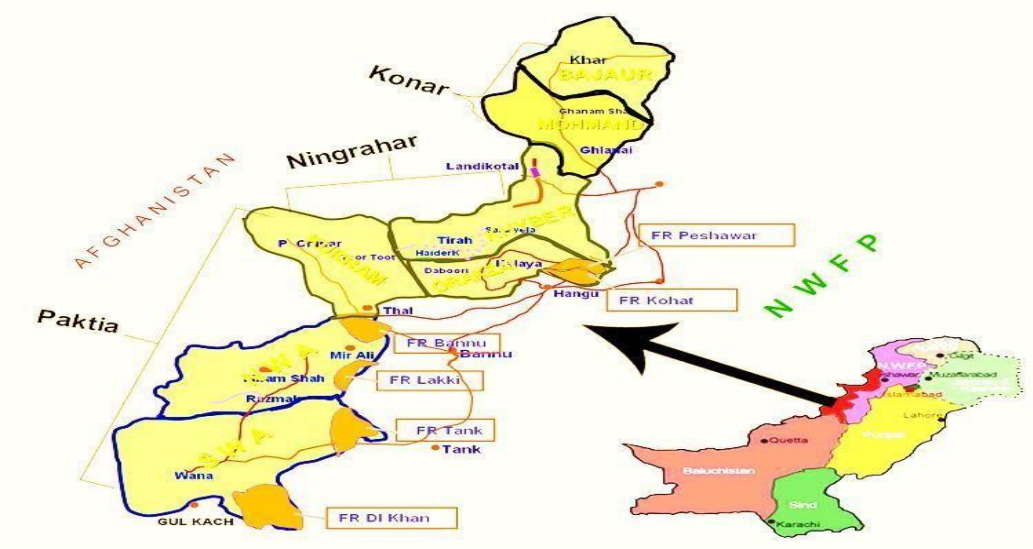
MATERIALS AND METHODS

Sample Collection

In the current studies the samples were collected from different regions of FATA, KPK. The geographical area is shown in Figure 3.1. The samples were collected from patients who agreed to participate in this study and signed the informed consent form.

The samples were collected by cleaning the skin lesions with cotton soaked in 70% ethyl alcohol and left to dry. It was followed by injecting the o.1ml normal saline into the active borders of the skin lesions using an insulin needle and then aspirating the fluid in sterile 1.5ml tubes. The samples were properly labelled and stored in 0.4ml absolute ethanol and stored at room temperature for further studies.

Figure 3.1 Geographical map of the study area in KPK



DNA Extraction

100mg of frozen ground tissue and 3ml chilled cell lysis solution was added to pestle and mortar and homogenized thoroughly using 50 strokes and was transferred to a 15 ml centrifuge tube then lysate was incubated at 65°C for 60 minutes. Proteinase k solution was added to the lysate and inverted 25 times and then incubated at 55°Cover night until tissue particulate dissolved. 15µl RNAse A solution was added to the cell lysate and mixed the samples by inverting the tube 25 times and incubated at 37°C for 60 minutes. Samples were cooled at room temperature by placing on ice for 3 minutes. 1 ml protein precipitation solution was added to the RNAse a treated cell lysate. To mix the protein precipitation solution uniformly with the cell lysate it was vortexed vigorously at high speed for 20 seconds and centrifuged at 2000 x g for 10 minutes. The precipitated protein formed a tight pellet. The supernatant containing the DNA was poured into a clean 15 ml centrifuge tube containing 3 ml 100% isopropanol. The samples were mixed by inverting the tubes gently 50 times and then centrifuged at 2000x g for 3 minutes the DNA was visible as a white pellet. The supernatant was poured and drained from tube on clean absorbent paper and 3 ml 70% ethanol was added and tube was inverted several times to wash the DNA pellet. Then centrifuged at 2000 x g for 1 minute and carefully poured off the ethanol. The tube was inverted and drained on clean absorbent paper and allowed to air dry for 15 minutes. 150µl DNA hydration solution was added. DNA was rehydrated by incubating sample for 1 hour at 65°C and stored at -20°C till further use.

Qualitative and Quantitative Analysis of DNA

1% agarose gel was used for the qualitative analysis of genomic DNA. The concentration of DNA was determined by Nano drop.

Cytochrome-B Gene Amplification

The leishmania Cytochrome-B (LCB) gene was amplified from the isolated genomic

DNA with primer set by PCR. The primer sequences of LCB are described below:

LCB forward 5′ - GGTGTAGGTTTTAGTTTAGG- 3 primer

LCB reverse 5′ - CTACAATAAACAAATCATAATATACAATT - 3′ primer

Dilution of Primers used to Amplify LCB Genes

Dilution for the primers of LCB were prepared, which were further diluted to make working solutions. Primer dilutions are given in Table 3.1:

Table 3.1 Primer dilutions for E6 and E7 genes

Oligo name	Stock Solution	Working Solution of primers (10 pmol)
LCB forward primer	300µl of PCR water was added And mixed well.	10µl of stock solution + 90µl of PCR water
LCB reverse primer	290µl of PCR water was added And mixed well.	10µl of stock solution + 90µl of PCR water

LCB Gene PCR Amplification

PCR reaction mixture was prepared with the following reagents Table 3.2.

Table 3.2 PCR reagents used for the amplification of LCB gene.

Reagents	Quantity
10X PCR buffer	2.0µl
25mM $MgCl_2$	2.4µl
10mM dNTPs	1.0µl
Forward primer	0.5µl
Reverse primer	0.5µl
Template	3.0µl
Taq DNA polymerase	1µl
Nuclease free water	9.6µl
Total reaction volume	10µl

The PCR was optimized and performed by the following reaction conditions Table 3.3. PCR products were analysed on 1.5% agarose gel.

Table 3.3 PCR optimization reaction conditions for LCB gene amplification.

Step	Temp	Time
Initial denaturation	95°C	3 minutes

Denaturation	95°C	45 seconds
Annealing	53°C	45 seconds
Extension	72°C	2.5 minutes
Final extension	72°C	10 minutes

Purification of PCR Product from Agarose Gel

The PCR product was excised and purified from agarose gel by Pure Link Quick Gel Extraction Kit (K2100-12).

Excising and dissolving the gel

Heat block was equilibrated to 50°C. Minimal area of gel containing the DNA fragment of interest was excised. The gel slice containing the DNA fragment was weighed using a scale sensitive to 0.001g. Gel solubilisation buffer L3 was added to the excised gel in the tube size indicated in the following Table 3.4. The tube with the gel slice and buffer L3 was placed into 50°C heat block for 10 minutes. The tube was inverted every 03 minutes to mix and ensure gel dissolution. After the gel slice appeared dissolved, the tube was incubated for additional 5 minutes.

Table 3.4 LCB amplified product cleaning protocol from the Gel

Gel	Tube	Buffer L3 Volume
<2% agarose	1.7ml polypropylene	3:1 (i.e., 1.2ml buffer L3: 400 mg gel piece)

Purifying DNA using a centrifuge

The DNA was purified using a centrifuge with the following method.

The dissolved gel piece was loaded onto a Quick gel extraction column inside a wash tube. The column was centrifuged at >12000 x g for 1 minute. The flow through was discarded and the column was placed into the wash tube. 500ul wash buffer (W1) was containing ethanol was added to the column. The column was centrifuged at 12000 x g for 1 minute. The flow through was discarded and the column was placed into the wash tube. The column was centrifuged at maximum speed for 2 minutes to remove ethanol. The flow through was discarded. The column was placed into a recovery tube and 30ul of elution buffer was added in the centre of the column. The tube was incubated for 1 minute at room temperature. The tube was centrifuged at >12,000 x g for 1 minute. The elution tube containing the purified DNA was stored at -20°C till further use.

Sequencing of PCR product

Sequencing of the PCR amplified fragments was performed using both gene specific reverse and forward primers of the LCB gene. Sequencing was performed according to the manufacturer's instructions (Big Dye Deoxy Terminators; Applied Bio systems, Weiterstadt, Germany). Sequencing with both forward and reverse gene specific primers was performed on automated sequencer (Applied Bio systems; 3100 DNA Analyzer). The reaction mixture for single reaction reagents for sequencing are reported in Table 3.5. The cycling profiles for LCB gene sequencing PCR are given in Table 3.6.

Table 3.5 The reaction mixture for single reaction reagents for LCB gene sequencing.

Reagents	Volume
Big Dye	1µl
5X Sequencing buffer	1.5µl
Primer	1µl
Sterile water	4.5µl

Template	2ul
Total Reaction Volume	10 µl

Table 3.6 The cycling profiles for LCB gene sequencing PCR

Step	Temp	Time
Initial Denaturation	96°C	1 minute
Denaturation	96°C	30 seconds
Annealing	50°C	15 seconds
Extension	60°C	4 minutes
Final Extension	60°C	4 minutes

Ethanol Precipitation of Sequencing PCR product

The sequencing PCR product was moved into plate. 2ul 3M NaEDTA and 2µl of 125M EDTA was added into wells. 25µl of absolute ethanol was added to each well. Plate was vortexed and centrifuged. It was incubated at room temperature for 30 minutes. Plate was centrifuged for 20 minutes at 13000 rpm at 4°C. Ethanol was removed by inverting the plate and pellet was washed with 50µl of 70% ethanol. The plate was again centrifuged for 15 minutes at 13000 rpm at 4°C. Ethanol was removed by inverting the plate and the pellet was air dried. The pellet was rehydrated in 13µl formamide and was incubated at 95°C for 5 minutes. It was kept on ice for 5 minutes. Then handed over to sequencing lab for sequence analysis.

Sequencing of *Cytochrome-B* gene

The beginning of Next Generation Sequencing (NGS) technology has provided the means to directly analyse the primary cells or tissues genetic material of any species in a high throughput method for mutagenesis effects of possible genotoxic agents. The gold standard for observing the sequence mutations in the DNA is Sanger Sequencing

[59]. the positive samples were selected for Sanger sequencing to confirm the Leishmania species based on cytochrome-B gene. The results of DNA sequencing were compared to other sequences through NCBI Blast for confirmation. The redundant sequences at the start and end were trimmed for the quality refinement of the sequences.

3.7. Cytochrome-B phylogenetic analysis

The sequence chromatograms were analysed using the Snap Gene 3.2.1[33]. Reference sequences selected from GenBank were used with the chromatogram data for maximum likelihood trees in parallel, Bayesian analysis. The sequences were aligned using CLC Genomic workbench version 20.0.4 [34] for single nucleotide polymorphisms (SNPs). The aligned sequences were selected for Maximum likelihood tree using the neighbourhood joining construction model. Jukes Cantor Nucleotide substitution model was used with bootstrap analysis of 1000 replicates. The cytochrome-B sequences from the current study have been submitted to GenBank (MW387226 - MW387249).

RESULTS

Data Collection

The data were collected from new merged district of Khyber Pakhtunkhwa included district Bajaur, Kurram, North Waziristan, South Waziristan, Mohmand and Khyber district respectively where the prevalence of leishmaniasis are more frequent through a proper questionnaire including age group of 1 to 20 years, both gender, district, treatment and vaccination. The positive samples were selected for data collection which are shown in table 4.1. The infection sites from which samples were collected are shown in Figure 4.1.

Figure 4.1 The infection sites from which samples were collected.

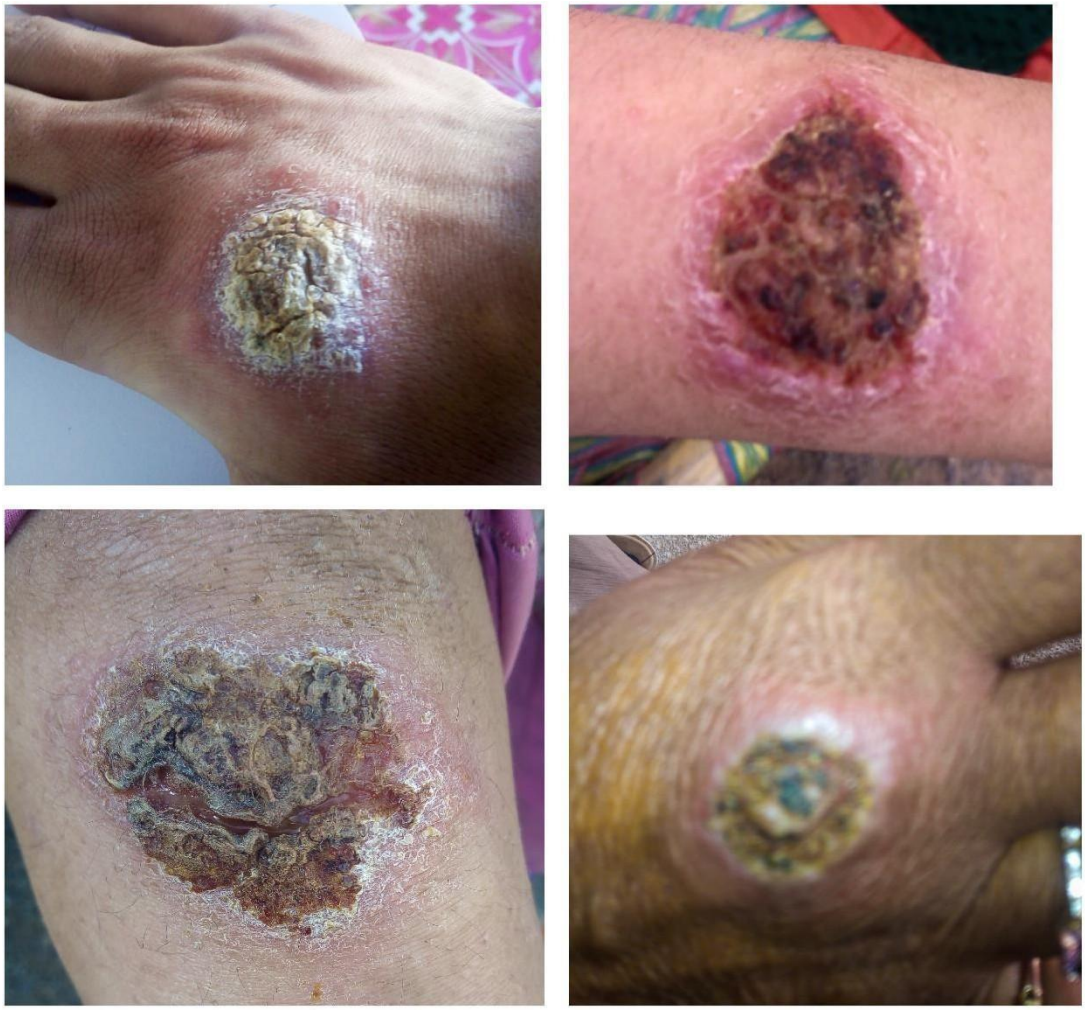


Table 4.1 Positive samples data which were selected for sample collection.

S.No	Accession Allotted	Name	Age	Gender	Sample Name	District/Provence	Body Part	Vaccination	Treatment
1	MW387226	Muddasir	10	Male	NW-1	North Waziristan/KPK	Leg	Glucantime	10 days
2	MW387227	Asad	12	Female	NW-2	North Waziristan/KPK	Arm	Glucantime	15 days
3	MW387228	Aftab	11	Female	NW-3	North Waziristan/KPK	Face	Glucantime	12 days
4	MW387229	Rohail	6	Female	NW-4	North Waziristan/KPK	Ear	Glucantime	13 days
5	MW387230	Shams	7	Female	NW-5	North Waziristan/KPK	Ear	Glucantime	9 days

6	MW387231	Naveed	9	Female	NW-6	North Waziristan/KPK	Face	Glucantime	6 days
7	MW387232	Muzamil	6	Male	NW-7	North Waziristan/KPK	Leg	Glucantime	6 days
8	MW387233	Bacha Khan	19	Male	NW-8	North Waziristan/KPK	Arm	Glucantime	7 days
9	MW387234	Dilbar	10	Male	NW-9	North Waziristan/KPK	Face	Glucantime	5 days
10	MW387235	Hossain	10	Male	NW-10	North Waziristan/KPK	Arm	Glucantime	4 days
11	MW387236	Rohail	5	Male	NW-11	North Waziristan/KPK	Face	Glucantime	17 days
12	MW387237	Rizwan	7	Male	NW-12	North Waziristan/KPK	Leg	Glucantime	16 days
13	MW387238	Sadaqat	8	Male	NW-13	North Waziristan/KPK	Nose	Glucantime	20 days
14	MW387239	Ali Khan	7	Male	NW-14	North Waziristan/KPK	Ear	Glucantime	nil
15	MW387240	Sadaqat	9	Male	SW-1	South Waziristan/KPK	Eye	Glucantime	nil
16	MW387241	Safeer	13	Male	SW-2	South Waziristan/KPK	Eye	Glucantime	5 days
17	MW387242	Jabran	14	Male	SW-3	South Waziristan/KPK	Arm	Glucantime	6 days
18	MW387243	Danish	11	Female	SW-4	South Waziristan/KPK	Arm	Glucantime	14 days
19	MW387244	Waleed	6	Female	SW-5	South Waziristan/KPK	Face	Glucantime	9 days
20	MW387245	Aisha	5	Female	SW-6	South Waziristan/KPK	Face	Glucantime	8 days
21	MW387246	Ruqayya	7	Male	SW-7	South Waziristan/KPK	Finger	Glucantime	12 days
22	MW387247	Munazza	8	Female	SW-8	South Waziristan/KPK	Face	Glucantime	11 days
23	MW387248	Maryam	9	Male	SW-9	South Waziristan/KPK	Face	Glucantime	days
24	MW387249	Khadija	10	Male	DAK-1	Darra Adam Khil/KPK	Face	Glucantime	10 days

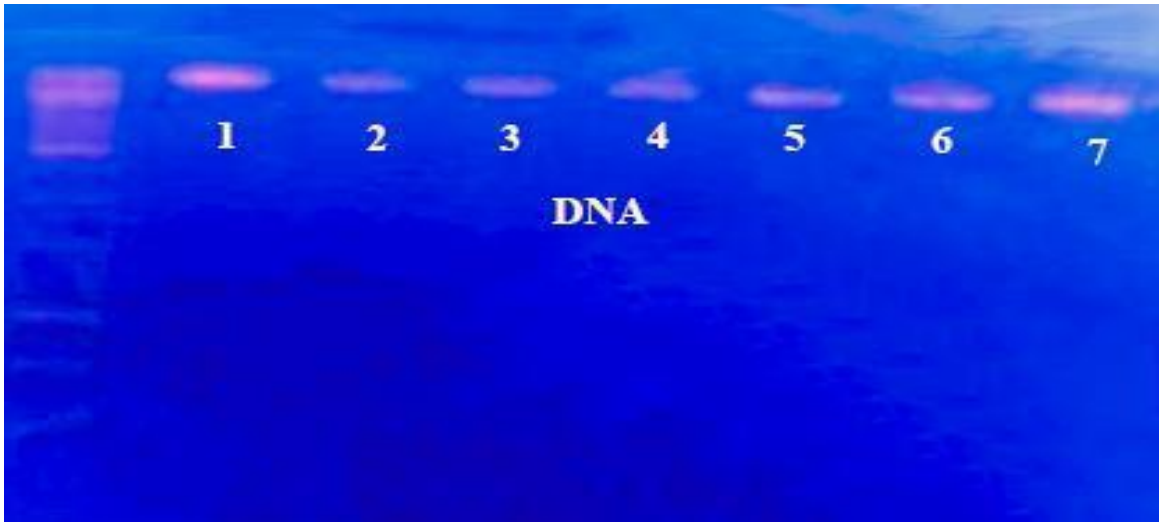
Quantitative analysis of DNA

DNA concentration was measured by Nanodrop. Absorbance of all DNA samples was measured. DNA concentration of all samples was ranging from 700-800 ng/µl.

Qualitative analysis of DNA

DNA was isolated from lesions of clinically suspected CL patients and was confirmed by 1.5% agarose gel electrophoresis (Figure 4.1). Clear intact bands were observed in each sample. DNA from all subjects was stored at -20°C.

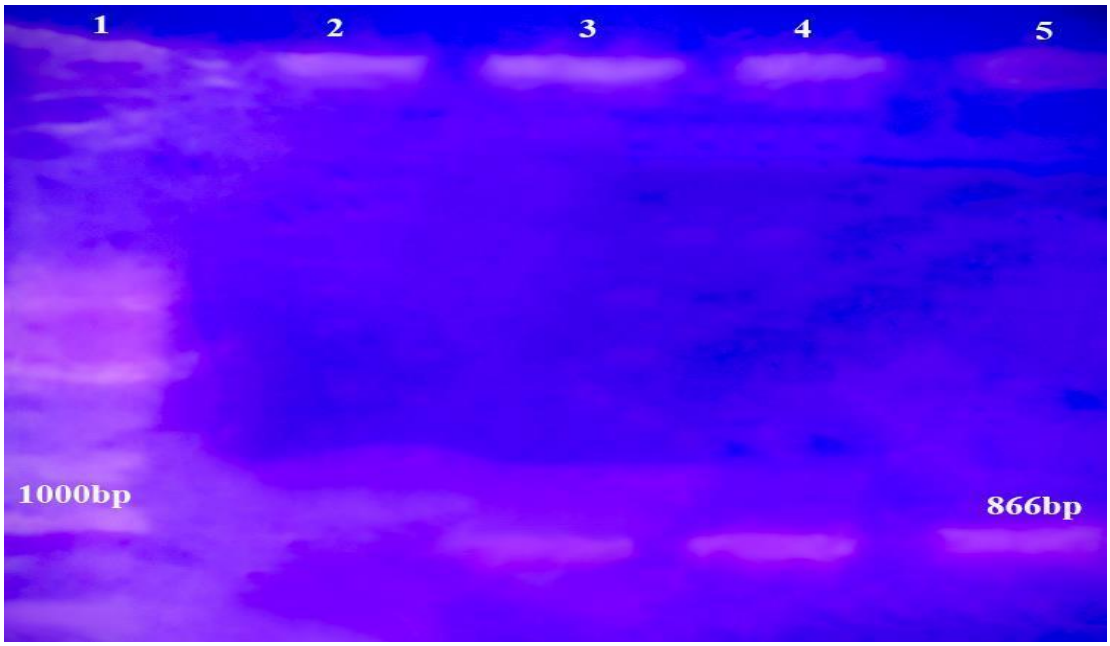
Figure 4.2 Agarose gel electrophoresis (1.5%) showing presence of DNA.



LCB Gene Amplification

LCB gene was amplified with annealing temperature of 52°C. The electrophoresis analysis showed single band which indicated the presence of positive PCR results. By comparing with DNA ladder the size of the PCR product was found to be 866 bp as shown in Fig. 3. Lane 1 has 1Kb DNA ladder (Thermo Scientific # SM1163), Lane 2 has negative control while Lane 3-5 contains PCR product present in CL patients. All 100 samples were positive for PCR. The sequencing of these PCR positive samples is in progress.

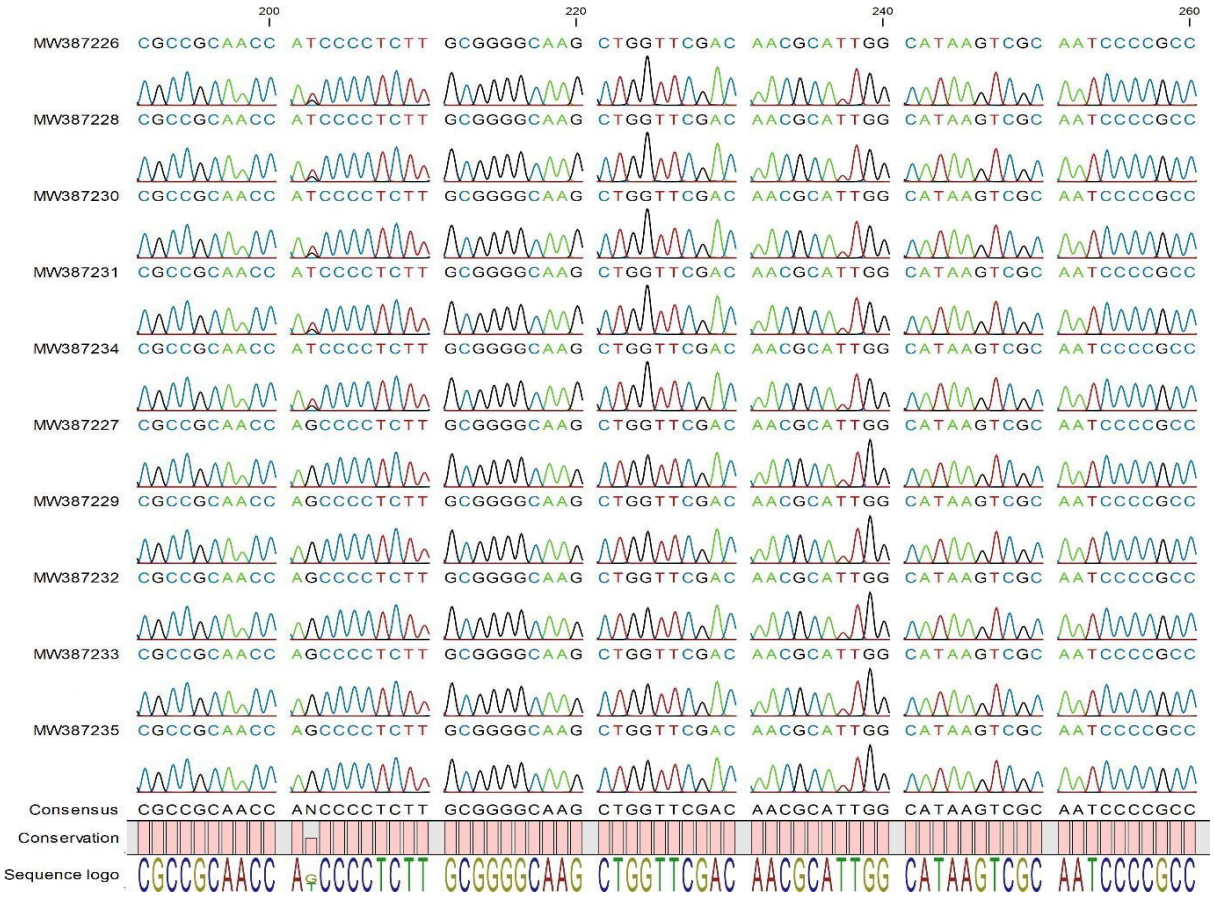
Figure 4.3 Amplification of LCB gene in CL patient's samples. Line 1 shows 1 Kb DNA Ladder (Thermo Scientific # SM1163), Line 2 shows Negative Control and Line 3-5 shows Patient's DNA.



Sequencing of *Cytochrome-B* gene

The Amplified PCR products after purification through gel cleaning kit were sent for Sanger sequencing to" Centre of Excellence in Molecular Biology". The chromatogram alignment of the sequences is reported in Figure 4.6.

Figure 4.410 samples sequences aligned of cytochrome-B gene.

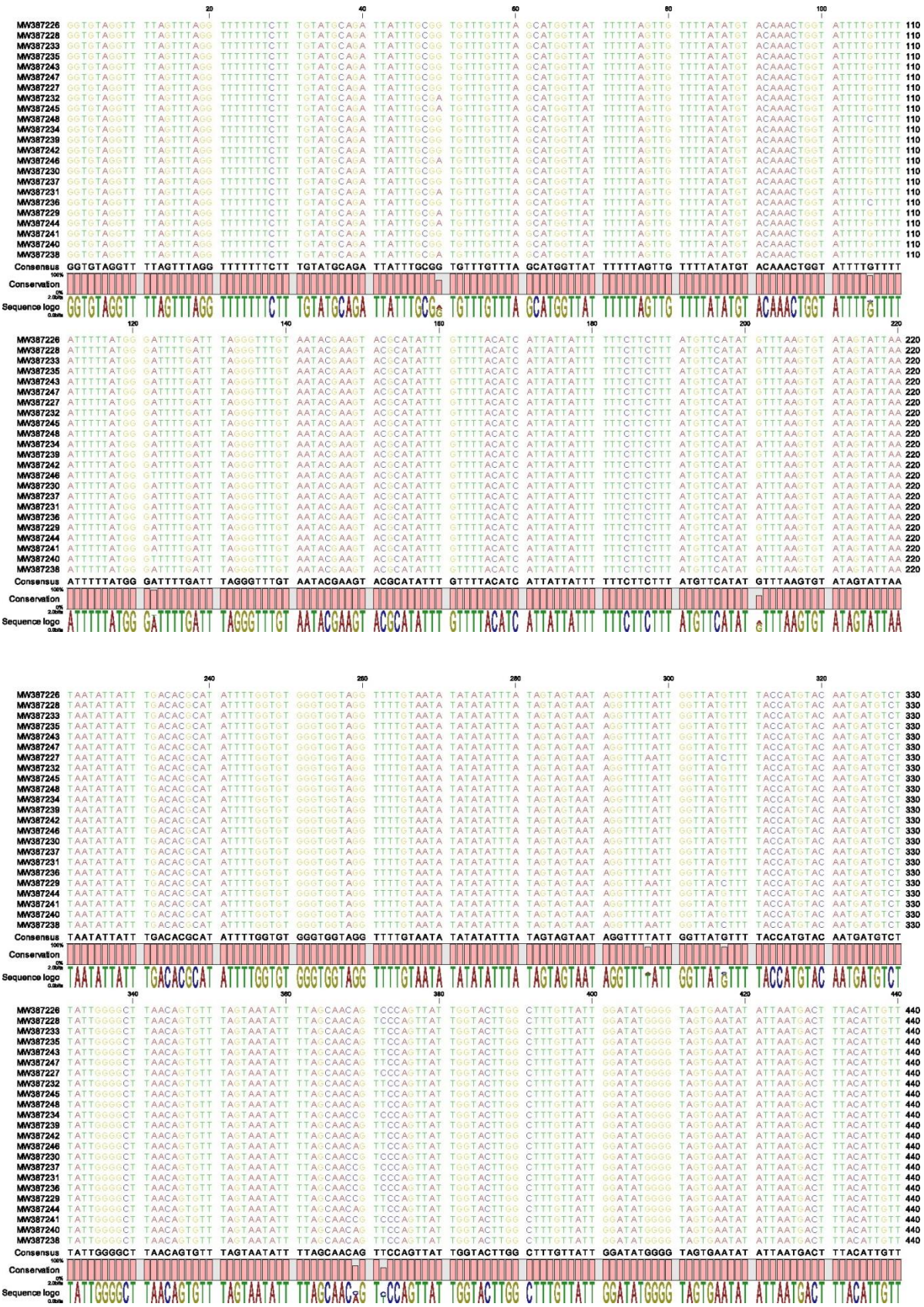


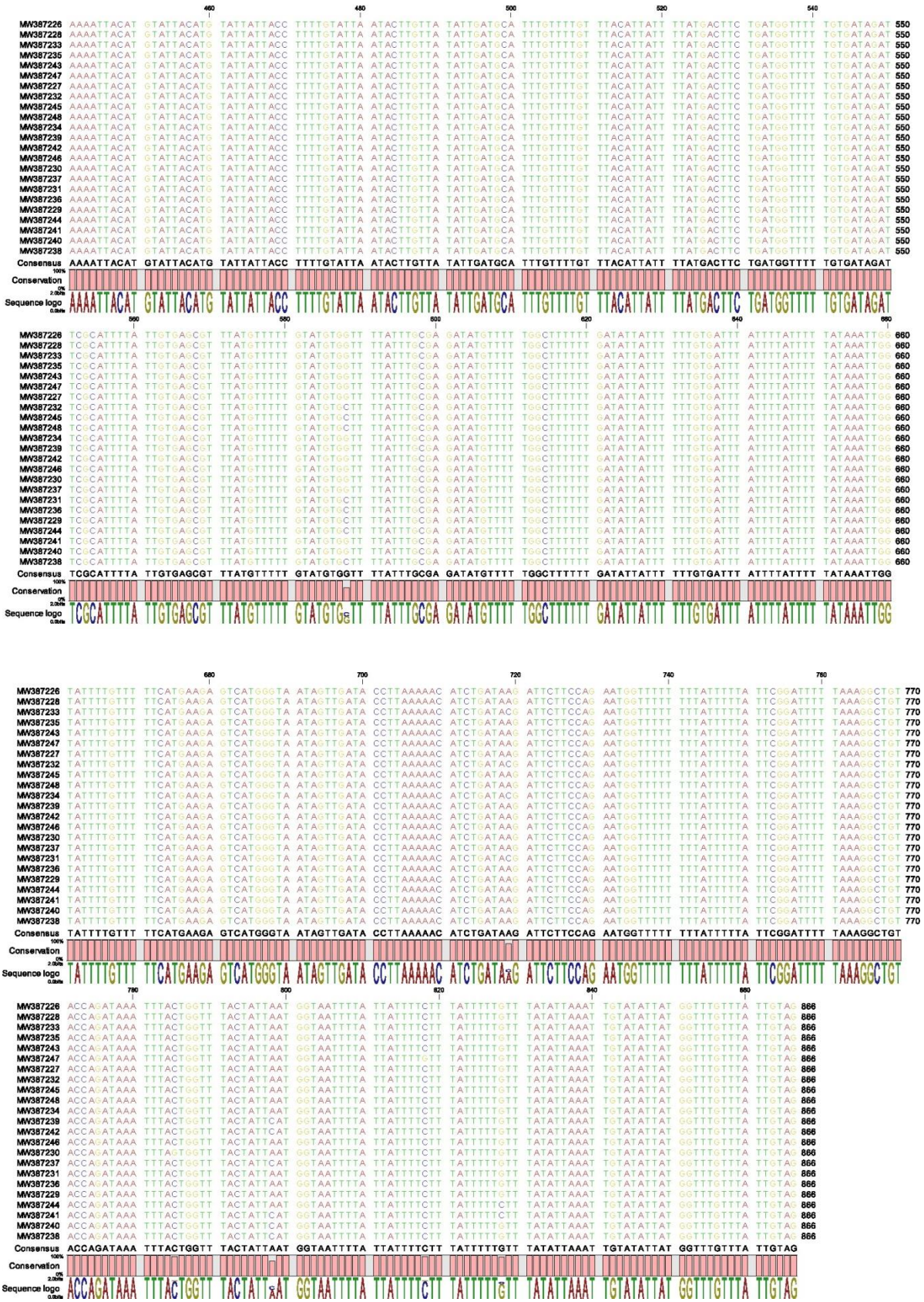
Sequence Alignment

After sequencing of cytochrome-B to examine the conserved region in the said gene with reference sequence available online in NCBI by accession number AB095959.

Sequence alignment was carried out by using Mega 6.06 manually as shown in figure.

Figure 4.5cytochrome-B sequence alignment.

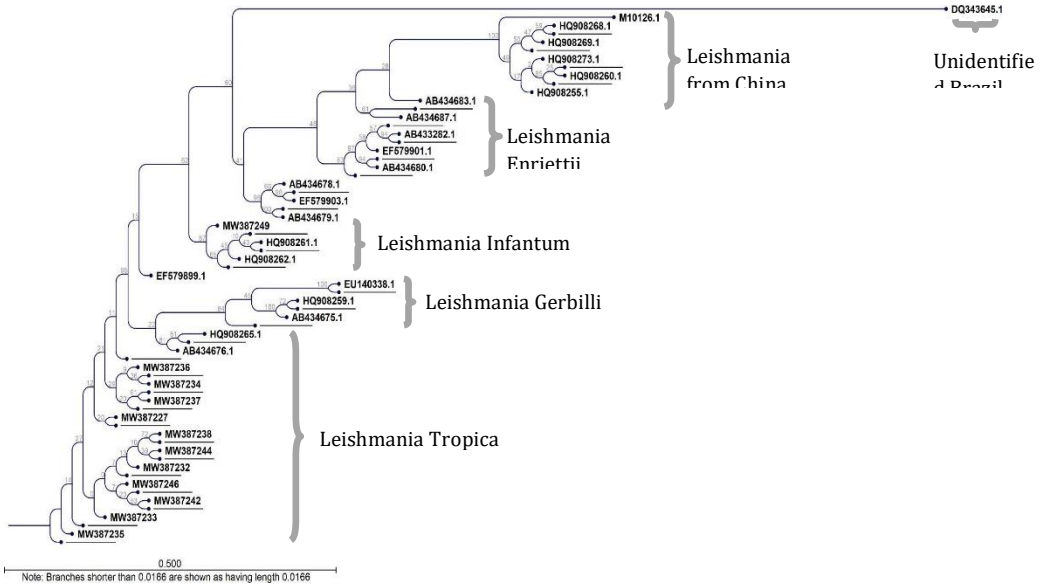



Consensus
Conservation
Sequence logo

Cytochrome-B phylogenetic analysis

Among all the Leishmania *cytochrome-B* gene sequences 65 were selected for the current study. All the 65 sequences were analysed in the present study (24 from the study and 41 from Genbank). Amongst all the *Leishmania Tropica cytochrome-B* sequences have variable sites in the 23 sequences alignment. The single nucleotide polymorphism (SNPs) within the *Leishmania Tropica* dataset categorized the samples into 5 groups. The most common genotype in the current dataset was *Leishmania Tropica* observed among 23 of 24 samples in the Khyber Pakhtunkhwa different tribal areas. One strain among 24 was observed as the *Leishmania infantum*. In Khyber Pakhtunkhwa tribal districts 2 genotypes of Leishmania are reported. In figure 4.5 sub- clusters of clones were reported in *Leishmania Tropica* strains although not supported by significant bootstraps for example > 50%. Overall *Leishmania Tropica* strains have homogeneity from different tribal districts.

Figure 4.6Phylogenetic tree constructed on neighbourhood joining method with 1000 boot strapping.



DISCUSSION

Leishmaniasis is a kind of disease which is vector-borne disease. The main reason of leishmaniasis disease is obligate intra macrophages protozoan which is called leishmania. It is a highly endemic disease that popularly occurs in large sub tropic and tropic region across the world. The diseases caused by the Leishmania spp range in severity with varied signs and symptoms ranging from pathologically asymptomatic to lethal severity, causing severe human suffering, death, and great economic loss. Different kind of study show various epidemiological ratio L. tropica in entire region of Pakistan [37]. The mortality ratio of CL in southern region in KP is may be attributed to immigration in high ratio of foreign moved individual from CL high mortality tribal area (FATA) [35] [36]. 23 known species of protozoan potentially cause Leishmaniasis Research show that in the early stage from district Dir. upper and Dir. lower L. Tropica has been molecularly studied. But due to not proper diagnosis system L. tropica widely spread in district Dir. [38] [1, 2]. Approximately more than 12 million individual are affected worldwide with about 7000,000 to 1 million new cases of various clinical forms and 20,000 to 30,000 died annually [5]. In Current study we extract the selected sample DNA the using PCR for amplification of that DNA and then did Sequencing for identification of leishmania species. L. tropica as dominant species in Southern region KP. Previous research don't show any epidemiological and molecular base study of Leishmania [38][36].The diseases caused by the Leishmania spp. range in severity with varied signs and symptoms ranging from pathologically asymptomatic to lethal severity, causing severe human suffering, death, and great economic loss [1, 2]. In the present study, the data show the prevalence of CL in tribal districts of Khyber Pakhtunkhwa Pakistan as the country went through several wars, interior conflicts, economic crises, and sanctions overthe previous 27 years, massive fluctuations in the number of reported CL cases can be noticed. The CL outbreak occurred in areas of tribal districts of Khyber Pakhtunkhwa Pakistan. The main and common form of leishmaniasis is CL causes skin lesions mainly ulcers on open parts of the body leaving life-long wounds and serious, debility and stigma. 95% of CL cases occur in America and the Middle East and Central Asia. In 2018 over 85% of new CL cases occurred in 10 countries like Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, and Pakistan [6].The present study

showed 100% prevalence rate of cutaneous leishmaniasis. Hayat [46] confirmed 93.5% positive cases, by PCR in Dir. district. Abd El-Salam [49]. PCR examination showed 87.61% positive results. The main causing factor of high incidence in our study reason could be that only clinically diagnosed patients were involved in our studies, who were already taking the treatment.

89% children under the age of 12 were infected in comparison to 11% teenagers. The greater prevalence of leishmaniasis disease quantity in younger age in the current study is maybe due to the unwell developed immune system. Similar rates are also recorded by Marri [38] and Bosan. The male: female ratio recorded in the present study was 2.4:1 that is similar to results observed by Sana-Ullah, Nisar [36] the high ratio in male's prevalence is due to cultural in males was probably due to the cultural practises of the region where the females use full-covered dresses, which reduce the risks of sand fly bites. Research conducted by AL Jawabreh which show that women are obliged to be home before evening [37]. In the current research study, all the cuts were present on the uncovered parts of the physique i.e. for the sandfly bites the sites available. Nisar [36] recorded similar results. In the present study single lesions were observed in most of the patients, which is supported by many. To our knowledge, this is the first record of a phylogenetic study in all districts of Khyber Pakhtunkhwa concerning *L.major* causing CL prevalence. The findings are also significant for future creation of vaccines against the *Leishmania* strain in Pakistan and it is important to understand the global prevalence and epidemiology of the *Leishmania* strains. Different area in Pakistan shows different prevalence like afghan refugee and timergera prevalence was investigated in 1997 [32]. Till Now no proper variation was originate between the kind of lesion renowned in local and Afghan refugee's individual of

Pakistan, who had pouring lesions frequently [36]. The high prevalence ratio of CL in Landi Kotal influence be due to colonisation of Afghan refugees though there is a threat to Spread CL to another region in Pakistan. The incidence ratio of leishmaniasis as find out by [33]. Exposed it in age group reaching from 1 to 5 and 5 to 10 year. In our current study show that the prevalence of infection in the group age of 11 to 20 years and show relatively high incidence beneath ten years of age in the sample. This result influence due to high rate of awareness and strong immune system in adults out insect such as sand flies. Research shows that for controlling the high spread rate of leishmaniasis to use bed Net dominancy. The Causes of leishmania is very high when individual is sleeping outside the room at nigh time [31]. The current study was conducted in all tribal districts *L.tropica* was found in 23 samples. 1 of the patient with diagnosis leishmania *L.infantum.* It is the major study that L. tropica circulating in all tribal district's region of KP. Rodents are the most common reservoirs of L. tropica. CL caused by L. tropica in Pakistan mostly in different region of tribal districts of KPK province and chamman border. It was typically studied to be anthroponomical.

The purpose of the present study was conducted due the high speed of leishmaniasis disease in 2019 as compared to last 20 year which is less then present epidemiology study. In mazar-e-Sharif province of Afghanistan it's zoonotic but mortality frequency of leishmaniasis high in every region of Afghanistan [29]. In KP province Phlebotomus paptasi and Phlebotomus sergeant these are the imagine the main vector of zoonotic CL and anthroponotic CL individually are generally spreading in different region of KP. The zoonotic CL address the three autochthonous *L.major* cases short of migrant history in Tribal area of KP which is waterless territory, seasonal crops, consuming irrigation systems of waterways and mechanisms that favors' condensed residents of paths and rodents. The great concentrations of the normal zoonotic CL animal reservoirs and its related sand-fly vector as the main origins in creating new infection foci. However, to date animal reservoirs are still unidentified in every region of Pakistan [36]. There is different human factor involve for zoonotic CL like mass movement of publics throughout battle within relocated societies in current incident and natural adversities, climate changes improvement of farming and business projects. Also, the main causing factor of Leishmaniasis disease in subjected area due to military operation that's Raddul Fasad and operation Rahe Nijjat because due to travelling of the individual the leishmania disease was spread in different tribal region. In early-stage study was investigated to find out the leishmania species in a specific region of KP but in the current study we selected all the region of tribal area of KP for the identification of leishmania species.

The current result approximately L. main expect in upcoming the chance of facing new widespread zoonotic CL centres in further southern Regions of KP. Since inside the particular polymorphisms were originate along with Pakistani main residents [37].as well as between *L.major* populations in, the Middle East, Africa and Central more research like iso enzyme electrophoresis, multi- locus sequencing method and microsatellite study are inexorable toward expose the source of *L.major* strains recognized in current inquiry . This sort of realities shows that a dynamic stage for coming Accusations of vectors, eco- epidemiologic picture, creature stores, opening in KP territory. This is the principal concentrate based on molecular openness of and L tropica exist one next to the other from FATA region of KP.

Its reality that our research work has shown that we find L.*tropica* supreme specie in tribal districts of KPK Pakistan. from previous three year different research were conducted at tribal area of KPK Pakistan for the evaluation of the epidemiological and molecular study of leishmania but no one identified the *L.tropica* spp. were find in the leishmania species at tribal districts. In addition, a piece of creature lake host ought to likewise be explored to prescribe to decide whether the occasions

set off by *L.major*.

2. CONCLUSION

In conclusion, the approval of genotype of the *L. tropica* strain will highlight our grateful of the epidemiological and molecular study of the subject disease. For future our research will show how to control the L. tropica disease and also our research will aware the population for the leishmania disease.Additionaly ,around the globe this area could be considered for the research work regarding *L.tropica* disease.in non endemic nation where there is also couple of reservoir hosts and sandfly vectors are present. The sequence analysis of the Leishmania Cyt B gene showed genetic polymorphisms in *L.major* and L. tropica and a reasonable connection among the genetic heterogeneity of the parasite, in human the result of the disease was performed geographically and experimentally. Furthermore, the cyt B gene sequencing on leishmania spp will be considered for the future study. We did phylogenetic analysis and Genetic polymorphism on based in our result. We conclude that various kind of clones of parasites on diverse populations are circulating in endemic areas.In tribal districts of Khyber Pakhtunkhwa Pakistan. It is significant contribution of our research work that we recognize a novel species of leishmania which is *L.tropica* at tribal districts Khyber Pakhtunkhwa as previous study did not show any presence of the above mentioned specie.

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