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## Molecular detection of cutaneous leishmaniasis in human at district Hangu Khyber Pakhtunkhwa, Pakistan

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**Abstract**

Cutaneous Leishmaniasis is parasitic disease which causes severe morbidity and mortality. The present study identified the leishmania infection caused by *Leishmania tropica* in Hangu. A total of 45 samples were examined through PCR, Microscopy which showed 87.61% (99/113), 53.98% (61/113) thus the sensitivity of PCR is very high as compared to microscopy. During the study 186 bp *Leishmania tropica* was identified through PCR and presence of *Leishmania tropica* showed the infected person serum through SDS method analysis respectively.

**Keywords:** Microscopy, PCR, and *Leishmania tropica*

**Introduction**

Cutaneous leishmania is a single-celled, parasitic protozoan. Each species of *Leishmania* is adapted to transmission in a specific species of sandflies, which act as biological Vectors, spreading the CL (*Leishmania tropica*) to humans. *Leishmania tropica* is the primary cause of Cutaneous leishmaniasis (also known as "Aleppo boil," "Baghdad boil," "Bay sore," "Biskra button," "Chiclero ulcer," "Delhi boil," "Kandahar sore," "Lahore sore," "Leishmaniasis tropica," "Oriental sore," "Pian bois," or kalaazar Symptoms, although most cases begin to show symptoms between two and six months. Symptoms of this disease can include a prolonged fever, a decreased appetite, weight loss, signs of anemia, abdominal distension, and abnormal enlargement of the spleen and liver. Coughing, darkening of the skin, chronic diarrhea, infection of the lymph nodes, and signs of chronic kidney disease may also symptomatic of cutaneous leishmaniasis. Without treatment most cases are fatal, usually due to a secondary infection or other complications. Patients who are treated for the infections will still carry the parasite *Leishmania tropica* and risk reoccurrence of the disease if they become immunosuppressed. Under a light microscope, *Leishmania tropica* intracellular form consists of a nucleus and a rod-like kinetoplast, and a homogeneous mass of cytoplasm. The extracellular form has an anterior flagellum. The intracellular form is between 2- and 4  $\mu$  and is generally round or ovoid in shape. The extracellular form is roughly 14-20 microns in length and 1.5-3.5 microns in breadth. Due to the size of *L. tropica* and the limited resolution of the light microscope, not much information is available on its fundamental cellular organization. The products of *Leishmania Donovan tropica* is metabolism are carbon dioxide, acetic acid, pyruvic acid, and succinic acid. The life cycle of *Leishmania tropica* has two distinct forms. One is a promastigote flagellar form that is found in the gut of the sandfly vector, and the other is an amastigote form, which develops intracellular in the human host. Only female sandflies can transmit the disease by inoculation of the promastigote form into the skin of the human. The *L. tropica* internalized by dendritic cells and macrophages in the dermis where they turn into amastigotes by losing their flagella. The amastigotes then multiply and destroy the host cell. The amastigotes spread through the lymphatic and vascular systems, and eventually spread to the bone marrow, liver, and spleen. The cutaneous Leishmaniasis (CL) in human being is caused by several *Leishmania* species and intensity and disease causing ability of which depends on the species which infect the specific area of the human population [1, 2]. No effective technique for diagnosis is found [2, 3].

The only technique used for diagnosis required the isolation and cultivation of the *Leishmania* organism from lesions [4]. Several techniques have been described for the identification and characterization of *Leishmania* at the molecular level. These techniques include Polymerase chain reaction (PCR), restriction fragment length polymorphism, sequence analysis of multicopy genes and inter genic spacer regions, DNA fingerprinting and randomly amplified polymorphic DNA, and PCR followed by reverse line blot hybridization [5-8].

Infection caused by *Leishmania* protozoa are of three types cutaneous, mucocutaneous, or visceral Leishmaniasis [9]. While most of the time diagnosis of CL is carried out for detection of parasite in the skin scrapings specimens by microscopy [10-12]. Techniques concerned with PCR are more accurate and precise and give more reliable results for leishmaniasis [13-16]. The healing of *Leishmania* infection depends on the early production of proteins. A protein that stimulates the production of IL-12 could be a significant key for an immunotherapeutic *Leishmania* infection as well as to be used in therapy as an adjuvant. Originally *Leishmania* proteins that induce the Th1 response in the *Leishmania* patient [17, 18].

## Materials and methods

### Area of Intervention

Samples were collected from the wound of a patient with suspected Cutaneous Leishmaniasis in Hangu region of Khyber Pakhtunkhwa province. The study was carried out from September, 2012 to June, 2013 further added that research work was funded from the HEC project "Epidemiology and molecular detection of Leishmaniasis in humans and canines in Khyber Pakhtunkhwa

### Sample collection

Specimens were collected from leishmania infected patients of cutaneous leishmaniasis. The skin scrapings were made with the help of scalpel in one direction till out of the blood from the lesion and an incision were given mostly in the inflamed border of lesion. Half of the sample was mixed in 1% formalin and stored at 4 °C until stained and were seen under the microscope and half were mixed with buffer at pH 7.2 and kept in sterile Eppendorf tubes for future processes.

### DNA extraction

The samples were subjected to DNA extraction by using GF-1 kit (vivantis). 200µl culture sample in eppendorf tube was mixed with 50µl of proteinase K and 200µl of buffer VL. They were mixed well with the help of vortex and then were incubated at 65 °C for 10 min in hot plates. The columns were centrifuged at 6000rpm for 1min and the flow through were discarded, then 400µl of wash buffer-1 was added and centrifuged at 6000rpm for 1 min again the supernatant was discarded. Then 400µl of wash buffer-2 was added and centrifuge at 6000rpm for 1min supernatant was discarded and again 400µl of wb buffer-2 was added and centrifuge at 14000rpm for 3min, flow through was discarded again and then the columns were transferred to new tubes and 30µl of elution buffer was added and placed for 2min at room temperature. After that centrifugation at 6000 rpm for 1min and was stored at -40 °C for DNA amplification.

### DNA Amplification PCR

The target DNA was amplified in 20µl reaction mixture containing 10x PCR buffer 2µM, 1µM deoxynucleoside triphosphate (500µM), 2.4µM MgCl<sub>2</sub> (25µM) 1 µM

primers(10pmol), target DNA 5 µl, and 0.3 unit of *Taq* DNA polymerase (5u/µl add deionized water up to 20 µl. DNA amplification at start of temperature for denaturing at 92 °C for 3 min, 25 cycles of 92 °C for 40 sec for to anneal at 50 °C for 40sec and extension at 72 °C for 60 sec. In the last stage extension at 72 °C for 7 min and hold at 4 °C for unlimited time, the designed program was saved as CL PCR.

### Settings of PCR Cycle

Stage	Cycle	Step	Temperature	Time
1	1	1	92 °C	3:00 min
2	25	1	92 °C	40 sec
		2	50 °C	40 sec
		3	72 °C	1:00 min
3	1	1	92 °C	7:00 min
		2	92 °C	Hold

Electrophoresis is a technique used in the laboratory that results in the separation of charged molecules. In this we are separating molecules of DNA that we got from Restriction Digestion DNA is a negatively charged molecule, and is moved by electric current through a matrix of agarose.

What exactly a gel is and what it has to do with agarose. By "making" a gel. Purified agarose is in powdered form, and is insoluble in water (or buffer) at room temperature. But it dissolves in boiling water. When it starts to cool, it undergoes is known as polymerization. Rather than staying dissolved in the water or coming out of solution, the sugar polymers crosslink with each other, causing the solution to "gel" into a semi-solid matrix much like "Jello" only more firm. The more agarose is dissolved in the boiling water, the firmer the gel will be. While the solution is still hot, we pour it into a mold called a "casting tray" so it will assume the shape we want as it polymerizes (otherwise it will just solidify in the bottom of the flask wasting the expensive agarose). Look through the sequence of images below to learn how to prepare a gel.

### Gel electrophoreses.

10µl of PCR product mixture was mixed with 5µl loading dye and loaded in a gel and 0.5µl ethidium bromide was added and poured into gel tray and combs were fixed. Combs were removed after gel was formed. Gel tray was placed in gel tank containing 1000ml 0.5X TBE buffer. 15µl of each sample was loaded in the wells and 15µl of DNA Ladder (100bp). The gel was run for 25 min at voltage of 130 volts and 500 ampere current. Gel was then examined by UV transilluminator.

### Results

For a correct result pcr is best way. We have prepared about 200 slides in which 35 were positive but in pcr techniques about 55 were positive.

### Identification of the parasite.

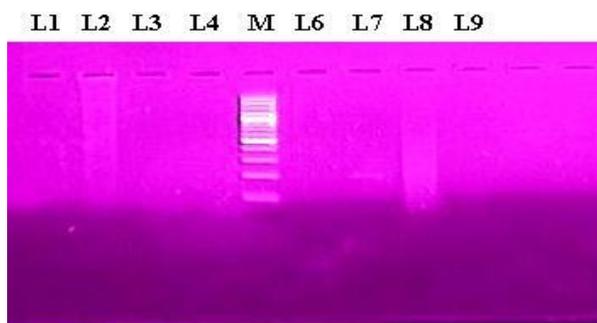
The results of cultivation, microscopic examination and PCR of the materials obtained from the patients having suspected CL lesions were given in Table 1. No amastigotes were observed in the slides microscopy. However during pcr some DNA were observed

**Table 1:** Prevalence of CL through PCR and Microscopy

Sample	Microscopy	PCR
Positive	35	55
Negative	165	185

**Table 2:** Gender wise CL prevalence of microscopy and PCR.

parameter	Number of patient	Male (%)	Female (%)	Total (%)
MICROSCOPY	200	20 (10%)	15 (7.5%)	35 (17.5%)
PCR	200	41 (20.5%)	14(7%)	55 (27.5%)



**Fig 4.** M; Marker 100bp  
L7; 186 bp band which is positive.

### Discussion

Molecular Characterization of *Leishmania* species which cause cutaneous leishmaniasis in Hangu Khyber Pakhtunkhwa, was reported in the present study. Sporadic of CL are also reported in other parts of Pakistan, Leishmaniasis is a serious disease that has an impact on millions of people worldwide. It has many faces. In certain forms it is a lethal killer, in others a cruel mutilator leaving its victims disfigured for life.

A parasite needs a so-called “vector” to fulfil its lifecycle. A vector is an organism that does not cause disease in itself, but does spread the parasite to other life forms. For example, mosquito’s (malaria), fleas (bubonic plague) and ticks (Lyme disease) are all well-known vectors. In the case of leishmaniasis the vector is a type of sand fly.

Our study stated that *L. tropica* cause leishmaniasis which was examined in all cases in the endemic region in Hangu. Out of 200 cases studied most were belonged to a poor socioeconomic class, living in overcrowded conditions. The infection was more common in male as compared to female and this may be due to ecological and environmental condition of the areas. The finding of the present study was similar to the report of [19].

Cutaneous leishmaniasis causes skin sores that, depending on the type of the parasite, can be localized on a specific place on the body (mostly on the limbs or the face) or spread. The skin sores usually develop within a few weeks after being infected. In specific varieties of cutaneous leishmaniasis these lesions can cause massive tissue damage, leaving the patient disfigured and the subject of social prejudice and stigma. Cutaneous leishmaniasis can be self-healing, although treatment is always highly recommended as the sores can spread, take months and even years to heal, or even cause death following secondary infections.

Diagnostic procedure is only remedy to identify the *Leishmania* species in hospital as well as in community level in epidemic areas and likewise sign were occurs due to other parasites and can cause the confusion in determination of the species diagnosis [20]. It was concluded that *L. tropica* (CL) species was present in the CL patients of endemic region, for detailed study of parasites; culture is needed to be carried out. The DNA PCR was the most accurate diagnostic assay and was recognized as a most reliable method in the diagnosis. The test may be standardized for the detection of parasite in patients of CL having negative microscopic examination and/or culture results.

For the *in vitro* culture and direct microscopic diagnosis high number of amastigotes of CL is required which is very low in skin lesions [20].

### Conclusion

Leishmaniasis has caused much sorrow in many lives, and yet it has not caught the attention of the broad public. Adding to the poignancy of the problem is the fact that treatment options are limited and that those most in need of help are the least likely to receive it as leishmaniasis mainly spreads in poor and remote regions. It was concluded from the present study that CL (*Leishmania tropica*) is present in Distt Hangu region having size of 186 bp observed through PCR and It has also been determined that PCR is more sensitive and specific than microscopy examination. It is suggested that further genomic study may be carried out to determine the strain and sub strain of the *Leishmania tropica* in the region and work should be stream line to develop the recombinant DNA vaccine against the *Leishmania* parasite.

### References

- Zuckerman A, Lainson R. in Parasitic Protozoa, ed. Kreier, J. P. (Academic, New York) 1977; 1:58-133.
- Lainson R, Shaw JJ. in Biology of Kinetoplastida, ed.Lumsden, W.H. R. & Evans, D. A. (Academic, London) 1979; 2:1-116.
- Chaves LF, Pascual M. Climate cycles and forecasts of cutaneous leishmaniasis, a nonstationary vector-borne disease. PLoS Med, 2006; 3:1320-28.
- Marsden P, Engl NJ. Med. 1979; 300:350-352.
- Salotra P, Sreenivas G, Beena KR, Mukherjee A, Ramesh V. Parasite detection in patients with post kala-azar dermal leishmaniasis in India: A comparison between molecular and immunological methods. J Clin Pathol. 2003; 56:840-843.
- Bañuls AL, Guerrini F, Le Pont F, Barrera C, Espinel I. Evidence for hybridization by multilocus enzyme electrophoresis and random amplified Polymorphic DNA between *Leishmania braziliensis* and *Leishmania panamensis guyanensis* in Ecuador. J Eukaryot Microbiol. 1997; 44:408-40111
- Mauricio IL, Stothard JR, Miles MA. *Leishmania donovani* complex: genotyping with the ribosomal internal transcribed spacer and the mini-exon Parasitology. 2004, 28:263-267.
- Nasereddin A, Bensoussan-Hermano E, Schönian G, Baneth G, Jaffe CL. Molecular diagnosis of Old World cutaneous leishmaniasis and species identification by use of a reverse line blot hybridization assay. J Clin Microbiol. 2008; 46:2848-2855.
- Bates PA. *Leishmania* In: Encyclopedia of life sciences. Hoboken (NJ), John Wiley & Sons, Inc; 2006
- Laskay T, Miko TL, Negesse Y, Solbach W, Rollinghoff M, Frommel D. Detection of cutaneous *Leishmania* infection paraffin-embedded skin biopsies using the polymerase chain reaction. Trans R Soc Trop Med Hyg. 1995; 89:273-275.
- Sreenivas G, Ansari NA, Singh R, SubbaRaju BV, Bhateja R, Negi NS *et al.* Diagnosis of visceral leishmaniasis Comparative potential of amastigote

- antigen, recombinant antigen and PCR. *Br J Biomed Sci.* 2002; 59:218-222.
12. Sreenivas G, SubbaRaju BV, Singh R, Selvapandiyan A, Duncan R, Sarkar D *et al.* DNA polymorphism assay distinguishes isolates of *Leishmania donovani* that cause Kala-azar from those that cause Post kala-azar dermal leishmaniasis. *J Clin Microbiol.* 2004; 42:1739-1741.
  13. De Brujin MHL, Barker DC. Diagnosis of new world Leishmaniasis Specific detection of species of the *Leishmaniabrazi liensis* complex by amplification of kinetoplast DNA *Acta Tropica.* 1992; 52:45-48.
  14. Arevalo J, Inga R, Lopez M. PCR detection of *Leishmania braziliensis*. In *Diagnostic molecular microbiology: Principles and applications*, D. H. 1993.
  15. Eresh SS, Mccallum M, Barker DC. Identification and diagnosis of *Leishmania mexicana* complex isolates by polymerase chain, 1994.
  16. Uliana SR, Nelson K, Beverley SM, Camargo EP, Floeter-Winter LM. Discrimination amongst *Leishmania* by polymerase chain reaction and hybridization with small subunit ribosomal DNA derived oligonucleotides. 41: 324-330 *rection. Parasitology.* 1994; 109:423-433
  17. Heinzl FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon  $\gamma$  or interleukin 4 during the resolution or progression of murine leishmaniasis: evidence for expansion of distinct helper cell subsets. *J Exp Med.* 1989, 169:59.
  18. Scott P, Natovitz P, Coffman R, Pearce E, Sher A. Immunoregulation of cutaneous leishmaniasis: T cell lines that transfer protective immunity or exacerbation belong to different T-helper subsets and respond to distinct parasite Antigens. *J Exp Med.* 1988; 168:1675.
  19. Sultan Ayaz, Sanaulah Khan, Shahid Niaz Khan, Sumaira Shams, Muhammad Saqalain, Jan Ahmad *et al.* Cutaneous leishmaniasis in Karak, Pakistan: Report of an outbreak and comparison of diagnostic techniques. *African Journal of Biotechnology.* 2011; 10(48):9908-9910.
  20. Kassi M, Afghan AK, Rehman R, Kasi PM. Marring leishmaniasis: the stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan. *PLoS Negl. Trop. Dis.* 2008; 2(10):259.