ISSN 2278-7763

Identification of Leishmania tropica by PCR and RFLP Techniques in Kohat Region of Khyber Pakhtunkhwa,Pakistan.

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ABSTRACT

Cutaneous Leishmaniasis is a worldwide public health and a social problem in many developing countries. This disease is also found in Pakistan in general and Khyber Pakhtunkhwa in particular. A total of 100 samples were examined from clinically suspected Cutaneous Leishmaniasis patients under sterilized conditions in village Sordag, District Karak, Khyber Pakhtunkhwa. The DNA was extracted and amplified through PCR and reconfirmed by RFLP technique. The PCR showed the result 53% (53/100) were positive, in which 67.30% (35/100) were female while 37.50% (18/100) male positive. These results were reconfirmed through RFLP which also showed 186bp amplified fragment. It is concluded that PCR-RFLP appears to be most sensitive and appropriate techniques for the detection of Leishmania tropica.

Keywords: Cutaneous Leishmaniasis, DNA, PCR and RFLP

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1 INTRODUCTION

Globally Leishmaniais is one of the most ignored tropical disease with high prevalence rate[1] caused by infectivity of protozoa of the genus Leishmania [2] in their different forms [3,4]. Leishmaniasis is transmitted by Female Phlebotomine Sand flies (Family: Psycodidae, Order: Diptera) [5, 6]. It has been reported that about 350 million people are at the risk of Leishmaniasis [7] in 88 countries among which 22 are in the new world and 66 in the old world [8] and annually 2,357,000 new cases are reported [7].

Approximately 90% of the Cutaneous Leishmaniasis cases came about in Pakistan, Saudi Arabia, Iran, Brazil, Syria, Afghanistan and Peru. Widespread areas of disease in Pakistan include the Karakoram and Hindukush sub-mountainous range (Gilgat, Dir and Chatral); the Toba Kakar sub-mountain range (Qila Abdullah, Quetta, Qila Saifullah, Pishin); the Suleman and Kirthar sub-mountain range (Jacobabad, D.G.Khan, Rajanpur, Derabughti, Khuzdar, Lasbela, Dadu and Larkana); the Himalayan sub-mountain range (Azad Kashmir, Mansehra, Rawalpindi, Abbotabad) [9].

Extra-cellular promastigotes and intra-cellular amastigotes are the two morphological forms of Leishmania. The elongated and motile flagellated promastigotes are initiated in the alimentary canal of the sandfly while nonmotile and ovoid amastigotes exists and reproduce in the phagocytosomes of host macrophages [10]. Taxonomy of *Leishmania* is as; Kingdom-*Protozoa;* Subkingdom-*Protista;* Phylum-*Sarcomastigophora;* Class-*Zoomastigophora*; Order-*Kinetoplastida*; Suborder-*Trypanosomatina*; Genus-*Leishmania*; Species: *L. tropica complex, L. donovani complex, L. mexicana complex, braziliensis complex* [11]. The lesions are generally found on the uncovered areas of the skin [12,13]. The lesions or ulcers leave a scratch mark on infected area [14]. Increase tissue demolition and disfiguring of the skin are caused by secondary fungal or bacterial infection of the ulcers [13]. Keeping in view the importance of the disease; research is design to carry out

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the identification of leishmania parasite through PCR and RFLP techniques.

2 MATERIALS AND METHODS

2.1 Sample Collection

Specimens were collected from leishmania infected patients of Cutaneous Leishmaniasis. The skin scrapings were made with the help of surgical blades in one direction till the blood oozes out of the lesion and an incision were given mostly in the inflamed border of the lesion. Distilled water was sprinkled with the help of sterilized syringe on the lesion and after that samples were collected in sterilized eppendorf tubes.

2.2 DNA Extraction

By using the DNAzole kit (Trizole USA), DNA was extracted from biopsies /skin scrapings through a standard protocol of the manufacturer. The following steps were involved,

2.3 Step.1 Cell lyses / Denaturation

300µl of sample was taken in a sterilized eppendorf tube and 500µl of trizole was added in it and mixed well through vortex. Then 160µl of chloroform was added in it and mixed well through vortex and Centrifuged at 12000rpm for 5 minutes. The upper layer (aqueous) above the middle layers was removed.500µl of 100% absolute ethanol was added in it and mixed well through vortex and again Centrifuged at 12000rpm for 5 minutes. The supernatant was discarded.

2.4 Step.2 Washing of DNA

500µl of trisodium citrate was added and centrifuged at 12000rpm for 5 minutes, and supernatant was discarded. Then incubated for 20 minutes in orbital shaker at roomtemperature.500µl of 70% ethanol was added and centrifuged at 12000rpm for 5 minutes and the supernatant was discarded. Incubated at room temperature for 10 minutes in orbital shaker. 500µl of 8mM of NaOH was added and centrifuged at 14000rpm for 5 minutes .From the supernatant 30µ l of DNA was taken in a new sterilized eppendorf tube and was kept at -40□C

2.5 DNA Amplification

The target DNA was amplified in 20µl reaction mixture containing 10x PCR buffer 2µl (ammonium sulphate), 1µl dNTPs (10mM), 2.4µ l MgCl2 (25mM),

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1µl of forward primer 5/-TTŕCTTTGGATGGGTTTCTGG-3/ (10pM), 1µl of reverse primer 3/-CAACACCAACGTAAGCGTAAC-5/(10pM), deionized water 8.3µl, target DNA 4ul and 0.3µl of *Taq* DNA polymerase (5µl). The designed program for leishmania for the DNA amplification was initial denaturation cycle1 at 92°c for 3 minutes,25 cycle at initial cycle 1 is on 92°c for40 second, cycle 2 for 50°c for 40 second, cycle 3 for 72°c for 1minute and finally extension at 72°c for 7 minutes.,

2.6 Restriction Fragment Length Polymorphism

The PCR products (amplified ITS1 region) from ITS1 PCR were digested using restriction endonuclease enzyme *Hae III* (*Haemophilis* III) as recommended by manufacture (BioLabs Inc, New England). Briefly, for clinical diagnostic samples that were amplified by ITS PCR, quantity of 15-20µl of DNA was incubated and restricted byaddition of 1µl (5 units) of Hae III enzyme and 5µl of corresponding 10 X N.E buffer and 5-10µl of deionized water and incubated at 37°C for 1.5-2 hours and gently vortex occasionally during the incubation procedure

2.7 Gel Electrophoreses

10µl of PCR product was mixed with 5µl of loading dye through pippting. Similarly 5µl of ladder was mixed with 5µl of loading dye. Then gel tray was placed in gel tank containing 1000ml 0.5X TBE buffer. The ladder was loaded in the 1st well and 10µl of each sample was loaded in the remaining wells. The gel was run for 20-25 minutes at a voltage of 120 volts and 500ampere current. Gel was then examined by UV Tran illuminator.

3 RESULTS

A total of 100 samples of skin scarping /biopsies were collected from the clinical patients in village Sordag, District Karak under sterile conditions and examined through poly merease chain reaction (PCR) and 53%(53/100) were found positive. Among these(37.50%) were male and (67.30%) were female and 186 bp DNA was amplified (Fig 1and table.1)The result of PCR was reconfirmed through RFLP and the result showed the same result as determined by PCR. (Fig.2 and Table.2)

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TABLE NO 1: PCR diagnostic ratio of Cutaneous leishmanisis in district Karak Khyber Pakhtunkhwa.

Samples	Sex		Percentage of Samples	
	Female	Male	Female	Male
Positive	35	18	67.30%	37.50%
Negative	17	30	32.70%	62.50%
Total	52	48	100%	100%

TABLE: 2. Comparison between PCR and RFLP diagnostic techniques.

Test	Positive	Negative	Total
PCR	53	47	100
RFLP	53	47	100

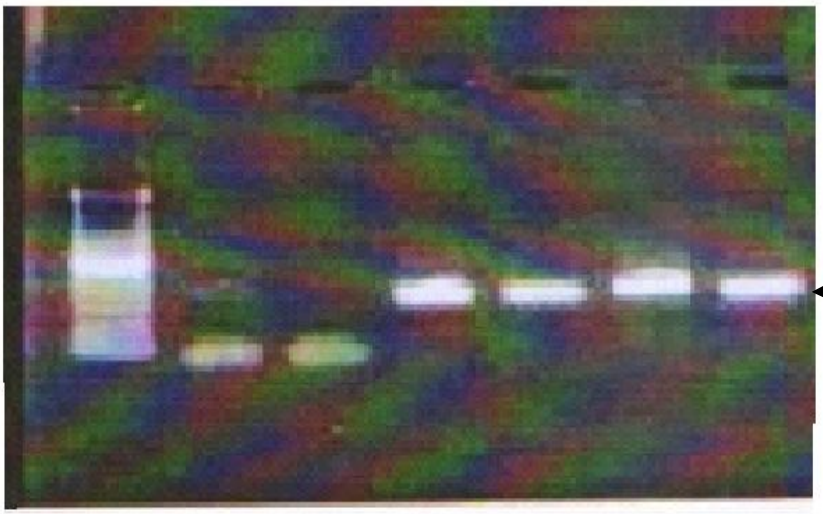

Fig.2; RFLP product showing the band of 186bp

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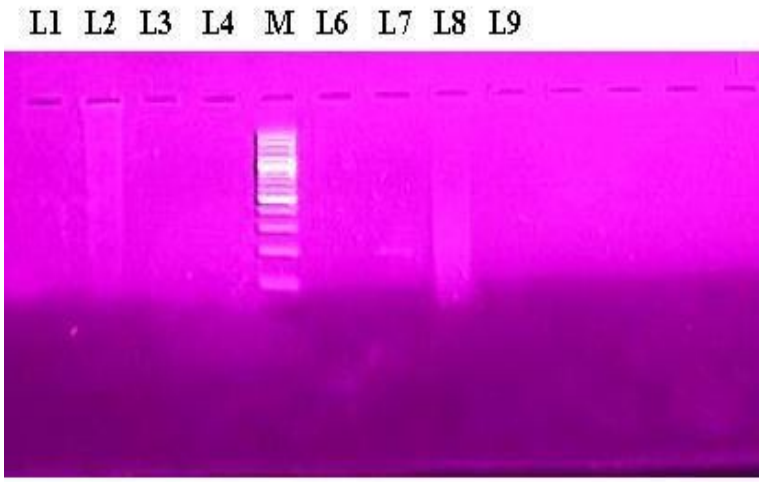

Fig 4. M; Marker 100bp
L7; 186 bp band which is positive.

4 DISCUSSION

Cutaneous Leishmaniasis is widespread in many tropical and sub-tropical areas of Equator [15]. Cutaneous Leishmaniasis are reported in Iran, Afghanistan, India and Pakistan where both Leishmania donovani and Leishmania tropica were found that initiate Leishmaniasis in challenging areas [16].

It is a main society health problem largely disturbing populations living in distant areas where essential services of life were not simply accessible and where the detection of Cutaneous Leishmaniasis was base on clinical characteristics. Affirmative laboratory tests are basically used and very limited facilities are available (Laboratory and Skilled persons). Direct microscopic detection of Leishmaniasis is cheap and easy but its sensitivity is very low, even if it is carried out by skilled persons [17, 18].

PCR offer certain compensation over conventional methods for the diagnosis and characterization of Cutaneous Leishmaniasis. When approximately applied, PCR can be more specific, sensitive, versatile and rapid than conventional methods; in addition genetic information can be obtained in the process [19].

Cutaneous Leishmaniasis is prevalent in Pakistan and has been reported from all the Provinces [20]. From the finding of

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the present study it has been revealed that Cutaneous Leishmaniasis is caused by a parasite, Leishmania tropica, in village Sordag, District Karak, Khyber Pakhtunkhwa.During the present study, a total of 100 samples were examined through PCR and reconfirmed by RFLP technique. The finding by the present study showed that out of 100 samples, 53% (53/100) samples were positive. Among these, 67.30% females and 37.50% males were positive.

This result showed that infection was more common in female than in male which is contrast to the report of [21,22] and similarities with the epidemic report CL in Khyber Pakhtunkhwa by [16]. Further added that this result showing more inclination toward females than males by the vector of parasite, Due to cultural ,environmental, adaptation of the norm value , managemental socioeconomic condition of the community.

5 Acknowledgement

The research study was facilitated from the Higher Education commission of Pakistan funded project “Epidemiology of Lishmensiasis in Canin and Human being in Khyber Pakhtunkhwa (NWFP)” to the Kohat University of Science and Technology Kohat, Pakistan.

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