

1 **Lesion aspirate isolation, culture, diagnosis and Molecular identification of Cutaneous**
2 **leishmaniasis in district Kohat, Khyber Pakhtunkhwa, Pakistan**

3
4 **Abstract**

5 Diagnosis and recognition of clinical and morphological features of *Leishmania* parasites
6 involved in the spread of disease is mandatory to design appropriate strategies for ecologic
7 control and preventive plans. Although clinical investigations and findings are necessary to
8 estimate about epidemiological findings but they are not sufficient for identification and
9 characterization of the particular parasite, as the transmission of the disease especially in
10 endemic areas is manifested by various *Leishmania* species. Samples from 108 patients with
11 suspected CL lesions, fulfilling the inclusion criteria irrespective of gender, age groups, location
12 and immune status were enrolled and examined. Aspirate smears and skin scrapings collected
13 from the active edge of the lesion using surgical blades, fine needle aspiration biopsy and/or
14 lancet were stained by Giemsa and confirmed via microscopy. Productivity and the potential
15 isolates of *Leishmania* samples inoculated in Biphasic Novy-MacNeal- Nicolle (NNN) and
16 cultured in Monophasic Roswell Park Memorial Institute (RPMI) 1640 media were used in
17 evaluating the efficiency for each sample under study. In order to identify *Leishmania* spp. a
18 definite molecular technique, Polymerase Chain Reaction (PCR) product analyzed by gel
19 electrophoresis using 2% agarose was adopted. Genomic DNA was extracted using proteinase k
20 and amplified by specific primers of kinetoplast DNA. Gel staining was performed using
21 ethidium bromide. The presence of 186bp fragment indicated *L. tropica* confirming it as the
22 prevalent species in Khyber Pakhtunkhwa, Pakistan, which has immense importance from an
23 epidemiological, transmission and treatment point of view. PCR is a very reliable and sensitive
24 method to detect *Leishmania* DNA as compared to Giemsa and culture techniques.

25
26 **Keywords:** Cutaneous Leishmaniasis, Giemsa stain, Microscopy, Polymerase chain reaction

Introduction

Leishmania is caused by single cellular, hemoflagellate protozoan parasites of the genus *Leishmania* transmitted by the bite of infected female sand fly. Only 60 of about 600 species are vectors for *Leishmania*, around which 20 *Leishmania* species are described as human pathogens (Vannier *et al.*, 2002). Leishmaniasis depends on the *Leishmania* spp. involved, site of bite, number of bites, type of sandfly, genus of parasite and acute predisposition of the host's genetic potential (Markle and Makhoul, 2004). Worldwide prevalence is about 12 million, endemic in 90 tropical and subtropical countries threatening approximately 350 million people each year approximately (Desjeux, 2001).

Categorically, *Leishmaniasis* is classified into three different clinical forms: Cutaneous, Mucocutaneous and *Visceral leishmaniasis* (Hamid *et al.*, 2012). Cutaneous leishmaniasis represents up to 75% of all the new cases with 1-1.5 million annual incidences commonly caused by *Leishmania major*, *L. tropica*, *L. aethiopica*, and *L. braziliensis* particularly in sub-continent and middle east (Hailu *et al.*, 2006). CL is characterized by a small red papule which becomes darker and turn into ulcer with raised edges after several weeks. Ulcers can be moist and exude pus with a crusted scab; sores usually appear on exposed body parts of the skin, especially on the face and extremities (Markle and Makhoul, 2004). Localized cutaneous leishmaniasis is characterized by lesions on face, nose, forehead and lower limbs that usually heal naturally. Another type of CL is the diffuse cutaneous leishmaniasis that produces symptoms like nodules, plates or lumps on the face, arms and legs and relapse after treatment (Davies *et al.*, 2003). The currently used parasitological diagnostic routine procedures involved the direct examination of amastigotes using stained smears via microscopic examination obtained from tissue lesions are based on clinical signs and symptoms and history of exposure to the disease (Culha *et al.*, 2006). Morphologically 2 forms of the parasite; (1) the amastigote form responsible for disease pathogenicity is found intracellularly in the vertebrate hosts including humans, and (2) the promastigote form, predominately found in the insect vector (Mohammed, 2008). Studies using axenically grown amastigotes are becoming an acceptable alternative as they combine the advantages of being rapidly screened using the clinically relevant stage of the parasitic life cycle (Romero *et al.*, 2009).

Pakistan is at important geographical location for the disease as it is located and surrounded by nations like Afghanistan, India and Iran where different forms of leishmaniasis

have been endemic for many years. Most studies in Pakistan on *Leishmania* spp. identification have been carried out in Baluchistan, Sindh and Azad Kashmir. However in KPK region very limited work has been done. Therefore, this study was carried out to identify the causative agent of cutaneous leishmania aiming to take attention on this serious health problem in district Kohat, KPK region.

Design of Study

Materials and Methods

All experimental procedures involving human tissue samples were obtained and conducted in accordance with the Code of ethics and Practice for Scientific purposes as approved by the Human Research Ethics Committee Centre guidelines for biomedical research, Peshawar centre. All chemicals, reagents, solvents purchased were of high-performance liquid chromatography (HPLC) analytical grade and maintained at given temperature and pH at given concentrations for reaction mixture as indicated by suppliers without further purification.

Patient's history

A descriptive, cross-sectional, prospective study based on patient's consent and history of both gender irrespective of age groups, location, immune and financial status were included. Altogether 108 clinically suspected patients met the reference standard for cutaneous leishmaniasis were referred to the Molecular Parasitology and Virology Research Laboratory, Department of Zoology, Kohat University of Science and Technology, Kohat where investigations were carried out to diagnose and identify the causative agent of the disease after the collection of sample specimen using protocol as follows.

Isolation of sample Lesion from Leishmania patients

Aspirate lesions of leishmania infected tissue were made clean, sterilized with ethanol and air dried. Dermal smear, ulcer scrapings were taken from nodular or raised part by making a small incision near the margin the active edge of a lesion with the help of sterilized lancet or blade as shown in figure 1. Insulin syringe (1cc) containing 0.9 percent sterilized normal saline tightly connected to a 0.5mm diameter needle syringe and slowly advanced with a negative pressure near the ulcer margin penetrating into the subcutaneous tissue in the direction of the ulcer. Suction was applied when the needle reached below the ulcer margin of the lesion and injects normal saline inside. The needle was detached, syringe was filled with air, needle was reattached and inserted again until the blood containing stinged fluid oozes out from the lesion and contents

were blown into the eppendorf and rapidly onto a clean alcohol-free microscope slides for further examination as shown in figure 2. (Mashhood *et al.*, 2005).

The following diagnostic investigations were performed for each case;

1-Microscopic examination using Geimsa stained smears from the lesion border

Smears were fixed in methanol and stained with Giemsa to examine for *Leishmania* parasites (amastigotes and promastigotes) based on their morphology with maximum cellular yield to look either for large macrophages containing the parasite or areas with predominance of mononuclear inflammatory cells as shown in figure 3 (Sharquie *et al.*, 2002).

2-Culture from skin lesion scrapes

Similar to the preparation of the slide smears, scraped tissue was inoculated on the liquid phase of Novy- McNeal-Nicolle (NNN) medium and cultured in RPMI 1640 (supplementad with 10% fetal calf serum plus 200 µg/ml streptomycin and 200 U/ml penicillin) of antibiotics. The cultures were incubated at 26°C and examined for parasite growth under optic microscope use every 4th day until promastigotes were seen or up to one month before being discarded as negative. The cultures were made at least in duplicates for each case. Slides were labelled accordingly to identify the patient and the sampling technique used.

3- DNA extraction and PCR amplification of stained specimens

The target DNA was extracted from exudate/skin scrapings using DNA zole kit (Trizole USA) and amplified in 20µl reaction mixture containing 10x PCR buffer 2µM, 1µM deoxynucleosidetriphosphate (500µM), 2.4µM MgCl₂, 1 µM primers (10pmol), target DNA 5 µl, and 0.3 unit of *Taq* DNA polymerase in a thermal cycler (Nyxtech USA) using Oligonucleotide, LSa (5' TCTTGCGGGGAGGGGGTG-3') and LSb, (5' - TTGACCCCCAACCACATTTTA-3') specific to kDNA of *L. tropica*. (Machado EMM, 2000). Reaction buffer without Leishmanial DNA was also included as a negative control. DNA amplification was initiated at temperature of 92°C for 180 sec for denaturing and annealing at 92°C for 40 sec and extension at 72°C for 60 sec. The designed program was carried out in Thermal Cycler (Nyxtech USA) for 2-3hrs and saved as CL PCR. PCR was conducted in a 25 µl of reaction mixture containing MgCl₂, 10 mM dNTPs and 2U of *Taq* DNA polymerase (Fermentas USA). The amplified product was run for 25 min at voltage of 130 volts and 500 ampere current on 2% agarose gel poured into gel tray in a gel tank consisting of 1000ml of 0.5x TBE buffer, 15µl of DNA Ladder (100 bp) visualized under UV

transilluminator using ethidium bromide (0.5 mg/ml). The CL specific band 186 bp was compared with 100 bp DNA ladder marker (Fermentas USA) (Romero *et al.*, 2001).

Results and Discussion

Culture methods make possible diagnoses by isolation of biopsies or aspirate lesions in patients with the reproduction of the parasite which can be overlooked by the staining method depending on the handling and experience of laboratory technicians (Pratlong *et al.*, 2009). In our study, the clinical diagnosis of cutaneous leishmaniasis was confirmed by direct microscopic examination of lesion aspirate as intracellular or extracellular amastigotes observed as colourless, round to oval small bodies containing nucleus and/or kinetoplast as shown in figure 3. These amastigotes which measure about 2-5µm are intracellularly present in monocytes and macrophages of infected vertebrate hosts and rarely in other cell types; i.e monocytes, dendritic cells and fibroblasts (Sharquie *et al.*, 2002). Promastigotes cultured from amastigotes were observed as a single anterior flagellum emerging from the cell body running parallel to the microtubules of the axoneme making these organisms functional and motile as shown in figure 4. Culture of promastigotes from infected tissues and/or direct identification of amastigotes in microscope smears have long been considered the standard for diagnosis (Liarte *et al.*, 2001). However, it takes days to weeks until parasites can be visualized, while some species are very difficult to culture and the quality of the growth medium preparation can be highly variable. Likewise, the percent success for microscopic identification of amastigotes in stained preparations varies depending on the number of parasites present and/or the experience of the person examining the slide. In those cases where promastigotes were cultured, additional serological, biochemical, biological, and other techniques had to be used to characterize the parasites (Singh and Sivakumar, 2003). Thus there is a need for a new sensitive molecular method for diagnosis rather than traditional ones such as microscopy, culture or clinical picture which all lack sensitivity and specificity (Culha *et al.*, 2006). So the identification of the parasite is the only tool to confirm the disease categorically. Therefore, differentiations among species require a more sensitive method for diagnosis and confirmation of the infection such as polymerase chain reaction (Murray *et al.*, 2005). In this study, we evaluated its potential in the identification and molecular characterization of cutaneous leishmaniasis and showed the presence of 186bp fragment

confirming *Leishmania tropica*, as the causative agent in all cases of cutaneous leishmaniasis examined in district Kohat, KPK, Pakistan as shown in figure 5. Vectors recognition by clinicians and pathologists need to rely on advanced techniques to identify clinical and morphological features of the infection remain of utmost importance as they are directly related to crucial value of disease containment.

Conclusion

Histopathological and serological methodologies have limited the diagnostic criteria due to their low sensitivity when employed in areas of endemicity. As the diagnosis is more challenging, enhanced PCR technique in chronic biopsies should be considered the preferred diagnostic method for evaluating prognosis. Also, study of species distribution can help us to design prophylactic preventive plan for disease control and effective therapy.

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Conflict of Interest: None declared



Figures. Tissue aspirate isolation of *Leishmania* infected patients with cutaneous leishmaniasis using sterilized scalpel (Fig. 1) and insulin syringe (Fig. 2 containing sterilized 0.9 percent normal saline)

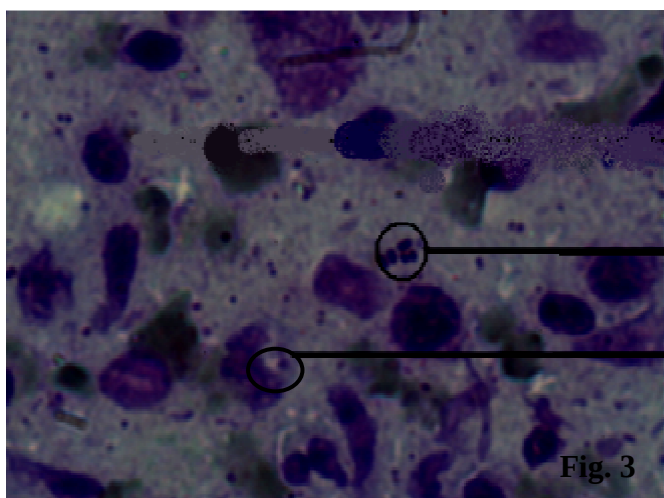


Figure 3. Giemsa stain image showing the amastigotes form of *Leishmania* inside and outside macrophages observed under microscope

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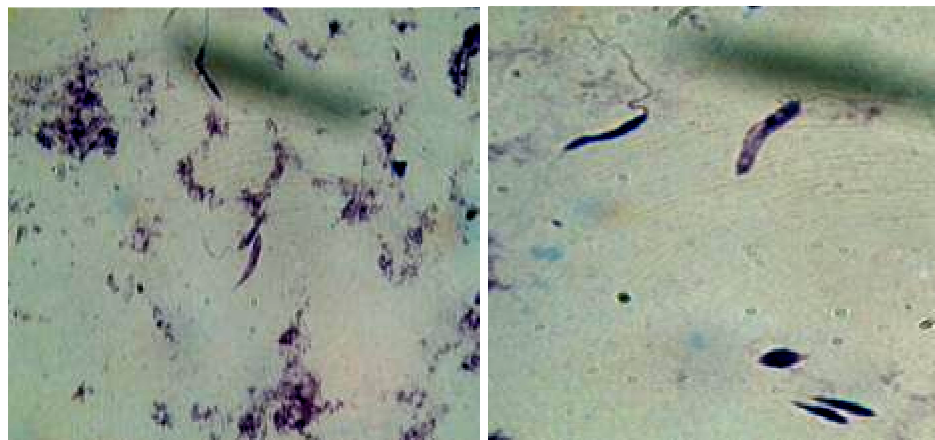


Figure 4. *In vitro* cultured promastigotes with the emersion of single anterior flagellum inoculated in Novy- McNeal-Nicolle (NNN) medium using Giemsa stain

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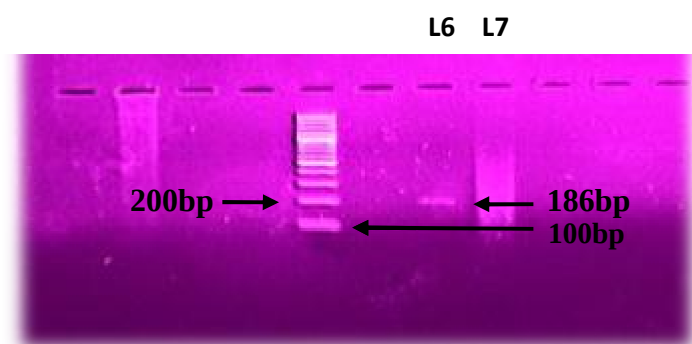


Figure 5. PCR-based amplification of kinetoplast DNA extracted from skin lesions of the patients. The bands shown on 2% agarose gel stained with ethidium bromide correspond to molecular weight markers. PCR amplified product of *L. tropica* in biopsy samples obtained from patients with Lanes 1, 3 and 4 = negative samples, lane 5= negative control, lane 6 = positive sample, lane 2 and 7 = positive control, M = Marker 100bp DNA ladder

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