

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

4 Abstract

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

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

25 Introduction

26 *Leishmania* is a tropical neglected disease caused by single cellular, hemoflagellate protozoan
27 parasites of the genus *Leishmania* transmitted by the bite of an infected female sand fly,
28 *Lutzomyia* and/or *Phlebotomus* (Handman and Bullen, 2002). Sandflies are the vectors of
29 *Leishmania* parasites that are distributed throughout intertropical and temperate regions of the
30 world (Vannier et al. 2002). Only 60 of about 600 sand flies species are vectors for *Leishmania*
31 around which 20 *Leishmania* species are described as human pathogens (Mehlhorn 2004).

Leishmaniasis depends on the *Leishmania* spp. involved, site of bite, number of bites, type of sandfly, genus of parasite and acute predisposition the host's defense system and genetic potential (Bari 2006; Markle and Makhoul 2004). Consolidated data are frequently not available, only estimates have been provided (Croft and Coombs 2003). Worldwide prevalence is about 12 million, (Desjeux 2001) endemic in 90 tropical and subtropical countries threatening approximately 350 million people each year approximately, 90% of which occurs in the Sudan and Indian subcontinent (Davis et al. 2003). In most countries the incidence numbers are probably highly underestimated, since many cases are not recognized and there is no obligation to report the disease (Desjeux 2004). Leishmaniasis ranges from self healing i.e. cutaneous leishmaniasis to progressive nasopharyngeal infections mucocutaneous leishmaniasis and finally to a life threatening infection called visceral leishmaniasis (Hamid et al. 2012). Cutaneous leishmaniasis is a dermal manifestation representing up to 75% of all the new cases with 1-1.5 million annual incidences commonly caused by *Leishmania major*, *L. tropica*, *L. aethiopica*, *L. mexicana*, *L. amazonensis*, and *L. braziliensis* (Hailu et al. 2006) in many parts of the world particularly in sub-continent and middle east (Donald 2003). 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 (Davies et al. 2003; Gilles 1999). Another type of CL is the diffuse cutaneous leishmaniasis (DCL) that produces symptoms like nodules, plates or lumps on the face, arms and legs never heal spontaneously and relapse after treatment (Davies et al. 2003; Mehlhorn 2004; Desjeux 1996). Mucocutaneous leishmaniasis (Espundia) is characterized by nasal obstruction and bleeding, disfiguration and generation of painful mucosal lesions and cartilage of the mouth, ear and pharynx (Vidyashankar and Agrawal 2007). Although cutaneous leishmaniasis is not lethal, but can cause significant morbidity and the course of the disease is often accompanied by psychological social repercussion, painful disfiguration and secondary dermal manifestations, neoplasms and sarcoidosis (Yanik et al. 2004; 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 based on clinical signs and symptoms and history of exposure to the disease (Culha et al. 2006). Morphologically

2 forms of the parasite i.e the amastigote form responsible for disease pathogenicity is found intracellularly in the vertebrate hosts including humans, and 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 a rapid primary screen using the clinically relevant stage of the parasitic life cycle (Serenio et al. 1997; Sundar and Rai 2005).

Most studies in Pakistan on *Leishmania* spp. identification have been carried out in Baluchistan, Sindh and Azad Kashmir where two species, *L. major* in rural areas and *L. tropica* in urban areas were being identified as responsible for leishmaniasis (Jaffarany and Haroon 1992; Rab et al. 1997). However in KPK region very limited work has been done. Therefore this study was carried out to identify the causative agent of Cutaneous Leishmania species in district Kohat, KPK region.

Materials and Methods

A descriptive, cross-sectional, prospective study based on patients of both sexes having variable age groups clinically suspected to cutaneous leishmaniasis were referred to the Molecular Parasitology and Virology Research Laboratory, Department of Zoology, Kohat University of Science and Technology, Kohat were investigated 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

The lesions and the adjacent normal-looking skin around the infected area were made clean, sterilized with 70% ethanol, and allowed to dry. Tissue smear, ulcer scrapings were taken from nodular part or raised edge 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

Small quantity of leishmania infected tissue obtained by skin scrapings was smeared onto clean alcoholic-free microscopic glass slides and fixed with methanol for 15-20 seconds and air dried. Slides were stained with Giemsa (diluted to 70%) for 15-20 minutes and slowly washed with gentle tap water and air dried. Geimsa-stained slides after application of cover slip were initially examined under light power (10x) and then high power (40x) objective to localize the fields with maximum cellular yield and to look either for large macrophages containing the parasite or areas with predominance of mononuclear inflammatory cells. The slides were then finally moved to oil immersion lens (100x) to identify *Leishmania* parasites based on their morphology (intracellular or extracellular, ovoid in shape, containing nucleus and/or kinetoplast) (Figure 3 and 4) Sharquie et al. 2002).

2-Culture from skin lesion scrapes

Similar to the preparation of the slide smears, a small amount of the 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 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 appropriately to identify the patient and the sampling technique used (Kalter 1994).

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 3 min 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 Cyclor (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 (100bp) 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; Marfurt et al. 2003).

Results and Discussion

Direct microscopic examination of lesion scraping is considered to be the diagnostic method most widely applied because of the ease of performance, low cost, and speed of this technique (Singh and Sivakumar 2003). The clinical diagnosis of Cutaneous Leishmaniasis according to WHO Recommended Surveillance Standards (1999) was confirmed by observing the amastigotes using Giemsa stained smear via microscopy as shown in figure 3 and 4. The amastigotes were observed as colourless, round to oval, small bodies having homogeneous cytoplasm under light microscope at 100x which measure about 2-5µm and found only in the macrophages of infected vertebrate hosts. As discussed by (Klaus and Frankenburg 1999; Ashford 2000) these amastigotes are intracellularly present in monocytes and macrophages and rarely in other cell types; i.e monocytes, dendritic cells and fibroblasts but they may also be found as extracellular parasites (Gilles 1999; Chan and Rodriguez 2001). *Leishmania* parasites colonize first in the gut of sand fly as flagellated promastigotes, then approaches to mammalian hosts, where they survive as obligatory intracellular amastigotes found in phagocytic cells. The rapidly dividing promastigotes are converted from non-infectious procyclic forms to the infectious metacyclic promastigotes, which finally develop into amastigotes in the mammalian macrophage (Handman and Bullen 2002). Smears were declared positive if at least one or two intracellular or extracellular amastigotes were found. (Sharquie et al. 2002). When no

amastigotes were seen after microscopic observations, the result was declared negative. Most of the CL patients were double checked to exclude the chances of error. Furthermore, the sensitivity of microscopic examination can be increased by increasing the number of samples taken per single lesion (Navin et al. 1990; Faber et al. 2003). Negative results may not always mean absence of *Leishmania* infection because the result of the test may be influenced by secondary bacterial or fungal infection. In well-equipped laboratories, cutaneous leishmaniasis parasites can be isolated by culturing of biopsies or aspirates lesion since it is performed easily depending on the handling, performance and experience of laboratory technicians or pathologist in patients infected with different *Leishmania* species (Romero et al. 1999; Weigle et al. 2002). Promastigotes 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 5. Promastigotes clusters may also be seen. (Herwaldt 1999; Ritting and Bogdan 2000). Culture methods make it possible to make diagnoses more comfortable with the reproduction of the parasite which can be overlooked by the staining method (Romero et al. 2009; Pratlong et al. 2009). However, contamination is a constant risk factor in culturing and can sometimes affect 30% of the samples even in well-established laboratory setups (Liarte et al. 2001). Additionally, 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 (Herwaldt et al. 1999). *Leishmania* parasites have similar morphology and sometimes cause similar clinical manifestations. Thus there is a need for new sensitive molecular methods for diagnosis rather than traditional ones such as microscopy, culture, clinical picture or travel/residence history which all lack sensitivity and specificity (Bensoussan et al. 2006). So the identification of the parasite is the only tool to confirm the disease categorically. Therefore, differentiations among species require molecular technique such as polymerase chain reaction (PCR) (Murray et al. 2005; Reithinger and Dujardin 2007). In this study, we evaluated its potential in the identification and characterization of Cutaneous Leishmaniasis in comparison to direct microscopy and culture-based methods. In this report, molecular characterization of cutaneous leishmaniasis showed the presence of 186bp fragment confirming *Leishmania tropica*, as the causative prevalent agent in all cases of cutaneous leishmaniasis is examined in district Kohat, KPK, Pakistan as shown in figures 6 and 7. In our study, the better detection rate by Giemsa stain as compared to culture was in agreement with the results reported by some

investigators (Ramirez et al. 2000) and in contrast with the findings of others who reported higher sensitivity for culture as compared with Giemsa stain (Weigle et al. 1978; Sharquie et al. 2002). In contrast to our study, culture method was previously reported to be the most sensitive for parasitologic diagnosis (Navin et al. 1990; Weigle et al. 1978). Even though the disease is usually diagnosed with microscopic examination of a dermal scraping or biopsy (Aviles et al. 1999), PCR was found to be a more sensitive method for diagnosis and confirmation of the infection (Culha et al. 2006; Romero et al. 2001).

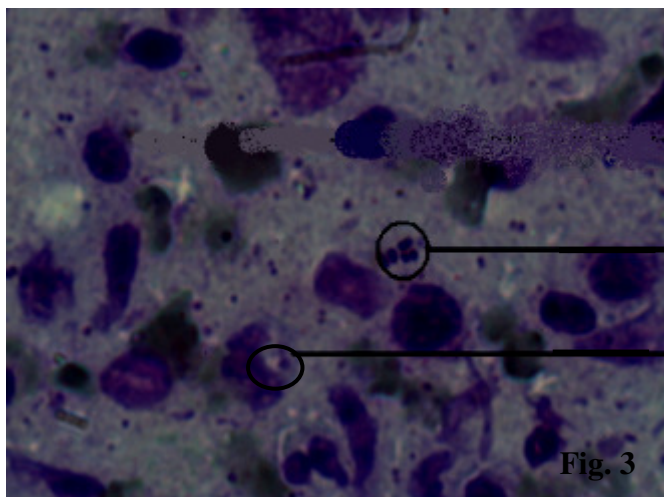
Conclusion

Histopathological and serological methodologies have limited the diagnostic criteria due to their low sensitivity, incompatible handling and with intercurrent bacterial or fungal superinfections. Our study showed the presence of *L. tropica* confirmed through PCR technique. In our study, PCR based method has been successfully applied for diagnosis and identification of *leishmania* parasite in number of cases in patients suspected with cutaneous leishmaniasis as compared to traditional microscopic and culture examination.



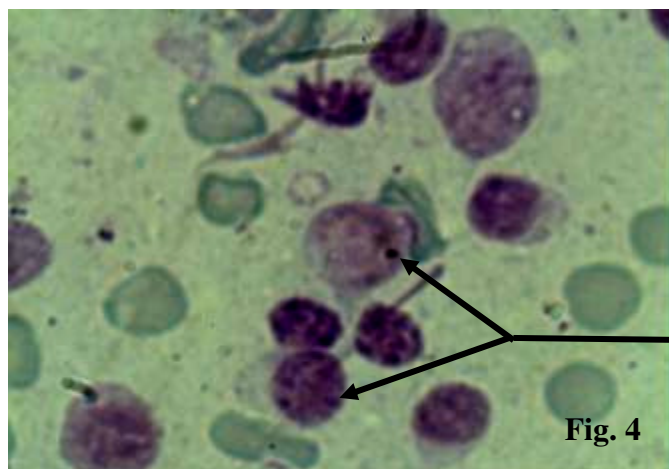


Figures demonstrate Tissue aspirate isolation of *Leishmania* infected patients with cutaneous leishmaniasis using sterilized scalpel (Fig. 1) and insulin syringe (Fig. 2) containing sterilized 0.9 percent normal saline



Amastigotes coming out from ruptured macrophages

Macrophages engulfed amastigote



Macrophages engulfed
Amastigotes

Fig. 4

Figures 3 and 4 demonstrate Giemsa stain image showing the amastigotes form of *Leishmania* inside and outside macrophages observed under microscope

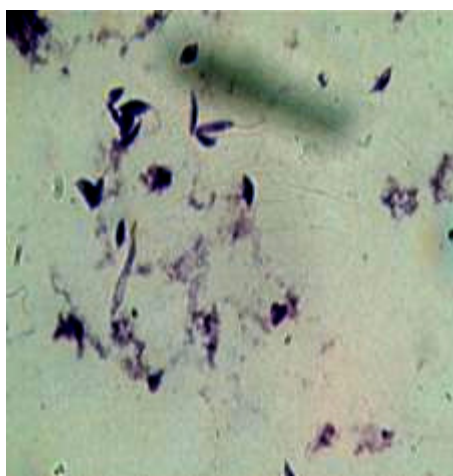
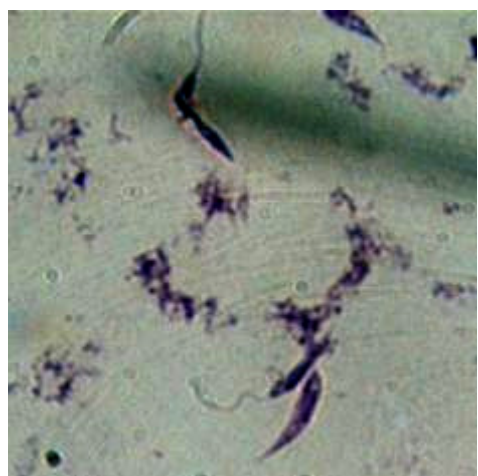
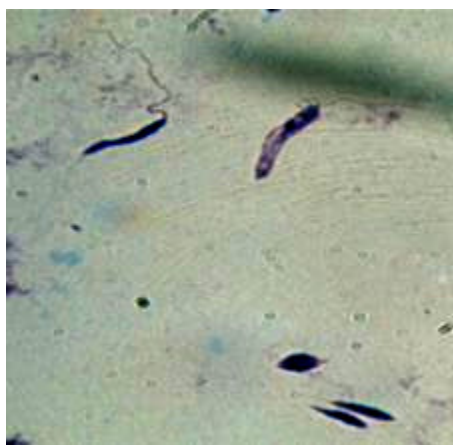
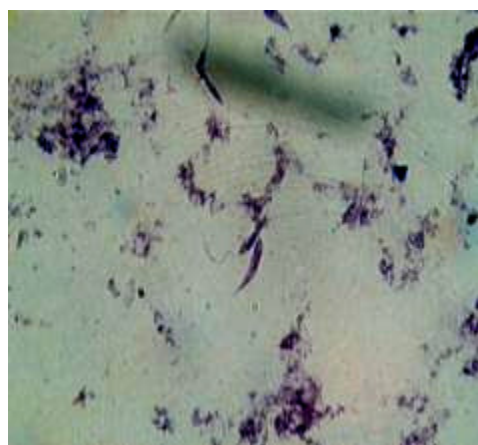


Figure 5 demonstrates *in vitro* cultured promastigotes with the emersion of single anterior flagellum inoculated in Novy- McNeal-Nicolle (NNN) medium using Giemsa stain

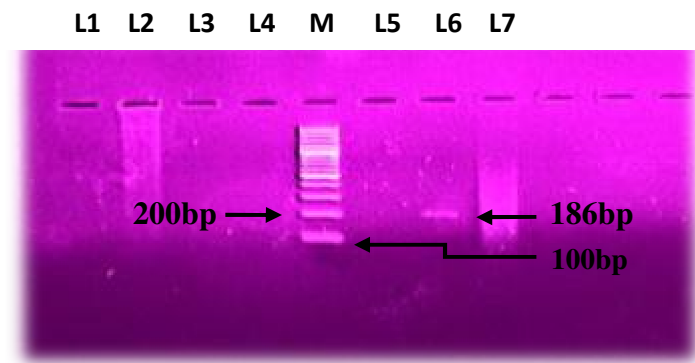


Figure 6. The result of the PCR-based amplification of kinetoplast DNA extracted from skin lesions of the patients. The bands shown on 2% agarose gel stained with ethidium bromide correspond to molecular weight markers. PCR amplified product of *L. tropica* in biopsy samples obtained from patients with 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

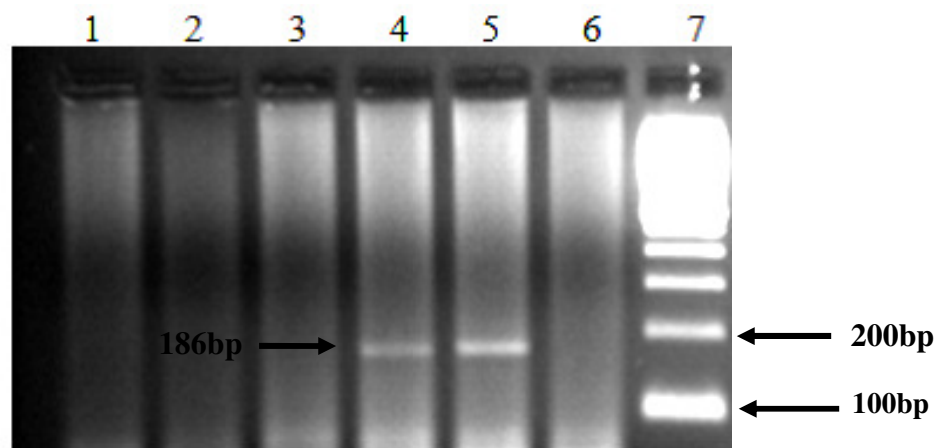


Figure 7. PCR amplified product of *L. tropica*. Lanes 1, 2 and 3 = negative samples; lane 6 = negative control, lane 4 = positive sample, lane 5 = positive control, lane 7 = 100 bp ladder DNA marker

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