Prevalence of Cutaneous Leishmaniasis in Humans and Dogs in Pakistan

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Abstract.- The study was undertaken from May 2007 to June 2008 to determine the prevalence of Cutaneous Leishmaniasis in humans and dogs in four regions of Pakistan. Humans and dogs were screened for the disease and potential positive cases were identified on the basis of skin lesions. Samples of blood and skin lesions were collected for thin smear and polymerase chain reaction (PCR) examination. PCR analyses of clinical samples were found to be positive for a single schizodeme of Leishmania tropica. It was also revealed that PCR could detect DNA from less than a single parasite and can be effectively used in epidemiological surveys. Both dry and muco-purulent cutaneous forms of the disease were found to be endemic in the North, South and West of Pakistan. The East and South-eastern regions were non endemic. No case of visceral form of disease was encountered during the period of study from any part of the country. In Northern Pakistan the disease was most prevalent in humans in November 2007 (661) and least prevalent during February 2008 (292) while in dogs the highest prevalence was during November 2007 (24%) and lowest prevalence in January 2008 (5%). In Southern Pakistan the highest human disease prevalence was in April 2008 (518 cases) and lowest disease detections in June 2007 with 308 cases. In dogs, the highest number of cases were in December 2007 (25% cases) and least number during July 2007 (9% cases). In Western Pakistan the human disease prevalence was highest in October 2007 (281 cases) and lowest during Feb. 2008 (66 cases) while in dogs most cases were detected in Nov. 2007 (21% cases) and the number of cases was lowest in Feb. 2008 (8%).

Keyword: Cutaneous leishmaniasis, PCR, dogs, humans.

INTRODUCTION

Leishmaniasis is vector transmitted zoonoses caused by more than 25 obligatory intracellular protozoan belonging to Leishmania species. Depending upon the species involved visceral, cutaneous and mucosal lesions are induced by involvement of macrophages in various organs and systems. Leishmaniasis represents a major health problem. An estimated one tenth of world’s population is at risk of infection, approximately 12 million people in 88 countries are infected and 2 million new cases occur each year (Anez et al., 1999). Leishmania: a genus of flagellate protozoa (suborder Trypanosomatidae, order Kinetoplastida) are parasites with worldwide distribution, several species of which are pathogenic for humans. Leishmania species are divided into two sub genera Vianna and Leishmania. Vianna comprises Leishmania that develop in mid and hind-gut (peripyralia reproduction) and Leishmania encompass forms that multiply in the fore-gut (suprapyralia reproduction) of the sand-fly (Barral et al., 1991). The United States Centers for Disease Control (CDC) describe leishmaniasis as either a disfiguring skin disease or a potentially fatal (if untreated) liver and spleen disease. Skin leishmaniasis may develop into a mucosal affliction of the nose and mouth. Drug resistance is reported in virtually all endemic areas and three quarters of annual occurrences are skin related. Of the 500,000 new cases reported annually worldwide, some 90% occur in five developing countries: Bangladesh, Brazil, India, Nepal and Sudan (Louzir et al., 1998). Leishmaniasis is mainly transmitted by blood sucking sand-flies or Phlebotomines of class Insecta, order Diptera, family Psychodidae. L. tropica has also been isolated from patients with visceral leishmaniasis in India and Israel (Lainson, 1982). L. donovani, L. infantum and L. chagasi are considered subspecies or members of a principal species or species complex called L. donovani-sensu lato. They can be distinguished easily by serological, enzymatic and molecular techniques.
The \textit{L. tropica} complex is found in the ‘Old World’ countries such as Afghanistan, Iran, Iraq, Israel, Kuwait and Uganda. The subspecies of \textit{L. tropica} are found in some Mediterranean countries including Greece, Tunisia and Turkey. \textit{L. major} exists in the Arabian Peninsula, Afghanistan, Algeria, Egypt, India, Iran, Iraq, Israel, Jordan, Libya, Morocco, Pakistan, Sudan, Syria and Turkey (Ashford \textit{et al.}, 1998). Ninety percent of new annual cases of visceral leishmaniasis occur in four countries: Brazil, India, Nepal and Sudan (Ayub \textit{et al.}, 2003). \textit{L. d. donovani} occurs in Bangladesh, India, Nepal and China. \textit{L. d. infantum} is found in Africa, Central Asia, the Mediterranean coast of Europe and Africa, Afghanistan, Saudi Arabia, Northwest China, Egypt, Iran, Iraq and Yemen. \textit{L. d. chagasi} is found in Northeastern Brazil, Northern Argentina, Bolivia, Columbia, and Mexico (Gurtler \textit{et al.}, 1991). Endemic areas of disease in Pakistan include the Hindukush and Karakoram sub mountain range (Chitral, Dir and Gilgit); the Himalayan sub mountain range (Mansehra, Abbottabad, Azad Kashmir, Rawalpindi); the Kirthar and Suleman sub mountain range (Lasbela, Khuzdar, Derabughhti, D.G.Khan, Rajanpur, Jacobabad, Larkana and Dadu); the Toba Kakar sub mountain range (Quetta, Qila Abdullah, Pishin, Qila Saifullah) (Ali and Afrin, 1997).

In the present study an attempt is made to study the prevalence of leishmaniasis in humans in endemic areas of Pakistan.

**MATERIALS AND METHODS**

\textit{Collection of blood and skin biopsy samples}

Pakistan was divided into four regions i.e. North, South, East and West for collection of samples. Samples from skin lesions were collected in the endemic areas during all seasons from human cases at private clinics and health centers during 2007-2008. Skin lesion samples were also collected from stray dogs with clinical signs of skin infection. Lesions were cleaned with soap and water and swabbed with ethanol. Skin-scrape samples were taken from the border of the lesion using a sterile scalpel. The sample was divided into two parts, one part was used to make a thin smear on a microscope slide and the other part was placed in an Eppendorf tube containing 500 µl of 4 M Guanidine thiocyanate (GuSCN) and 0.25 M EDTA. GuSCN lysates were stored in a refrigerator for PCR analysis. Thin films of blood and lesion material were placed on glass slides, labeled and fixed in alcohol and the slides were placed in slide boxes for transportation. The samples were brought to the University of Veterinary and Animal Sciences, Lahore, for further processing.

\textit{Staining of thin films}

Dry thin films were prepared from blood and skin lesions from infested humans and dogs and were stained with Leishman’s stain for microscopic identification of protozoan parasites; (Leishman Donovan bodies) using a compound microscope. A monthly record of the identified parasites was maintained according to geographic region.

\textit{Preparation of DNA samples}

Template DNA was extracted from aliquots of 50, 250, and 100 µl of the GuSCN lysates. Briefly, the sample was bound to diatomaceous earth in the presence of 6 M GuSCN; washed with ethanol and acetone and eluted with 50 µl of 10 mM Tris-HCl (pH 8.4). One µl of template was used in the first round PCR. DNA was prepared from 30 confirmed samples and amplified by PCR at least three times. Each replicate batch was prepared independently from previous batches with fresh sets of reagents. DNA of reference strains was prepared by standard methods.

\textit{PCR conditions}

External primers CSB2XF (C/G A/GTA/GCAGAAAC/TCCCGTTCA) and CSB1XR (ATTTTTCG/CGA/TTTT/CGCAGAACG) were designed after identifying suitable regions around conserved sequence blocks 1 and 2 in accordance with kDNA sequences from \textit{L. major}, \textit{L. infantum}, \textit{L. donovani} and \textit{Leishmania tropica}. The primers were designed to be external to primers 13Z (ACTGGGGGTTGTTGTGAAAAATAG) which is homologous to conserved block 3 and LiR (TCGCAGAAACGCCCT) which is complementary to conserved block 1. The conserved block 1 was too small for two independent primers; therefore as a result, the 10 3’ bases of CSB1XR are the same as...
the 10'5' bases of LiR. First round PCR mixtures contained 2.0 mM MgCl₂, 200 µM Deoxynucleoside triphosphates, 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl (pH 9.0), 0.01% Tween, 0.4 U of Red Hot Taq and 40 ng each of primers CSB2XF and CSB1XR in a final volume of 20 µl. The cycling conditions were 94°C for 300 seconds, followed by 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 90 seconds in a thermocycler. One µl of a 9:1 dilution in double distilled water of the first round product was used as template for the second round in a total volume of 30 µl under the same conditions as those for the first round, except with primers LiR and 13Z. Three µl of the second round PCR product was loaded onto a 1% Agarose gel to confirm amplification. Positive samples were digested by the addition of 1 U of restriction enzyme, 1.5 µl of restriction enzyme buffer, and 1.4 µl of water to 12.5 µl of PCR product and incubated for 16 hours. The restriction digests were separated on a 1.5% 1:1 agarose gel to visualize the schizodeme patterns.

**DNA sequencing**

DNA for sequencing was prepared by the PCR. The first round product was reamplified with primers LiR and 13Z in a total volume of 100 µl. Primers and deoxynucleoside triphosphates were removed by centrifugation, the DNA was precipitated with ethanol and the sample was processed for cycle sequencing with primers LiR and 13Z on a cycle sequencer (Hyde, 1993).

**RESULTS**

**Seasonal and area-wise prevalence of Cutaneous Leishmaniasis**

The collected samples were brought to the laboratory and positive samples were identified by presence of Leishman Donovan Bodies in thin smears (Fig. 1). Cutaneous form of the human disease was found to be endemic in the North, South and West of Pakistan characterised by both dry and muco-purulent forms. The East and South-Eastern regions were non endemic. No case of the visceral form of the disease was encountered from any part of the country during the period of study. In Northern Pakistan the disease was prevalent throughout the year in human populations, with 375 positive cases of leishmaniasis in May 2007. Most cases occurred in November 2007 (661) and the least number of cases were detected during February 2008 (292) (Table I, Fig. 2A).

The disease was also prevalent throughout the year in dogs of the same region with 10% positive cases during May 2007. The disease reached a maximum in November 2007 (24%) and minimum in January 2008 with 5% positive cases (Fig. 5).

In Southern Pakistan the disease was prevalent throughout the year in human population, with positive 315 cases of leishmaniasis during May 2007. Human cases were most prevalent in April 2008 (518 cases) and the least number of cases (308) occurred in June 2007 (Table II). Cases reached a maximum in December 2007 (25% cases) and minimum during July 2007 (9% cases) (Fig. 3B). The disease was also prevalent throughout the year in dogs of the same region with 13% positive cases during May 2007.

In Western Pakistan the disease was prevalent throughout the year in the human population, with 219 positive cases of leishmaniasis during May 2007. Cases were most prevalent in October 2007 (281 cases) and the lowest number of cases were recorded during February 2008 (66 cases) (Table III, Fig. 2C).
The disease was also prevalent throughout the year in dogs of the same region with 10% positive cases recorded during May 2007. The highest prevalence occurred in November 2007 (21% cases) and the lowest prevalence was in February 2008 (8%) (Table IV, Fig. 3).

Specificity of polymerase chain reaction

The primer set designed was tested on DNA from a group of Leishmania species that are capable of infecting humans and dogs. It generated a single major product from representatives of all major complexes of Leishmania. L. tropica generated the
prevalence of cutaneous leishmaniasis in humans and dogs

Fig. 2. Seasonal prevalence of leishmaniasis in humans in northern (A) southern (B) and western(C) Pakistan.

largest PCR product 750 bp; which could be distinguished from L. infantum 680 bp and L. major 560 bp. It was therefore possible to identify the Leishmania complexes on the basis of size alone (Fig. 4).

Sensitivity of PCR

Decimal dilutions 500 pg to 1 ag of Leishmania tropica MOHM/EG/06/RTC-67 were amplified by PCR and digested with Hae III for determination of the limit of detection. The limit of
detection was 0.1 ag equivalent to 1/500 of Leishmania genome. Fingerprints of 100 fg and 10 fg were complex, whereas 1 fg and 0.1 fg were simple (Fig. 5).

**Detection of parasites by PCR**

The sensitivity of the PCR was tested on twenty samples collected from patients with lesions of cutaneous leishmaniasis collected from different endemic areas. Three replicate DNA extractions were prepared from 50, 250 and 100 µl aliquots; drawn from the 500 µl original sample volume. PCR on these replicate DNA preparations produced 14, 15 and 12 positives, respectively, from the 20 samples. All of the PCR products were of equal size and of the same size as the L. tropica reference strain MOHM/EG/06/RTC-67. This was also confirmed by similar schizodeme patterns in all of the samples. DNA was extracted from aliquots of each of the twenty samples at least three times. Of 60 DNA preparations, 41 were positive and 19 were negative for Leishmania kDNA. Of the total volume of the sample of 500 µl., aliquots of 50 µl. gave a greater number of negative results and less number of positives results compared to using aliquots of 250 µl. When 250 µl. was used there were less negative and more positive results. It was concluded that this was probably due to the volume used for the test.

**Schizodeme analysis of samples**

The 14 positive samples from the first set of replicates were digested with Hae III to prepare DNA fingerprints. Five samples had complex fingerprint patterns. There were also five simple fragments. The detection of simple fingerprints suggests that the PCR could detect a fraction of the DNA released from a single parasite (Table V, Fig. 6).

**DISCUSSION**

Endemic areas of disease in Pakistan were the districts of Chitral, Dir, Swat and Gilgit; Mansehra, Skardu, Chilas, Abbottabad, Rawalpindi and Azad Kashmir; Lasbela, Khuzdar, Derabughti, D.G.Khan, Rajanpur, Jacobabad, Larkana and Dadu; Quetta, Qila Abdullah, Pishin and Qila Saifullah. The above mentioned areas are foot hills of mountainous ranges that are present in the North, West and South-Western Pakistan, which cover all the four provinces including Azad Kashmir. The South-Eastern areas of Pakistan are non-endemic according to Ali and Afrin (1997).
The endemic areas provide optimal conditions for growth and development of the vectors. After the advent of the war in Afghanistan, large segments of the Afghani population were displaced and resettled in camps setup in these areas by relief agencies. They also brought with them diseases that were endemic to their native homeland. Before the migration of the Afghan refugees only sporadic cases of leishmaniasis were seen but now these areas have become established areas of endemincity involving the local Pakistani population Mujtaba and Khalid (1998).

During the period of study no case of visceral leishmaniasis was encountered in the endemic and non-endemic areas of the disease. In Pakistan the cutaneous form of disease was encountered throughout the year in human and dog populations as described previously by Fazal et al. (2003). The prevalence of disease was found to vary in different parts of Pakistan during the period of study.

In the northern region, the highest numbers of positive human cases were encountered during November 2007 (661 cases). Similarly the highest number of cases in dogs was also encountered during November 2007 (24% cases). In the western region, most human cases occurred during October 2007 (281 cases) and the highest number of cases in dogs was also during November 2007 (21% cases). In the southern region, of the highest prevalence in humans was during April 2008 (518 cases) and this reflected the highest prevalence in dogs also during April 2008 (25% cases).
The results indicate that there is a definite relationship between human and dog prevalence. It was observed that dogs were seen wandering in bushes and damp places during the day to rest. Such places were often found to be the hide-outs of sand-flies and therefore the dogs were bitten when they disturbed the insects as reported by Flynn (1973). Further, human dwellings had no insect proof screens to prevent sand-flies biting humans during the night.

The PCR detected extremely small amounts of *Leishmania* kDNA consistent with the work of Heath (1997) which was shown by sequencing to belong to an individual minicircle class. The primers were expected to amplify all minicircles classes present in the DNA template, but if one minicircle class was present in the template, only that class would be amplified. On the other hand if more than one minicircle classes were present then all those classes would be amplified. This entire phenomenon depends on the DNA sample template. Sometimes one minicircle class can suppress amplification of other classes due to the presence of an extensive secondary structure resulting in false negatives as found by Raja et al. (1988).

Fifteen samples from the endemic areas were positive in one of three replicates of DNA. Five samples were negative because the biopsy material may have contained zero or only a small number of parasites. Therefore the negatives reflect an absence of parasites in the samples. *L. tropica* generated the largest PCR product 750 bp, which could be distinguished from *L. infantum* 680 bp and *L. major* 560 bp. It was therefore possible to identify the Old World *Leishmania* complexes on the basis of size alone. All of the PCR products were of the same size as one another and of the same size as the *L. tropica* reference strain. This was also confirmed by similar schizonte patterns in all of the samples (Qiao et al., 1995). The detection of simple fingerprints suggests that the PCR could detect a fraction of the DNA released from a single parasite. Schizonte analyses revealed that all the positive samples belonged to same schizonte of *L. tropica*. This shows that PCR is extremely sensitive and can accurately detect *Leishmania* parasites and is an effective tool for diagnosis of diseases during epidemiological surveys.

**REFERENCES**


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