CUTANEOUS LEISHMANIASIS IN HUMANS AND DOGS IN PAKISTAN

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The study were done from May 2007 to June 2008 to find out prevalence of Cutaneous Leishmaniasis in human and dogs in four regions of Pakistan .The prevalence of cutaneous leishmaniasis in human and dogs was studied. Screening of positive cases in humans and dogs was done on the basis of skin lesions. Samples of blood and skin lesions were collected for thin smear and 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.Cutaneous form of the disease was found to be endemic in the North, South & West of Pakistan with characteristics of dry and muco-purulent forms. The East & 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 prevalent maximum in November 2007 (661) and were lowest during February 2008 (292) while in dogs maximum prevalence was during November 2007 (24%) & minimum in January 2008 (5%). In Southern Pakistan the disease was maximum in April 2008 (518 cases) and lowest in June 2007 with 308 cases while in dogs cases reached a maximum in December 2007 (25% cases) & minimum during July 2007 (9% cases). In Western Pakistan the disease maximum in October 2007 (281 cases) & lowest during Feb.2008 (66 cases) while in dogs cases reached a maximum in Nov. 2007 (21% cases) and were 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 & mucosal lesions are induced by involvement of macrophages in various organs & systems. Leishmaniasis represents a major health problem. 1/10 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*) comprising parasites of worldwide distribution, several species of which are pathogenic for humans. *Leishmania* species are divided into two sub genera *Viannia* and *Leishmania*. *Viannia* comprises of *Leishmania* that develop in mid and hind-gut (peripylaria reproduction) and *Leishmania* encompassing forms that multiply in the fore-gut (suprapylaria reproduction) of the sand-fly. (Barral *et al.,*. 1991). The U.S. Center for Disease Control (CDC) estimate that leishmaniasis occurs as either a disfiguring skin or 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, $3/4^{th}$ of annual occurrences are skin related. Of the 500,000 new cases reported annually worldwide, some 90% occur (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 & Israel(Lainson, 1982). *L.donovani*, *L.infantum* & *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 & molecular techniques. The *L.tropica* complex is found in the Old World, Afghanistan, Iran, Iraq, Israel, Kuwait & Uganda but the subspecies of *L.tropica* are found in some Mediterranean countries Greece, Tunisia & Turkey. *L.major* exists in the Arabian Peninsula, Afghanistan, Algeria, Egypt, India, Iran, Iraq, Israel, Jordan, Libya, Morocco, Pakistan, Sudan, Syria, & Turkey(Ashford *et al.,*1998). Ninety percent of new annual cases of visceral leishmaniasis come from five countries: Brazil, India, Nepal and Sudan(Ayub *et al.,*.2003). *L.d.donovani* occurs in Bangladesh, India, Nepal and China. *L.d.infantum* Africa, Central Asia, Mediterranean coast of Europe and Africa, Afghanistan, Saudi Arabia, Northwest China,

Egypt, Iran, Iraq, Israel and Yemen. *L.d.chagasi* is found in Northeastern Brazil, Northern Argentina, Bolivia, Columbia, and Mexico.(Gutler *et al.,* 1991). Endemic areas of disease in Pakistan were Hindukush & Karakoram sub mountain range (Chitral, Dir & Gilgit etc); Himalayan sub mountain range (Mansehra, Abbottabad & Azad Kashmir, Rawalpindi etc); Kirthar & Sulaman sub mountain range (Lasbela, Khuzdar, Derabughti, D.G.Khan, Rajanpur, Jacobabad, Larkana & Dadu etc);Toba Kakar sub mountain range (Quetta, Qila Abdullah, Pishin, Qila Saifullah etc) 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

Collection of blood and skin biopsy samples

Pakistan was divided into four regions i.e. North, South, East & West for collection of samples .Samples from skin lesions were collected in the endemic areas during all seasons from human population 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. Samples were taken by using a sterile scalpel from the border of the lesion. 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 on glass slides were labeled and fixed in alcohol, the slides were placed in slide boxes for transportation. The samples were brought to University of Veterinary and Animal Sciences, Lahore, for further processing.

Staining of Thin films

Dry thin films were prepared from blood & ski lesion samples of humans and dogs were stained with Leishman's stain for identification of protozoan parasites; (Leishman Donovan bodies) by using compound microscope according to method .Monthly record of the identified parasites was maintained area-wise.

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).1 µl of template was used in the first round PCR. DNA was prepared from 30 confirm 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 .

PCR conditions

External primers CSB2XF (C/GA/ GTA/GCAGAAAC/TCCCGTTCA) and CSB1 XR(ATTTTTCG/CGA/TTTT/CGCAGAACG) were designed after identifying suitable regions around conserved sequence blocks 1 and 2 in accordance with kDNA sequences from *L. major, L. infantum, L.donovani & Leishmania tropica.*The primers were designed to be external to primers 13Z (ACTGGGGGTTGGT GTAAAATAG) which is homologous to conserved block 3 and LiR (TCGCAGAACG CCCCT) 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 $MgCl_2$, 200 µM Deoxynucleoside triphosphates, 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl (pH 9.0), 0.01% Tween, 0.4 U of Red Hot Taq and 40 ng each of primers CSB2XF & 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 & 13Z. 3 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 incubation 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 disease was found to be endemic in the North, South & West of Pakistan with characteristics of dry and muco-purulent forms. The East & 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 prevalent throughout the year in human populations, with 375 positive cases of leishmaniasis in May 2007. Cases were maximum in November 2007 (661) and were lowest during February 2008 (292) (Table 1,Fig.2).

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%) & minimum in January 2008 with 5% positive cases (Figure 5).

In Southern Pakistan the disease was prevalent throughout the year in human population, with positive 315 cases of Leishmaniasis during May 2007. Cases reached a maximum in April 2008 (518 cases) and lowest in June 2007 with 308 cases (Table 2). The disease was also prevalent throughout the year in dogs of the same region with positive 13% cases during May 2007. Cases reached a maximum in December 2007 (25% cases) & minimum during July 2007 (9% cases) (Figure 3).

In Western Pakistan the disease was prevalent throughout the year in human population, with 219 positive cases of leishmaniasis during May 2007. Cases reached a maximum in October 2007 (281 cases) & lowest during Feb. 2008 (66 cases) (Table 3,Figure 4).

The disease was also prevalent throughout the year in dogs of the same region With 10% positive cases during May 2007.Cases reached a maximum in Nov.2007(21% cases) and were lowest in Feb.2008(8%)(Table 4,Figure 5)

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 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 (Figure 6).

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 .(Figure 7)

Sequencing of PCR products

The products of PCR of 1 fg of MOHM/EG/06/RTC-67 gave a sharp band after digestion with *Hae* III indicating that this sequence has been obtained from a single minicircle class *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; out of 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 the same size as one another and of the same size as *L. tropica* reference strain MOHM/EG/06/RTC-67. This was also confirmed by similar schizodeme patterns in all of the samples. As DNA was extracted from aliquots of each of the twenty samples at least three times. Therefore out of 60 DNA preparations, 41 were positive and 19 were negative for *Leishmania* kDNA. As the total volume of the sample was 500 µl. therefore aliquot of 50 µl. gave more number of negatives and less number of positives where as aliquot of 250 µl. gave less number of negatives and more positives; this was probably due to its volume.

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 5 & Figure 8).

Figure 1: *Leishmania tropica* from cutaneous lesions in man.

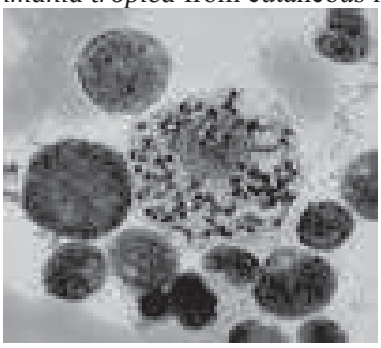


Figure 2: Seasonal prevalence of Leishmaniasis in humans, Northern Pakistan.

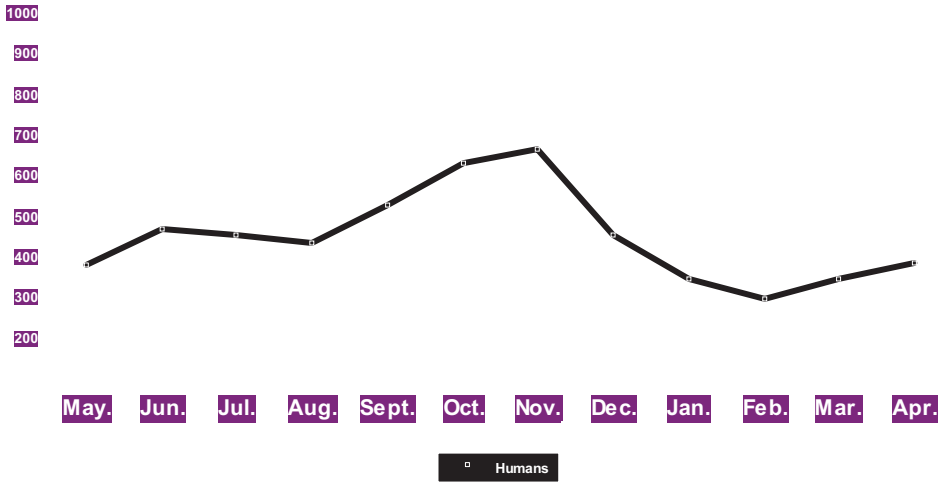


Figure 3: Seasonal prevalence of leishmaniasis in humans Southern Pakistan.

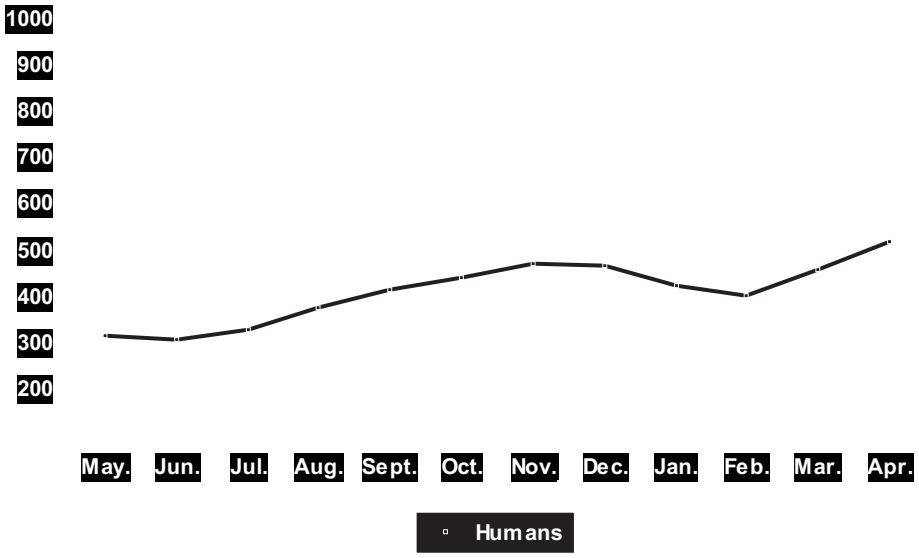


Figure4: Seasonal prevalence of Leishmaniasis in humans, Western Pakistan.

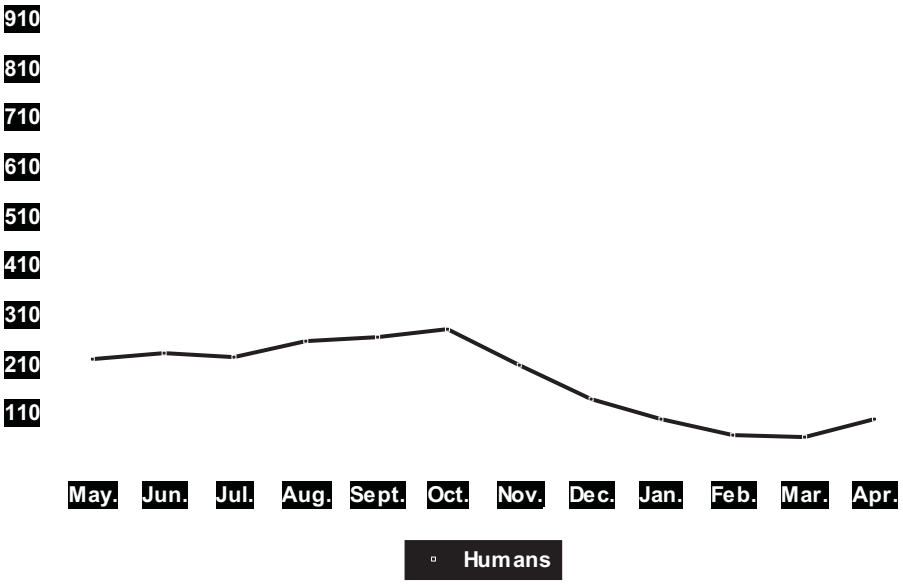


Figure 5: Seasonal prevalence of Leishmaniasis in dogs, Pakistan.

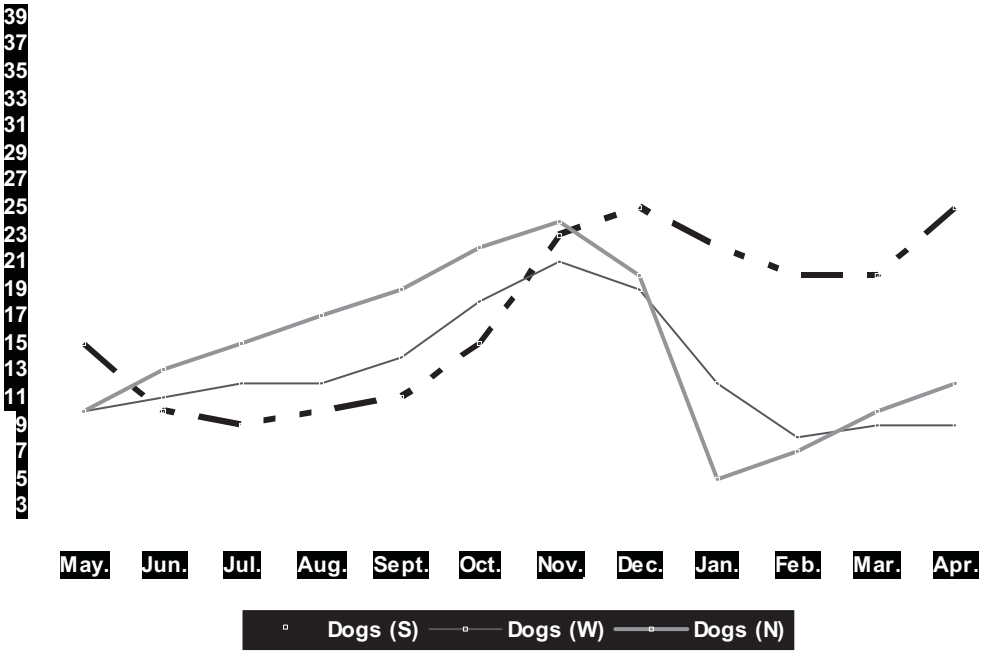
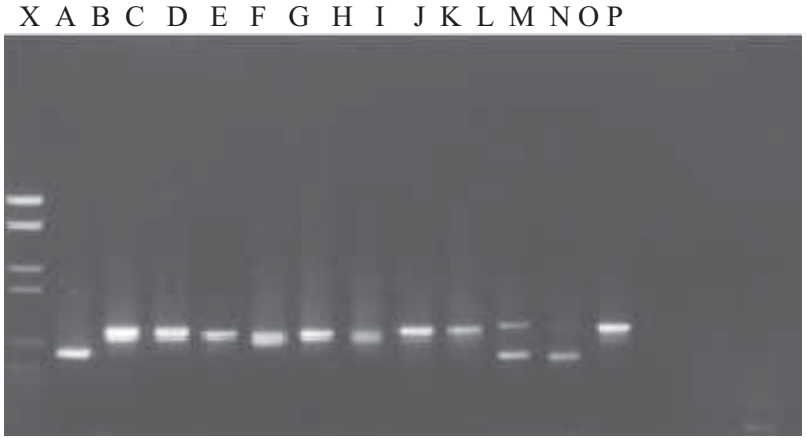


Figure 6 Products of PCR on Agarose gel 1.5%.



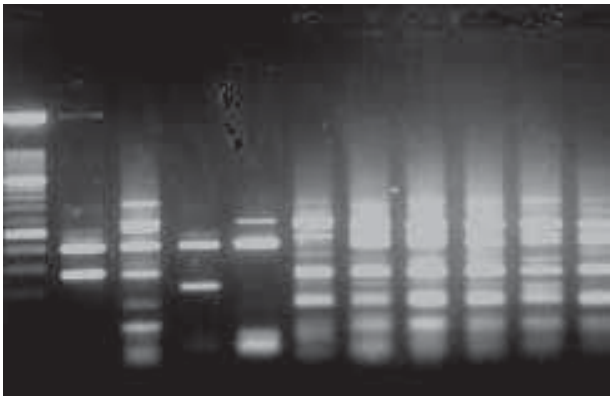
lane X, Boehringer Mannheim molecular weight marker set VI (100 bp. ladder).
lane A, *L.tropica* MOHM/EG/06/RTC-67.
lane B, *L.tropica* MOHM/EG/06/RTC-73.
lane C, *L. tropica* MHOM/IR/89/ARD22.
lane D, *L. tropica* MHOM/PK/97/37\13.
lane E, *L. Major* MOHM/EG/06/RTC-64.
lane F, *L. infantum* MHOM/HN/87/HN29.
lane G, *L. tropica* MHOM/PK/07/07/39W.
lane H, *L. donovani* MOHM/CN/80/801.
lane I, *L. major* MHOM/ET/95/FV1.
lane J, *L. infantum* MHOM/TN/80/IPT1.
lane K, *L. major* MHOM/ET/XX/LV305
lane L, *L. tropica* MHOM/PK/07/11/22N.
lane M, *L. tropica* MHOM/PK/07/08/10N.
lane N, Negative control.
lane O, *L. tropica* MHOM/PK/07/12/42W.
lane P, *L. tropica* MHOM/PK/08/04\02W.

Figure 7. Decimal dilution series of *L.tropica.*

Schizodemes prepared from kDNA amplified from 100, 10, 1& 0.1 fg of total DNA and digested with *Hae* lll. Lane X, 100 bp ladder molecular size marker. (Boehringer Mannheim)

Figure 8. Schizodemes of kDNA from samples.

X 8 9 16 19 10 11 1 14 18 2



Schizodemes of kDNA amplified from samples collected from endemic areas & digested with *Hae* lll. The lane numbers refer to the sample numbers in table ---.
Lane X, 100 bp ladder molecular size marker.
(Boehringer Mannheim)

Table I. Seasonal prevalence of Leishmaniasis in humans, Northern Pakistan on the basis of thin films

	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	Total
Skardu	30	45	34	36	38	55	42	21	12	11	14	17	355
Gilgit	24	44	30	39	37	53	57	23	10	8	10	14	349
Chitral	36	46	41	31	48	49	58	25	12	3	7	14	370
Dir upper	37	45	50	35	46	47	47	24	17	11	9	19	387
Dir lower	33	47	32	36	42	52	55	32	28	16	12	10	395
Swat	44	20	42	35	33	47	49	36	22	20	23	34	405
Kohistan	34	33	37	37	48	54	55	40	29	19	27	33	446
Batgram	35	30	32	30	39	47	56	37	24	17	23	42	412
Mansehra	27	34	39	37	38	47	51	49	40	42	37	31	472
Abbottabad	37	35	34	29	38	49	57	47	41	47	31	39	484
A.J.Kashmir	20	66	58	74	98	102	77	67	64	56	75	43	800
Rawalpindi	18	20	19	12	18	23	57	48	41	42	75	85	458
Total	375	465	448	431	523	625	661	449	340	292	343	381	5333

Table II. Seasonal & area-wise prevalence of Leishmaniasis in humans, Southern Pakistan.

	May 2007	Jun 2007	Jul 2007	Aug 2007	Sep 2007	Oct 2007	Nov 2007	Dec 2007	Jan 2008	Feb 2008	Mar 2008	Apr 2008	Total
D.G. Khan	22	19	21	26	24	28	33	35	31	30	36	38	343
Rajanpur	19	15	23	28	27	31	34	36	30	27	34	40	344
Jacobabad	20	18	19	21	25	29	32	34	29	28	32	38	325
Larkana	28	26	30	35	37	41	43	40	36	34	38	40	428
Dadu	27	25	32	35	39	43	45	44	38	45	47	51	471
Lasbela	34	39	41	44	46	50	52	49	46	40	42	48	531
Khuzdar	26	29	33	36	42	44	48	45	43	40	47	52	485
JhalMaghsi	40	38	36	42	48	45	49	43	36	39	45	56	517
DeraBughti	39	37	34	39	46	44	48	45	42	38	47	52	511
Barkhan	28	32	30	38	43	42	47	53	50	46	49	56	514
MusaKhel	32	30	29	35	39	43	40	45	43	38	40	47	461
Total	315	308	328	379	416	440	471	469	424	405	457	518	4930

Table III. Seasonal & area-wise prevalence of Leishmaniasis in humans, Western Pakistan.

	May 2007	Jun 2007	Jul 2007	Aug 2007	Sep 2007	Oct 2007	Nov 2007	Dec 2007	Jan 2008	Feb 2008	Mar 2008	Apr 2008	Total
Zhob	34	32	42	47	53	56	48	32	27	18	14	24	427
QilaSafulla	41	46	40	51	54	59	40	29	22	12	15	21	430
QilaAbdulla	43	50	47	44	59	55	39	24	19	09	11	15	415
Pashin	45	47	40	51	48	56	37	22	12	10	13	16	397
Quetta	56	60	57	66	53	55	45	33	21	17	10	23	496
Total	219	235	226	259	267	281	209	140	101	66	63	99	2165

TABLE 1V: Leishmaniasis in dogs on the basis of thin smears in three regions.

	May 2007	Jun 2007	Jul 2007	Aug 2007	Sep 2007	Oct 2007	Nov 2007	Dec 2007	Jan 2008	Feb 2008	Mar 2008	Apr 2008
North	10%	13%	15%	17%	19%	22%	24%	20%	5%	7%	10%	12%
South	15%	10%	9%	10%	11%	15%	23%	25%	22%	20%	20%	25%
West	10%	11%	12%	12%	14%	18%	21%	19%	12%	8%	9%	9%

Table V: Results of PCR test on 20 samples.

Sample No.	**Strain designation**	**Microscopy results**	**Duration (months)**	**No. of lesions**	**PCR positives**	**Replicate No.**	**Schizo. pattern**
1	MHOM/PK/07/05/21N	+	3-4	2	3	3	csn
2	MHOM/PK/07/05/90S	+	6-7	1	3	3	ncc
3	MHOM/PK/07/06/05W	+	4-5	3	3	3	css
4	MHOM/PK/07/06/69S	+	9	2	3	3	ncs
5	MHOM/PK/07/07/39N	+	7-8	2	2	3	-sn
6	MHOM/PK/07/07/68W	+	10	1	3	3	ssc
7	MHOM/PK/07/08/10S	-	2-3	2	0	3	---
8	MHOM/PK/07/08/40S	+	6-7	4	3	3	nss
9	MHOM/PK/07/09/16W	+	2	3	3	3	scn
10	MHOM/PK/07/10/52N	+	8	3	2	3	-cc
11	MHOM/PK/07/11/22W	+	5-6	1	3	3	ccn
12	MHOM/PK/07/12/42N	-	6	2	0	3	---
13	MHOM/PK/07/12/89S	+	8	1	2	3	nn-
14	MHOM/PK/08/01/06N	+	1-2	4	3	3	cnc
15	MHOM/PK/08/01/44S	-	5-6	3	0	3	---
16	MHOM/PK/08/02/07W	+	8-9	4	3	3	snc
17	MHOM/PK/08/02/60W	-	3	2	0	3	---
18	MHOM/PK/08/03/26N	+	5-6	1	2	3	-nc
19	MHOM/PK/08/03/52N	+	6	1	3	3	ssc
20	MHOM/PK/08/04\02S	-	7	2	0	3	---

n: > 10 fragments.
c: < 6 fragments.
s: simple fragments.

Discussion

Endemic areas of disease in Pakistan were districts of Chitral, Dir, Swat and Gilgit; Mansehra, Skardu, Chilas, Abbottabad, Rawalpindi and Azad Kashmir; Lasbela, Khuzdar, Derabughti, D.G.Khan, Rajanpur, Jacobabad, Larkana & 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 Afghan war, large segment of Afghan population was displaced and got

settled in the 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 Afghan population only sporadic cases of leishmaniasis were seen but now these areas have become established areas of endeminicity involving the local Pakistani population. Mujtaba and Khalid (1998).

Sampling of dogs was done at random; dogs with skin lesions characteristic of leishmaniasis were sampled. Human samples were taken from patients showing skin lesions .. Sampling was done at various private and Govt. health centers. 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. Fazal *et al.* (2003). The prevalence of disease was found to vary in different parts of Pakistan during the period of study.

In North; maximum numbers of positive human cases were encountered during November 2007 (661 cases); similarly maximum numbers of cases in dogs were encountered during November 2007 (24% cases). In West; maximum numbers of positive human cases were encountered during October 2007 (281 cases); similarly maximum numbers of cases in dogs were encountered during November 2007 (21% cases). In South; maximum numbers of positive human cases were encountered during April 2008 (518 cases); similarly maximum numbers of dogs were encountered during April 2008 (25% cases). The results indicate that there is a definite relationship between human and dog prevalence. It was experienced 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, therefore the dogs were bitten, upon agitation of flies. Flynn, (1973). Human dwellings were also not adequately built providing easy access of sand-flies to humans during night. The PCR detected extremely small amounts of *Leishmania* kDNA, Heath, (1997) which was shown by sequencing to belong to an individual minicircle class. Since 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 are present then all those classes would be amplified. This entire phenomenon will depend on the DNA sample template. Sometimes one minicircle class can suppress amplification of other class due to presence of an extensive secondary structure resulting in false negatives. 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 might be having scanty number of parasites. Therefore the negatives reflect 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 *L. tropica* reference strain. This was also confirmed by similar schizodeme 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. Schizodeme analyses reveal that all the positive samples belong to same schizodeme 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.

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