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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 *Viannia* and *Leishmania*. *Viannia* comprises *Leishmania* that develop in mid and hind-gut (peripylaria reproduction) and *Leishmania* encompass forms that multiply in the fore-gut (suprapylaria 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.

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The *L. tropica* complex is found in the 'Old World' countries such as Afghanistan, Iran, Iraq, Israel, Kuwait and Uganda. The subspecies of *L. tropica* are found in some Mediterranean countries including Greece, Tunisia and Turkey. *L. major* exists in the Arabian Peninsula, Afghanistan, Algeria, Egypt, India, Iran, Iraq, Israel, Jordan, Libya, Morocco, Pakistan, Sudan, Syria and Turkey (Ashford *et al.,*1998). Ninety percent of new annual cases of visceral leishmaniasis occur in four countries: Brazil, India, Nepal and Sudan (Ayub *et al.,* 2003). *L. d. donovani* occurs in Bangladesh, India, Nepal and China. *L. d. infantum* is found in Africa, Central Asia, the 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 (Gurtler *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, Derabughti, 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

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.

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.

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.

PCR conditions

External primers CSB2XF (C/GA/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 *L. major, L. infantum, L.donovani* and *Leishmania tropica.*The primers were designed to be external to primers 13Z (ACTGGGGGTTGGTGTAAAATAG) which is homologous to conserved block 3 and LiR (TCGCAGAACGCCCCT) 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 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).

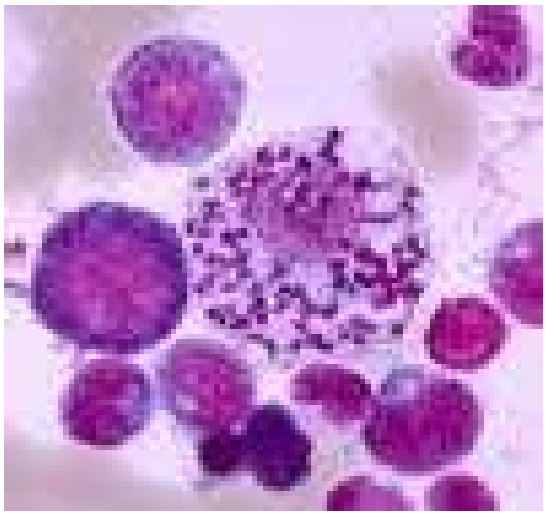


Fig. 1. *Leishmania tropica* from cutaneous lesions in man.

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).

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 and geographic area 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

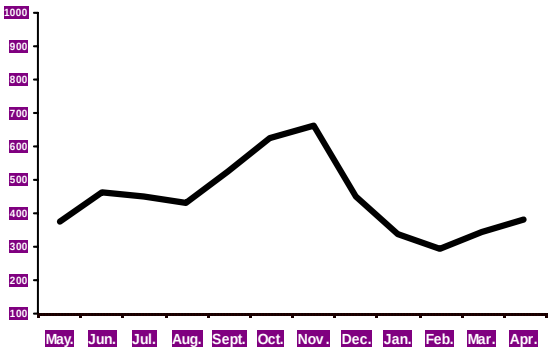
Table III.- Seasonal and geographic area prevalence of leishmaniasis in humans, Western Pakistan.

	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

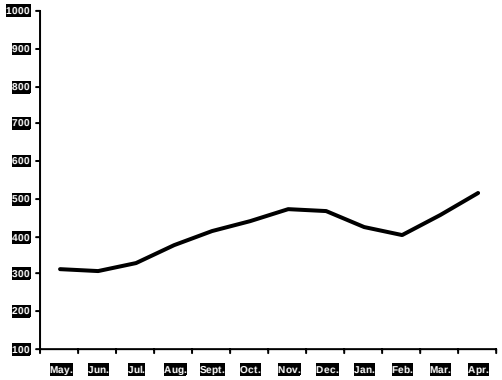
The disease was also prevalent throughout the year in dogs of the same region with 10% positive cases recorded during May 2007. The highest prevalence occurred in November 2007 (21% cases) and the lowest prevalence was in February 2008 (8%) (Table IV, Fig. 3).

Specificity of polymerase chain reaction

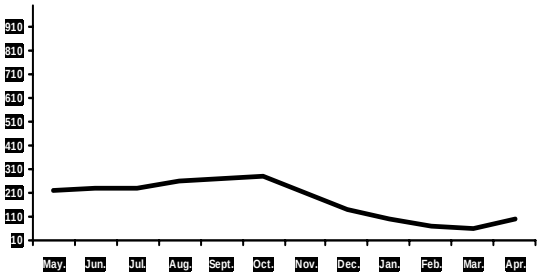
The primer set designed was tested on DNA from a group of *Leishmania* species that are capable of infecting humans and dogs. It generated a single major product from representatives of all major complexes of *Leishmania. L. tropica* generated the



A



B



C

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

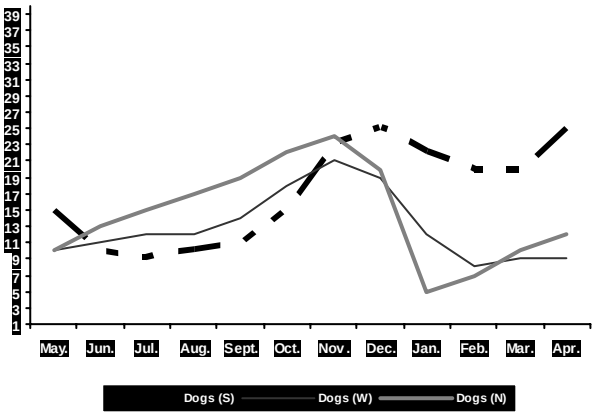


Fig. 3 Seasonal prevalence of leishmaniasis in dogs in Pakistan.

X A B C D E F G H I J K L M N O P

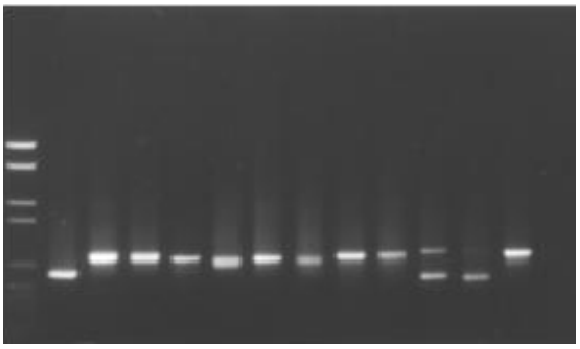


Fig. 4. Products of PCR on agarose gel 1.5%. lane X, Boehringer Mannheim molecular weight marker set VI (100 bp. ladder); Lane A, *L.tropica* MOHM/EG/06/RTC-67; Lane B, *L.tropica* MOHM/EG/06/RTC-73; Lane C, *L. tropica* MHOM/IR/89/ARD22; Lane D, *L. tropica* MHOM/PK/97/37\13; Lane E, *L. Major* MOHM/EG/06/RTC-64; 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.

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).

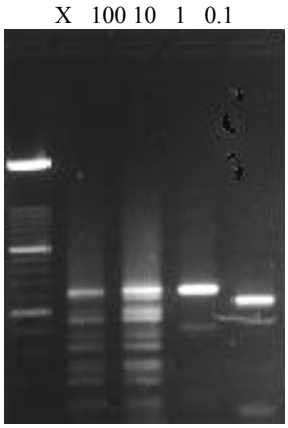


Fig. 5. Decimal dilution series of *L.tropica.* Schizodemes prepared from kDNA amplified from 100, 10, 1 and 0.1 fg of total DNA and digested with *Hae* lll. Lane X, 100 bp ladder molecular size marker (Boehringer Mannheim)

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; 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).

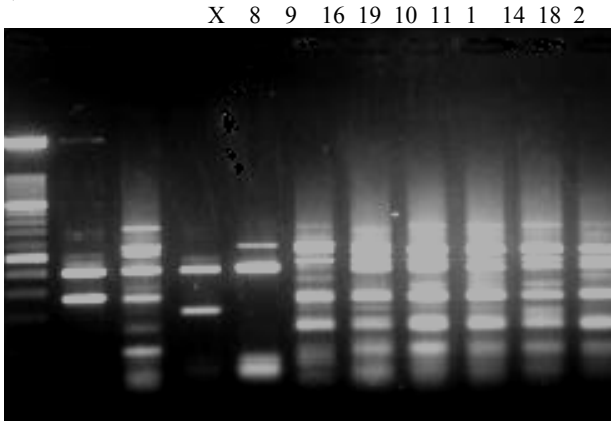


Fig. 6. Schizodemes of kDNA from samples. Schizodemes of kDNA amplified from samples collected from endemic areas and digested with *Hae* lll. The lane numbers refer to the sample numbers in Table V. Lane X, 100 bp ladder molecular size marker (Boehringer Mannheim)

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).

Table IV.- Leishmaniasis in dogs on the basis of thin smears in three regions of Paksitan.

	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.

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 Afghanistan refugees 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).

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 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 revealed that all the positive samples belonged 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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