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Prevalence of *Leishmania* in Sand Fly in Pakistan

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Abstract.- The study was conducted from May 2007 to June 2008 to cover all the four seasons. In this study the highest prevalence was recorded in the south of Pakistan (789) followed by the west (580) and northern region (533). The sand flies were collected, reared in a laboratory and their entire life-cycle studied. The species of sand-flies found in Pakistan belong to *Genus Phlebotomus,* and the species identified were *P. sergenti; P. papatasi; P. orientalis; P. major; P. longipes; P. bergeroti; P. alexandri; P.argentipes and P. pedifer*. Under laboratory conditions the entire life-cycle was completed in 20 to 40 days. The flies collected from the endemic areas were dissected and the promastigotes were collected from salivary glands and fore-gut. The infective stages could not be revealed from mid and hind-guts or from the reproductive systems. The promastigotes belonged to the genus *Leishmania.* Rabbits were experimentally infected to examine the progression of lesions and the resultant pathology. Out of 80 rabbits 78 developed characteristic lesions of cutaneous leishmaniasis at the site of subcutaneous injection (88%).

Keywords: Sand flies, *Leishmania*, promastigotes.

INTRODUCTION

Vector transmitted protozoans belong to the most important human diseases world wide (Garith *et al.,* 2001). Arthropods may physically carry the infectious agent to its primary or secondary host (mechanical vectors) or may act as biological vectors. As a biological vector, the infectious agent completes its required life cycle or multiplication before transmission to natural or secondary hosts (Avila *et al*., 1990). The epidemiology of leishmaniasis is determined by the reservoir of the infection (animal or human being) and by specific vectors (one or more species of Phlebotomines). Anthroponotic forms (without an animal reservoir) are found in India and East Africa. In the Mediterranean, Central Asia and South American countries, canines (*e.g.* dogs, foxes and jackals) act as reservoirs. In Africa the reservoirs are mainly rodents (Baker, 1997). Leishmaniasis is mainly transmitted by blood sucking sand-flies or phlebotomines. Phlebotomines are in the genus *Phlebotomus* in the Old World and the genus *Lutzomyia* in the New World (Barriga, 1997). There are about 700 species of Phlebotomine sand-flies of which about 70 are considered to transmit diseases to people. Of these, 37 species have been identified in Pakistan (Jones *et al*., 1997). Cutaneous leishmaniasis is caused by the *L. tropica* complex which is transmitted by *P. sergenti, P. papatasi, P. caucasicus, P. longipes* and *P. pedifer* in the endemic areas. Visceral leishmaniasis is caused by the *L. donovani* complex and transmitted by *P. argentipes, P. orientalis, P. martini, P. perniciosus, P. major, P. caucasicus, P. chinensis,* and *P. sergenti* in the endemic areas (Heath, 1997). New World cutaneous leishmaniasis is caused by *L. maxicana* complex and *L. braziliensis* complex. Visceral leishmaniasis is caused by *L. donovani* complex (*L. chagasi*) and transmitted by eight species of genus *Lutzomyia* (Kalter,1989).

The sand-flies are obligatory vectors and insect hosts of *Leishmania* species (Cotran *et al*., 1999). Only females suck blood and feeding usually takes place after sunset and before sunrise, when the flies leave their refuges in earth holes, rock crevices, tree caves or other dark, calm and moist places, storage places, latrines and stables. The activity of sand-flies is especially high under conditions of calm weather and high humidity. During blood sucking the sand-flies ingest free amastigotes or parasitized phagocytes (Ashford *et al.,* 1995). Parasitic protozoans are transformed into promastigotes in the fore-gut, mid-gut or hind-gut of the flies. The protozoans are 10-15 mμ in size and they multiply rapidly by binary fission. After 7-9 days, the infectious metacyclic stages are found in

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the proboscis, ready to be injected into a new host during feeding. These forms are leptomonad forms or the flagellated forms. Shortly after a bite by an infected sand-fly, a primary skin lesion (Leishmanioma) may develop *i.e.* itching papule with an erythematous rim 1 to 2 cm in diameter which may narcotize and ulcerate (Bhutto *et al*., 2003).

Knowing the zoonotic importance of leishmaniasis, the present study was conducted to find the prevalence of *Leishmania* in sand fly in four regions of Pakistan. The study also included experimental trials for rearing sand flies in captivity to develop recommendation on control measures for sand flies.

MATERIALS AND METHODS

Collection of sand flies

Samples were collected from four regions *i.e.* North, South, East and West of Pakistan. For this experiment blood sucking flies were collected each month from May 2007 to June 2008 using specially designed net traps. The collected flies were brought live to the laboratory for identification by using taxonomic notes adopted from Jones *et al.* (1997). The identified specimens were kept in especially designed cages. The data obtained was analyzed and processed to determine the prevalence of vectors both regionally and seasonally (Symth, 1994).

Rearing of collected sand fly specimens

The identified sand fly specimens were segregated into species by monthly collection, kept in specially designed net cages and provided with semi-decaying fruits and vegetables. Flies were also fed periodically on rabbit blood. The developmental stages of their life cycle were studied under laboratory conditions and compared with those occurring naturally as described by Clements (1972).

Dissection of sand flies

The flies were anaesthetized in jars having cotton soaked in chloroform and dissected by using mounted needles under a dissecting microscope in a few drops of phosphate buffer saline (PBS) at pH. 7.2 (on a microscopic slide). The head was separated from the thorax and the salivary glands were identified trailing along the head. The salivary glands were detached and placed on a slide in a few drops of PBS and teased into small fragments using a needle. One drop of this mixture was transferred to another slide and examined under microscope for the leptomonad forms which are the infective stages of protozoa (Crampton *et al.*, 1997). The same procedure was adopted for the fore; mid; hind-guts and reproductive systems. The mouth parts of the flies were examined for morphology and microphotographs were taken.

Experimental infection in rabbits

The infectious stages of protozoa from the salivary glands and fore-guts of flies diluted in PBS were injected (0.2 ml) subcutaneously into the backs of two or three previously shaven rabbits. The development of lesions was studied and confirmed by making smears of biopsy material on 3rd, 5th, 7th, 9th, 11th, 13th, 15th and 19th day post inoculation. Eighty rabbits were used in all for this part of the study.

RESULTS

Prevalence of sand fly

A total of 20,683 sand flies were belonging to Genus *Phlebotomus* were collected (Table I). The species identified were *P. sergenti, P. papatasi, P. orientalis, P. major, P. longipes, P. bergeroti, P. alexandri, P. argentipes* and *P. pedifer*, 30% of these were *P. papatasi,* 17% *P. major,* 15% each *P. argentipes* and *P. orientalis,* 8% *P. alexandri,* 5% *P. sergenti*, 4% *P. longipes,* 3% each *P. bergeroti* and *P. pedifer.*

In northern Pakistan a total of 483 sand-flies were collected in the month of May 2007, which increased to 582 in August 2007). The highest numbers of (851) sand-flies were identified In November 2007 and the lowest numbers (314)were recorded in February 2008.

In southern Pakistan a total of 425 sand-flies were identified in the month of May 2007, which gradually increased in the subsequent months and the highest number (592) was identified in October 2007. The highest numbers (789) of sand-flies were

Table I.- Monthly collection of sand flies by geographic region.

Regions of 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
North	483	522	517	582	654	726	851	703	317	314	481	533	6683
South	425	452	415	464	480	592	741	770	654	520	673	789	6969
East*	0	0	0	0	0	0	0	0	0	0	0	0	0
West	526	558	564	573	590	663	702	643	584	480	568	580	7031
Total	1434	1532	1496	1619	1724	1981	2294	2116	1555	1314	1722	1896	20683

*No flies identified as sandfly from East of Pakistan .

identified in April 2008 and the lowest numbers (415) were recorded in July 2007 .

In western Pakistan a total of 526 sand-flies were identified in the month of May 2007, which gradually increased in the following months and the highest number (573) was identified in August 2007. The highest numbers (702) of sand-flies were identified in November 2007 and the lowest numbers (480) were recorded in February 2008.

In eastern Pakistan no flies were recorded.

Growth and development of sand flies

The optimal conditions for growth and development of sand-flies were found to be at 28°C and 40% humidity. It was difficult studying the natural life cycle of sand-flies because the larvae were tiny and didn't live in well defined places, like mosquito larvae. Under laboratory conditions the entire life cycle took 20-40 days.

The female laid 30-70 eggs by scattering them around a potential breeding site. They hatched within 1-2 weeks. The larvae were fed on dead organic matter provided in cages. Pupal development took 5-10 days. Adults emerged from the pupae in darkness, often just before dawn. Only the female, suck blood, the food being used for egg production. Both males and females fed on sugary secretions from plants. The adults were mainly active in the early morning, evening and at night although they could bite during the day if disturbed. Sand-flies were able to survive in dry environments by withdrawing to cool, humid resting sites during the day and then becoming active at night when ambient temperatures dropped and humidity increased.

Prevalence of Leishmania *in sand flies*

Seventy eight out of one hundred and twenty flies (65%), carried the infective leptomonad forms of *Leishmania promastigotes* in their salivary glands and fore-guts. None of the dissected flies carried the infective stages in their reproductive systems.

Infection in rabbits

A lesion appeared as a reddish papule on the 3rd day post inoculation and developed a covering of dry scales by the 5th day. The lesion became moist from the formed crust on the 7th day and if the crust was removed a shallow ulcer was observed. The ulcer gradually enlarged and by the 19th day, had sharp-cut, raised edges surrounded by an indurated area. Secondary bacterial infection of the sore was observed after the 19th day. Sores healed spontaneously in untreated animals after four to six months, leaving a de-pigmented scar. Out of 80 rabbits, 78 developed characteristic lesions of cutaneous leishmaniasis at the site of subcutaneous injection (88%). The rabbits which didn't develop any lesions also didn't show any hypersensitivity reaction, indicating that they didn't have any previous immunity against the disease. One possibility for this result could be that the inoculum was sterile *i.e.* free of promastigotes/dead promastigotes.

DISCUSSION

Leishmaniasis is caused by flagellate protozoa genus *Leishmania* of family Trypanosomatidae. Life cycle of the parasite is simple (Ciaramella *et al*., 1997). The amastigotes

exist within the definitive host and the promastigotes exist in the salivary glands of the vectors (Barral *et al.*, 1991). The vectors in this case are small flies of family Phlebotomidae, genus *Phlebotomus*. The flies ingest the amastigotes while feeding on the blood of an infected host and once inside the vector's gut the amastigotes become motile by developing flagellum and thus transform into promastigotes. These are the infective forms and they travel to the salivary glands from where they are transmitted to a new host during the next blood meal. Once inside the definitive host the promastigotes loose their flagella and enter the macrophages, where they multiply inside the parasitophorous vacuole as described by Yeager and Wilcock (1993). The results of this study confirmed these findings.

The genus may be divided into two sub-genera. Species that develop in the fore-gut of their vectors are in *Leishmania* (suprapylaria reproduction) genus. Species that develop in the mid-gut and hind-gut are in the *Vianna* genus (Bittencourt and Barrel. 1991).

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 as described by Bhattacharia and Gosh (1983). The above mentioned areas are in the 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 as confirmed by Ali and Afrin (1997).

In this study the highest prevalence was recorded in the southern region (789) followed by the western (580) and the northern areas (533). The species of sand-flies found in Pakistan belong to Genus *Phlebotomus* previously described by Jones *et al.* (1997). The species identified were *P. sergenti, P. papatasi, P. orientalis, P. major, P. longipes, P. bergeroti, P. alexandri, P. argentipes and P. pedifer.*

In Pakistan sand-flies were caught year round from breeding sites in the endemic areas. The flies migrated from sub mountainous terrain to valleys and vice versa to avoid harsh weather as indicated by Iftikhar *et al.* (2003). Varying numbers of flies were caught during the study period from the endemic areas. In the northern and western area, November 2007 was the month in which the highest numbers of sand-flies were identified (851 and 702, respectively). In the Southern area, April 2008 was the month in which the highest number of sand flies were identified (789). It was evident from the results that with the increase in the number of flies there was a corresponding increase in the number of cases in both dog and human populations.

It was difficult studying the natural life cycle of sand-flies because the larvae were tiny and didn't live in well defined places as mosquito larvae do. Similar observations were made by Gurtler *et al.* (1991) under laboratory conditions. He captured flies, identified them and reared them in especially designed net cages and studied their entire life cycle which was completed in 20-40 days. In natural habitats, the adults were mainly active in the early morning, evening and at night although they could bite during the day if disturbed. When inactive, adult sandflies have habitat-specific resting sites that are characteristic of particular species as described by Jaffernay and Nighat (2001). Resting sites were often similar or near to the larval breeding sites and were usually places that were cool, humid and dark. Sand- flies were able to survive in dry environments by withdrawing to cool, humid resting sites during the day and then becoming active at night when ambient temperatures dropped and humidity increased. Similar observations were made by Kuzeo (1993). Seasonal activity of adults was affected mainly by temperature and rainfall. Kolaczinski *et al.* (2004) also confirmed these characteristics. Seventy eight out of one hundred and twenty sand-flies (65%) dissected, carried the infective leptomonad forms naturally *(Leishmania promastigotes)* in their salivary glands and fore-guts only (suprapylaria reproduction). This confirmed that these flies belonged to sub-genus *Leishmania* previously described by Armijos *et al*. (1997). None of the dissected flies carried the infective stages in their mid-guts, hind-guts and reproductive systems. Experimental transmission demonstrated that growth of *Leishmania* in the tissues of skin lead to hypertrophy of the stratum corneum with

hypertrophy and proliferation of the papilla as described by Zlylstra *et al*. (1995). A lesion developed at the inoculation site and secondary bacterial infection of the sore was observed after the 19th day post inoculation. Out of 80 rabbits 78 developed characteristic lesions of cutaneous leishmaniasis at the site of subcutaneous injection (88%).

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