Cutaneous Leishmaniasis : Iso-enzyme characterisation of Leishmania Tropica

Pages with reference to book, From 270 To 273

Mohammad Abdur Rab (Health Services Academy, Ministry of Health, Islamabad.)
Laila Al Rustamani, David A. Evans (Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London, Nishtar Medical College, Multan.)
Riaz Ahmed Bhutta, Mobammad Tariq Mahmood (Health Services Academy, National Institute of Health, Islamabad.)

Abstract

In order to identify and characterise the organisms responsible for Cutaneous Leishmaniasis, parasites were isolated from active lesions, grown in-vitro cultures and identified by iso-enzyme characterisation. Thirteen isolates from different patients were typed as L. tropica. Seven of these isolates were from Afghan refugees encamped in the suburbs of Islamabad, 3 were from patients in Multan, 1 was from a patient from Azad Jammu and Kashmir and 1 was from Besham (Swat, NWFP). The study confirms the presence of anthroponotic Cutaneous Leishamaniasis caused by L Tropica in Pakistan (JPMA 47:270,1997).

Introduction

It is generally believed that various species of Leishmania, both of humans and animals are morphologically indistinguishable and that both the amastigote and promastigote forms are remarkably similar. Nevertheless, subtle morphological deviations in size and ultra structure of the organisms have been demonstrated by researchers between species[1]. The importance of identifying the Leishmania species is fundamental not only from an epidemiological point of view, but also has obvious pertinence to clinical illness, therapy and management[2]. The Leishmania species can be differentiated from each other by enzyme profiles excretory factors[3,4], kinetoplast DNA buoyant density[5,6] and by the use of kDNA pmbes[7,8] and eDNA probes[9]. As a result of the recent advances in parasite identification techniques, the epidemiology of cutaneous leishmaniasis has been redefined bothinthe New as well as the Old World. Classically, three types of Old World leishmaniasis have been defined, namely visceral cutaneous and muco-cutaneoust[10-12]. The adult human visceral leishmaniasis (VL), caused by L. donovani is found in eastern Indian sub-continent and eastern China and in parts of subSaharan Africa by its variantL. donovani s.l. The infantile VL is spread overa wide geographical belt from Western China to the Atlantic coast of Southern Europe and North Africa. The parasite causing this disease is L. infantum. Cutaneous leishmaniasis (CL) caused by L. major and L. Tropica parasites is mainly found in the western Indian sub-continent, central Asia and the Middle East. The former is a zoonosis and is usually found in rural or semi-urban areas, while the latter causes anthroponotic CL and is found in urban towns and cities. The diffuse cutaneous leishmaniasis caused by L. aethipica is restricted to eastern sub-Saharan Africa[11-13]. CL is now also known to be caused by Leishmania parasites other than L. major and L.tropica. Cutaneous manifestations of L. donovani are long known to occur in the form of post-kala-azar dennal leishmaniasis[14,15] and these serve as human reservoir of the disease[16]. L. infantum and its variants manifest purely as a cutaneous disease[8,17-20] and occasionally may also cause muco- cutaneous leishmaniasis[21,22]. Similar muco-cutaneous lesions have been caused by both L. donovani s.l. and L. major[23] and visceral disease caused by L. tropica infections[24,25]. In Pakistan, both VL (infantile kala-azar) and CL are endemic. Infantile kala-azar has been reported from the Northern

Areas[26,27] (N.A.). Azad Janunu and Kashmi? (AJK) and Baluchistan[29,30] ~ L. infantum has been identified as the causative organism of this disease, in patients from AJK and N.A.[31,32]. The same parasite has also been identified in isolates obtained from domestic dogs from AJK and N.A.[33]. Two types of CL, zoonotic CL and anthroponotic CL are endemic in different parts of the country[34] and the classification of CL in Pakistan has been based mainly upon the clinical features, epidemiology and sandfly fauna[34-36]. The identity of parasites causing CL in Pakistan has notyet been ascertained and this is the first report describing the isolation and characterisation of Leish.mania parasites from patients with cutaneous disease.

Materials and Methods

Isolation of Leishmania parasites from CL patients

Patients with suspected cutaneous leishmaniasis were referred to the National Institute of Health (NIH), from different hospitals in Islamabad for parasitological diagnosis, isolation and culture of the parasites. Samples were obtained from the indurated ulcer margins using a small serrated dental probe and inoculated in the bi-phasic NNN medium was also sent to Multan where aspiration/biopsy material from the lesions was inoculated into the medium and dispatched by couricr service to NIH in Islamabad, where the inoculates were maintained initially at NIH, Islamabad, until their shipment to the London School of Hygiene and Tropical Medicine, where they were frozen and stores in liquid nitrogen until further use.

Isoenzy me characterization

Princciples of enzy me electrophoresis: Soluble enzymes are extracted fron the parasite and are placed in a gel matrix containing a buffer, the pH of which is pre-selected to render the isoenzymes a negative charge. When a direct current is passed through the gel, the iso-enzymes migrate through the matrix according-to their overall or net charge. As the net charges may be quantitatively different, isoenzymes with the greatest negative charge move the farthest towards the anode, while those with the least negative charge travel the shortest. The bands of isoenzymes are then visualized by appmpriate staining techniques.

Preparation of samples

The isolates stored in liquid nitregen were taken out, quickly, thawed quickly and inoculated in bi-phasic NNN medium37. In most cases the cultures grew well, however, in cases where the parasite revival was slow, inoculation in Sloppy Evans medium[37] was carried out. As soon as the organisms began to thrive and multiply rapidly in the initial agar medium, they were transferred to liquid medium (MEM:EBLB:FCS[37]) for mass culture. In cases where growth was slow, the MEM:FEBLB:FCS medium was supplemented by the addition of 1% human urine obtained from a healthy individual and filtered through a 0.2 um Milipore filter (Sartonus AG) This greatly enhanced the growth of the promastigotes[38]. Log phase promastigotes (1x10 7) were harvested and washed 2x in PBSS (Proline Balance Salt Solution). The pallet was mixed with an equal amount of enzyme stabiiser (a mixture containing 2 mM each of Ethylene diamine tetra acetic acid, E- aminocaproic acid and Dithiothreitol). The final mixture was snap frozen and thawed 3x in liquid nitrogen and centrifuged at 10,000 0 for half an hour. The pallet was discarded and the supematant was stored as 15 uL beads (lysate) in liquid nitrogen until use.

Starch Gel Electrophoresis

Thin layer starch gel electrophoresis was carried out according to the previously described technique39. The gels were prepared using hydrolysed potato starch (Connaught Laboratories Limited), 7.5 gm/l00 ml, in appropriate in gel (tank) buffer solutions. Linear slots were made in a straight line at regular intervals across the gel and samples applied using cotton thread (Anchor stranded embroideiy cotton)

cut in pieces of 8nun length and soaked in the lysate. The preparation of tank buffers, running time and the voltage requirements for electrophoresis was carried out as already defmed[39].

List of enzymes studied.

i)Alanine amino transferase (EC 2.6. 1.2), ii) Aspartate amino transferase (EC 2.6.1.1), iii) Superoxidase dismutase (EC 1.15.1.1), iv)Esterase (EC 3.1.1.1), v) Purine nucleoside hydrolase (EC 3.2.3.1), vi) Mannose phosphate isomerase (EC 5.3.1.8), vii) Glucose phosphate isomerase (EC 5.3.1.9), viii) Malate dehydrogenase (EC 1.1.1.37), ix) 6-Phosphogluconate dehydrogenase (EC 1.1.1.44), x)
Phosphoglucomutase (EC 2.7.5.1), xi) Proline iminopeptidase (EC 3.4.11.5) and xii) Pyruvate Kinase (EC 2.7.1.40).

Results

Inoculation of material from cutaneous lesions was carried out in 27 patients with CL. The age range was between 5 to 60 years. Clinically, all the lesions appeared to be of diy or urban (anthroponotic) type and the majority of these were either on the face or the hands and forearms. Parasites were grown in inoculates obtained from 16 patients. Seven isolates were from Afghan refugees encamped in the suburbs of Islamabad, 3 from Multan, 1 from a village north east of Rawalpindi, 1 from a patient from Bagh, AJK and 1 was from a patient who came from Besham in Swat (Kohistan district of NWFP). This patient had actually acquired his infection while working in Kuwait. Isoenzyme characterisation of 13 isolates was performed. Standard WHO isolates of L. tropica and L. major were used as controls. The isoenzyme pattern of the isoenzymes of L. donovani, L. infantum, L. tropica and L. major parasites on thin layer starch gel electrophoresis are graphically shown (Figure).

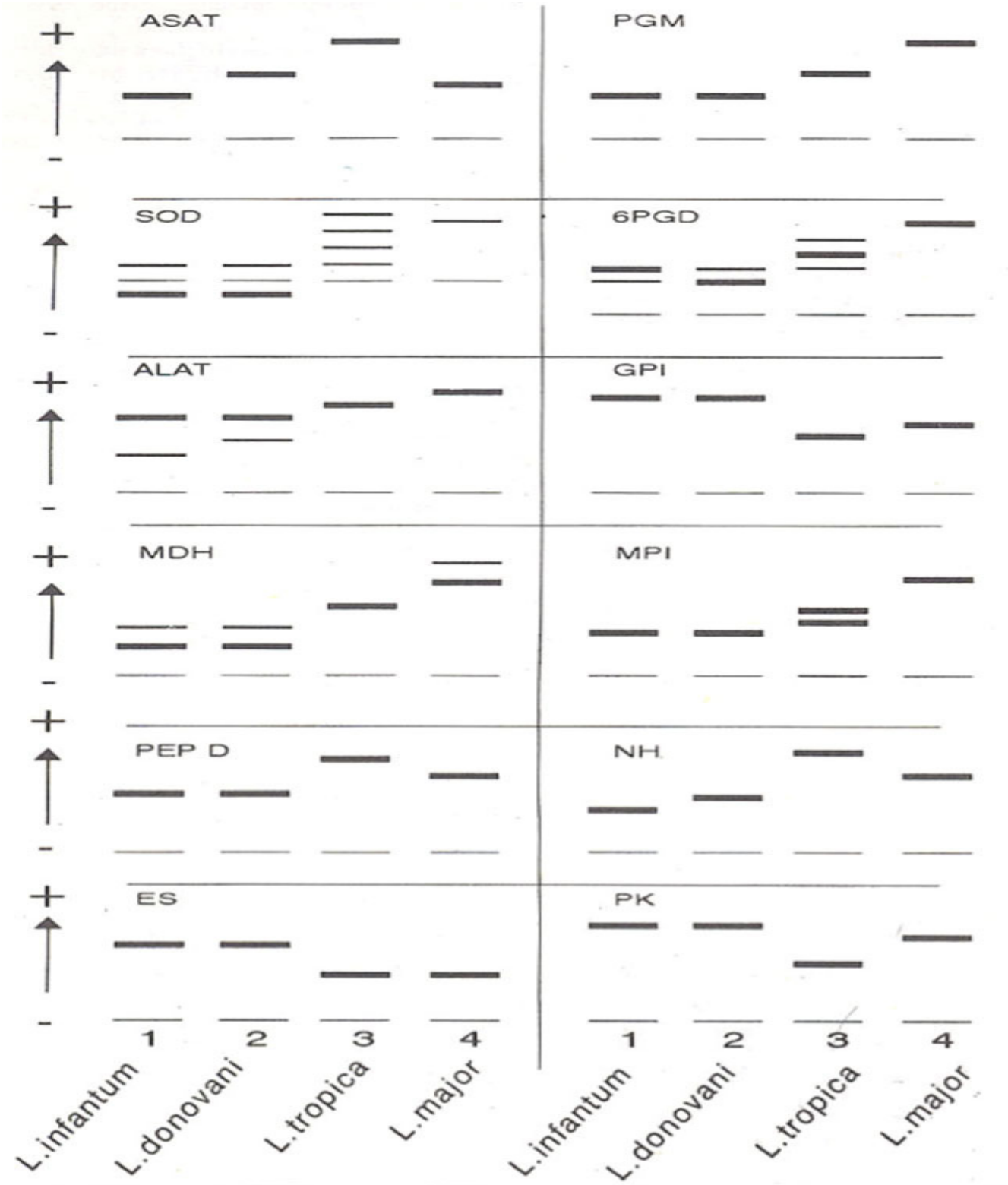

Figure. Isoenzyme patterns of leishmania parasites seen on thin layer starch gel electrophoresis.

All thirteen isolates were characterised as L tropica (Table).

Table. Isoenzyme characterization of Leishmania isolates obtained from patients with cutaneous leishmaniasis.

No.	Strain	Age	Sex	Place	Result
1.	MHOM/PK/90/RAB3	18	M	Bagh (AJK)	L.tropica
2.	MHOM/PK/90/RAB3	21	M	Besham (NWFP)*	L.tropica
3.	MHOM/PK/91/ABDULLA	5	M	Islamabad**	L.tropica
4.	MHOM/PK/91/BOMBER	12	M	Islamabad**	L.tropica
5.	MHOM/PK/91/HAWA	35	F	Islamabad**	L.tropica
6.	MHOM/PK/91/PARIGUL	35	F	Islamabad**	L.tropica
7.	MHOM/PK/91/CHERAMAT	4	M	Islamabad**	L.tropica
8.	MHOM/PK/91/NAMRO	12	F	Islamabad**	L.tropica
9.	MHOM/PK/91/SHAZIAMN	18	F	Multan	L.tropica
10.	MHOM/PK/91/SAJJADMN	21	M	Multan	L.tropica
11.	MHOM/PK/91/TASEER	12	M	Islamabad**	L.tropica
12.	MHOM/PK/91/SHIRIN	17	F	Rawalpindi	L.tropica
13.	MHOM/PK/91/ZAHIDMN	9	M	Multan	L.tropica

* This patient came from Besham (Swat, NWFP), but had acquired his infection in Kuwait.
** Afghan refugees residing in the suburbs of Islamabad.

Discussion

The Leishmania parasites causing visceral disease in Pakistan both in humans and in the dogs have been identified as L. infantum[31-33]. This report identifies L. tropica isolates obtained from patients with cutaneous lesion by isoenzyme characterisation. Isolates obtained from patients of Afghan origin living in the vicinity of Islanmbad were typed as L. tmpica. Anthroponotic cutaneous leishmaniasis, the causative organism of which is L. tropica, is endemic in Afghanistan[12,14], and the isolation of the parasite among the Afghan refugees suggests that the disease has been imported in this region from Afghanistan. The duration of the lesions in all these patients was under 8 months, therefore, the transmission of disease in these cases appeared to be indigenous, as the affected individuals had been residing in Pakistan for the last over eight years. Cases have also been observed among the locals and at least one isolate obtained from a young native girl has been typed as L. tropica. CL has also been described from the city of Multan in Southern Punjab, where the disease is endemic and occasionally may reachepidemic proportions[34]. Isolates obtained from this area have also been typed as L. tropica, thus confirming the endemic parasite in this area. Similarly, this parasite has also been identified from atleast one case of CL from AJK. One isolate from a patient from Besham (Swat, NWFP) was also identified as L. tropica. This patient had acquired his infection while working in Kuwait, from where he had fled following the Iraq conflict. This parasite is endemic in most of the Middle East region including Saudi Arabia and Iraq[40,41] and therefore, the finding was not surprising, although L. major is also endemic in Kuwait[42]. It is not unusual forthe disease tobe imported into areas where it is not known to exist[43]. This has been made possible due to high frequency of international travel and lucrative facilities that are now available in some endemic countries, particularly the Middle East. Phiebotomus sargnti, the vector of L. tropica is found in most parts of Paldstan[44,45], including the suburbs of Islamabad (personal communication, Dr. Mohamniad Arif Munir,NIH, Islamabad). The potential therefore, of spread of the disease in areas of the countiy hitherto non- endemic is high. The case from Besham (Swat, NWFP) was seen in 1991 and subsequently in. 1994, during the investigation

of a cholera outhreakinthe same area, one of the authors (MAR) saw acase of indigenously transmitted CL in an adult male.

In the present study we did not identify L. major among our isolates, although it has been isolated and identified in strains obtained from Baluchistan alongwith L. tropica (personal communications from Dr. David A. Evans and M.A. Shahid DESTO Laboratories, Karachi, Pakistan). This parasite has been isolated from both humans and rodents in Western India, Central Asia, Middle East and Northern Africa[11,12,14,46] and infected rodents have been found in Baluchistan[47]. In conclusion, anthroponotic cutaneous leishmaniasis caused by L. tropica has been continued from both northern and southern Punjab, as well as AJK. The disease in northern Punjab appears to be imported from Afghanistan, however, it is endemic in the southern Punjab and AJK. Zoonotic CL is also highly endemic in many parts of the country, further studies are therefore needed to characterise and document L. major in Pakistan. Since L. infantum also causes cutaneous manifestations and is endemic in Pakistan, its role in the epidemiology of cutaneous leishmaniasis if any, needs.to be ascertained. Furthermore, the role of dogs and rodents in cutaneous leishmaniasis in the countzy also needs investigation.

Acknowledgements

The authors wish to acknowledge the staff of the Dermatology Department of the Pakistan Institute of Medical Sciences who referred some of the patients. The supportby the staff of the Immunology Department is also gratefully acknowledged. This work received financial support from the UNDPIWor1d Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR).

References

1. Chance, M.I. Problems in identification of parasites and theirvector. Symposia of the British Society for Parasitology Vol. 17. In: Eds. Taylor, A.E.R. and Muller, R. The identification of leishmania. Oxford, Blackwell Scientific Publication, 1979, pp. 55-74.

2. Peters, W., Eans, D.A. and Lanham, S. Importance of parasite identification in cases of leishmaniasis. J. R. Soc.Med., 1983;76:540-542.

3. Schnur, L.F, Chance, M.L., Ebat, F. et al. The biochemical and serological taxonomy ofvisceralising leishmania. Ann. Imp. Med. Hyg., 1981:75:131-141.

4. Mansour, N.S., Awadalla, H.N., Youssef, F.G, et al. Characterisation of Leishmania isolates from children with visceral infections contracted in Alexandria, Egypt. Trans. R. Soc. Trop. Med. Hyg., 1984;78:704.

5. Peters, W., Chance, M.L., Mutinga, M.J. et al. The identification ofhuman and animal isolates of Leishmania from Kenya. Ann. Trop. Med. Parasitol., 1977;71:501-502.

6. Chance, M.L., Schnur, L.F., Thomas, S.C. etal. Thebiochemical and serological taxonomy of Leishmania from the Aethiopian zoological region. Ann. Trop. Med. Parasitol., 1978;72:533-542.

7. Barker, D.C. and Butcher, J. The use of DNA probes in the identification of leishmaniasis: Distinction between isolates of L. Mexicans and L. Braziliences complexes. Trans. R. Soc. Trop.Med. Hyg., 1983;77:285-297,

8. Ben-lsmail, R., Smith, D.F, Ready, P.D. eta!. Sporadic cutaneous leishmaniasis in north Tunisia: Identification ofthe causative agent as Leishmania infantumby the use of diagnostic deoxyribonucleic acid probe. Trans. R. Soc. Trop. Med. flyg., 1992;86:508-510.

9. Howard, MK., Kelly, J.M., Lane, R.P. eta!. A aensitive repetitive DNA probe that is specific to the Leishmania donovani complex and its axe as an epidemiological agent. Mo!. Biochem. Parasitol., 1991 ;44:63-72.

10. Lysenko, A.J. Distribution of leishmanissis in the Old World. Bull. WHO., 1971 ;44:5 12-520.

11. Desjeux, P. Human leishmaniasis: Epidemiology and public health aspects. World Health Stat. Q., 1992;45:267-275.
12. WHO Technical Report Series No. 973. Control of the leishmaniasis: Report of a WHO expert committee, Geneva, WHO, 1990.
13. Le Blancq, S.M., Belehu, A. and Peters, W. Old World 2: The distribution of L. aethiopica zymodemes. Trans. R. Soc. Trop.Med.Hyg., 1986;80:360-366.
14. Ashford, R.W. and Bcttini, S. Ecology and Epidemiology: Old World. In: The leishmaniasis in Biology and Medicine, edited by Peters, W. and Killick-Kenddck, R. London, Academic Press, 1987.
15. Beaver, P.C., Jung, R.C. and Cupp, E.W, Clinical Parasitology. 9th Edition.London, Lea and Feabiger, 1984, pp. 70-77.
16. Addy, M. and Nandy, A. Ten years of kala-azsr in West Bengal. Part I. Did post kala-azar dermsl leishmaniasis initiate the outbreak in 24-paragnas. Bull. WHO., 1990;70:341-346.
17. Kumar, P.V., Sadeghi, E. and Torabi, S. Kala-azar with disseminated dermal leishmaniasis. Am. J. Trop. Med. Hyg., 1989;40: 150-153.
18. Rioux, J.A., Lanotte, G., Petter, F. et al. Cutaneous leishmaniasis in Westem Mediterranean basin. An eco- epidemiological analysis of three 'foci' in Tunisia, Morrocco and France. Edited by Rioux,J,A. Montpellier, Institute Mediterranean Epidemioloques et d'Etudes (IMEE), 1986, pp. 365-369.
19. Rioux, J.A., Moreno, G., Lanotte, G. et al. Two episodes of cutaneous leishmaniasis in man caused by different zymodemea of Leishmania infantum s.I. Trans. R. Sdc. Trop. Med. Hyg., 1986;80: 1004-1006.
20. Gramaccia, M., Gradoni, L. and Pozio, E. Leishmania infantum a.I as an agent of cutaneous leishmaniasis in Abruzzi region (Italy). Trans. R. Soc. Trop. Med.Hyg., 1987;81:235-237.
21. Borzoni, N.K., Gradoni, F.B.L., Gramacia, M et al. A case of lingua! and palatine localisation of a viscerotropic Leisbmania infantum zymodeme in Sardinia, Italy. Trop. Med. Parasitol., 1991 ;42: 193-194.
22. Alvar, J,, Bsllesteros, J.A., Soler, R. eta!. Muco-cutaneous leishmaniasis due to Leishmania infantum: biochemical characterization. Am. J. Trop. Med. Hyg., 1993;43:614-618.
23. Ghalib, H.W, Eltoum, E.A., Kroon, CC. et al. Identification of Leishmania from mucosa! Icishmaniasis by recombinant DNA probes. Trans. R. Soc. Trop. Med. Hyg., 1992;86:158-160.
24. Mebrahtu, Y., Lawyer, PG., Githur, J. I. et a!. Visceral leishmaniasis unresponsive to Pentostam caused by Leishmania tropica in Kenya. Am. J. Trop. Med. Hyg., 1989;41:289-294.
25. Magil, A.J., Grog!, M., Gasser, R.A. et a!. Visceral infection caused by Leishmania tropica in veterans of operation desert storm. N. EngI. J. Med., 1993;328:1383-1387.
26. Ahmed, N. and Burney, MI. Leishmanissis in Northem Areas of Pakistan. Armed F.Med.J.,1962;12:1-11.
27. Bumey, M.I.,Lari, F.A. and Khan, MA. Status of visceral leishmanissis in Northern Pakistan. Trop.Doct., 1981; 11:146-148.
28. Saleem, M., Anwar, C.M. and Malik, l.A. Visceral leishmaniasis in children: A new focus in Azad Kashmir J. Pak. Med. Assoc., 1986;36:230-233.
29. Perver, Y., Bugti, A.N., Azeem, R. et al. Ka!a-Azar (Visceral Leishmaniasis) in Baluchistan. Pak. Pacd. J., 1992;16:225-227.
30. Nagi, AG. and Nasimullah, M. Visceral leishmanissis in Baluchistan. Pak. Paed. J., 1993;17:7-10,
31. Rab, M.A., Iqbal, J., Azmi, F.H. et a!. Visceral leishmaniasis: A seroepidemiological study of 289 children from Endemic foci in Azad Jammu and Kashmir by Indirect Fluorescent Antibody Technique. J. Pak. Med. Assoc., 1989;39:225-228.
32. Rab, MA. and Evans, D.A. Leishmania infantum in Himalayas.Trans. R. Soc. Trop. Med. Hyg., 1995;89:27-32.
33. Rab, MA., Frame, LA. and Evans, D.A. The role of dogs in the epidemiology of humanviscera! leishmaniasis. Trans. R Soc. Trop. Med. Hyg., 1995;89:612-615.

34. Bumey, MI. and Lan, F.A. Status of cutaneous leishmaniasis in Pakistan. Pak. J.Med. Rca., 1986;25:101-108.
35. Jan, SN. Cutaneous leishmaniasis in Baluchistan. Pak. J. Med. Rca., 1984;23:64-69.
36. Rajper, G.M., Khan, MA. and Hafiz, A. Laboratory investigation of cutaneous leishmaniasis in Karachi. J. Pak. Med. Assoc., 1983;33 :248-250.
37. Evans, D.A., Godfrey, D., Lanham, S. et a!. Leishmania: In: Handbook on isolation, chsracterisation and cryopreservation ofLeishmania. Ed. Evans, D.A. Switzerland, UNDP/World Banki'TDR, 1989.
38. Howard, M.K., Pharoah, M.M., Ashall, F. et al. Human urine stimulates growth of leishmania in-vitro. Trans. R. Soc. Trop. Med. Hyg., 1991 ;85 :477-479.
39. Godfrey, D.G. and Kilgour, V. Enzyme clectrophoresis in characterising the causative organism of Gambian Trypanosomiasis. Trans. R. Soc. Trop. Med. Hyg., 1976;70:219-224.
40. Le Blancq, S. and Peters, W. Leishmania in the Old World: 2. Isoenzyme charactenisation of Leishmania tropics. Trans. R. Soc. Trop. Med. Hyg., 1986;80:113-119.
41. A!jebori, TI. and Evans, GA. Leishmania spp: in Iraq. Electrophoretic isoenzymepattems IL Cutaneous leishmaniasis. Trans. R. Soc. Trop. Med.Hyg., 1980;74: 178-184.
42. Al-Taqi, M. and Evans, D.A. Characterization of Leishmania app. from Kuwait by isoenzyme electrophoresia. Trana. R. Soc. Trop. Med. Hyg., 1978;72:56-65.
43. Nautunne, T.D., Rajakulanderan, S., Abeywickreme, W. et a!. Cutaneous !eishmathasis in Sri Lanka. An imported disease linked to the Middle East and Africa employment boom. Trop. Geogr. Med., 1990;42:72-74.
44. Lewis, D.J. The phlebotomine sandflies of West Pakistan (Diptera: Psychodidae). Bull. Br. Mus. (Nat. Hist.) Entomol., 1967;19:1-75.
45. Nasir, AS. Sandflies as vector of human disease in Pakistan. Pak. J. Health, 1964;14:26.
46. Peters, W., Chance, ML., Chowdhry, A.B. eta!. The identity of some stocks of Leishmania isolated in India. J. Trop.Med. Parasitol., 1981;75:247-249.
47. Rab, MA., Armi, FA., Iqbal,J. et aL Cutaneous leishmaniasis in Baluchiatan: Reservoir host and sandfly vector in Uthal, Lasbella. J. Pak. Med. Aasoc., 1986;36:134-138.