Cutaneous Leishmaniasis: Iso-enzyme characterisation of Leishmania Tropica

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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 Leishmaniasis 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. 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.

The Leishmania species can be differentiated from each other by enzyme profiles, excretory factors, kinetoplast DNA buoyant density and by the use of kDNA pmbes and eDNA probes. As a result of the recent advances in parasite identification techniques, the epidemiology of cutaneous leishmaniasis has been redefined both in the New as well as the Old World. Classically, three types of Old World leishmaniasis have been defined, namely visceral cutaneous and muco-cutaneous. The adult human visceral leishmaniasis (VL), caused by L. donovani is found in eastern Indian sub-continent and eastern China and in parts of sub-Saharan Africa. The infantile VL is spread over a 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.

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 dennai leishmaniasis and these serve as human reservoir of the disease. L. infantum and its variants manifest purely as a cutaneous disease and occasionally may also cause muco-cutaneous leishmaniasis. Similar muco-cutaneous lesions have been caused by both L. donovani s.l. and L. major and visceral disease caused by L. tropica infections. In Pakistan, both VL (infantile kala-azar) and CL are endemic. Infantile kala-azar has been reported from the Northern
Areas\textsuperscript{26,27} (N.A.), Azad Janunu and Kashmi? (AJK) and Baluchistan\textsuperscript{29,30} – L. infantum has been identified as the causative organism of this disease, in patients from AJK and N.A.\textsuperscript{31,32} The same parasite has also been identified in isolates obtained from domestic dogs from AJK and N.A.\textsuperscript{33} Two types of CL, zoonotic CL and anthroponotic CL are endemic in different parts of the country\textsuperscript{34} and the classification of CL in Pakistan has been based mainly upon the clinical features, epidemiology and sandfly fauna\textsuperscript{34-36}. The identity of parasites causing CL in Pakistan has not yet been ascertained and this is the first report describing the isolation and characterisation of Leishmania 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 courier 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.

**Isoenyme characterization**

Principles of enzyme electrophoresis: Soluble enzymes are extracted from 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 appropriate staining techniques.

**Preparation of samples**

The isolates stored in liquid nitrogen were taken out, quickly, thawed quickly and inoculated in bi-phasic NNN medium\textsuperscript{37}. In most cases the cultures grew well, however, in cases where the parasite revival was slow, inoculation in Sloppy Evans medium\textsuperscript{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\textsuperscript{37}) for mass culture. In cases where growth was slow, the MEM:EBLB: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\textsuperscript{38}. Log phase promastigotes (1x10\textsuperscript{7}) were harvested and washed 2x in PBSS (Proline Balance Salt Solution). The pallet was mixed with an equal amount of enzyme stabiliser (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 g 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 technique\textsuperscript{39}. 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 embroidey 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 defined^3^.

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

The Leishmania parasites causing visceral disease in Pakistan both in humans and in the dogs have been identified as L. infantum. 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 Islamabad were typed as L. tropica. Anthroponotic cutaneous leishmaniasis, the causative organism of which is L. tropica, is endemic in Afghanistan 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 reach epidemic proportions. 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 at least 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 and therefore, the finding was not surprising, although L. major is also endemic in Kuwait. It is not unusual for the disease to be imported into areas where it is not known to exist. 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. Phlebotomus sargenti, the vector of L. tropica is found in most parts of Pakistan, including the suburbs of Islamabad (personal communication, Dr. Mohammadi, NIH, Islamabad). The potential therefore, of spread of the disease in areas of the country hitherto non-endemic is high. The case from Besham (Swat, NWFP) was seen in 1991 and subsequently in 1994, during the investigation.

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

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

* This patient came from Besham (Swat, NWFP), but had acquired his infection in Kuwait.
** Afghan refugees residing in the suburbs of Islamabad.
of a cholera outbreak in the same area, one of the authors (MAR) saw a case 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 along with 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\textsuperscript{11,12,14,46} and infected rodents have been found in Baluchistan\textsuperscript{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 country also needs investigation.

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References