Cutaneous leishmaniasis caused by *Leishmania donovani* in the tribal population of the Agasthyamala Biosphere Reserve forest, Western Ghats, Kerala, India

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Received 4 April 2014
Accepted 3 December 2014

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Cutaneous leishmaniasis (CL), a neglected tropical disease, is reported to be prevalent in tribal villages located in the Agasthyamala Biosphere Reserve forests of Western Ghats, Kerala state, India. We carried out an investigation to characterize the species of *Leishmania* parasites involved in these infections prevalent among one of the oldest human tribal populations in India. Skin aspirates collected from 13 clinically diagnosed cases were subjected to histopathological investigations, serological rapid tests using ‘rk39’ and molecular diagnostics. Clinical manifestations recorded among the patients were hypo-pigmented erythematous nodules/papules on limbs and other parts of the body. Histopathological investigations of these skin lesions among patients showed Leishman–Donovan bodies in macrophages. None of the patients were found to be positive for rk39 tests, which detect active visceral leishmaniasis. Using three different genetic markers [kinetoplast minicircle DNA, 3’ UTR region of heat-shock protein 70 (Hsp70) and Hsp70 gene] we identified the parasite species involved in these infections to be *Leishmania donovani*. The 6-phosphoglucanate (6-PGDH) gene sequences of the parasite isolates from Western Ghats indicated close genetic relatedness to *L. donovani* isolates reported from Sri Lanka, also causing CL. This could be cited as another instance of ‘local endemism’ of organisms in this single ‘bio-geographic unit’.

INTRODUCTION

Leishmaniasis is prevalent in 98 countries and about 310 million population live under the risk of infection of this neglected tropical disease globally. About 12 million people are infected, with an annual incidence of about 1.3 million cases. Cutaneous leishmaniasis (CL) is the most common clinical manifestation, affecting about 1 million people every year (WHO, 2013). However, visceral leishmaniasis (VL) remains the serious form of infection. Also, this is the most important variety of leishmaniasis infection in India. Bihar, West Bengal, Jharkhand and Uttar Pradesh are the endemic states of VL in India (Alvar et al., 2012). In addition, sporadic cases have been recorded from the foothills of the Himalayas (Desjeux, 1991), Gujarat and Kerala (Kesavan et al., 2003; Sharma et al., 2007). A total of 33 187 and 20 600 cases of VL were recorded in India during 2011 and 2012, respectively [National Vector Borne Disease Control Programme (NVBDCP), Government of India, http://www.nvbdcp.gov.in/ka-cd.html]. *Leishmania donovani* is the major parasite involved in VL, and *Phlebotomus argentipes* is reported as the major vector species involved in transmission (Kumar et al., 2001).

CL is reported mainly from the north-western region of India bordering Pakistan (Rajasthan and Punjab) (Sharma et al., 1973; Aara et al., 2013), and in some other small pockets in Himachal Pradesh (Sharma et al., 2005). The parasites involved in the infection are *L. tropica* and *L. donovani* (Sharma et al., 2005; Kumar et al., 2007). Mucocutaneous forms of leishmaniasis cases caused by *L. donovani* have been reported from Himachal Pradesh (Sharma et al., 2005). CL caused by *L. donovani* has been recorded as a major problem in the adjacent country, Sri Lanka (Siriwardana et al., 2007; Ranasinghe et al., 2012; Gajapathy et al., 2013).

Abbreviations: CL, cutaneous leishmaniasis; k-DNA, kinetoplast DNA; LD, Leishman–Donovan; VL, visceral leishmaniasis.

The GenBank/EMBL/DDBJ accession numbers for the Hsp70 gene sequences of our *Leishmania donovani* isolates are KC884001, KF562068, KF562069, KF562070 and KF562071, and for the 6-PGDH sequences are KJ461872 and KJ461873.

076695 © 2015 The Authors

Printed in Great Britain

DOI 10.1099/jmm.0.076695-0

Journal of Medical Microbiology (2015), 64, 157–163

076695 © 2015 The Authors

Printed in Great Britain

DOI 10.1099/jmm.0.076695-0
A recent preliminary study reported the prevalence of CL in two tribal settlements in the Western Ghats of Thrivananthapuram District, Kerala state, India (Simi et al., 2010). They reported 12 active CL cases from the region based on clinical examinations. Also, histopathological examinations showed Leishman–Donovan (LD) bodies in four cases. Based on this report, we initiated a study to elucidate the epidemiological factors involved in the indigenous transmission of this neglected disease in the tribal belt located in the protected Agasthyamala Biosphere Reserve forest region of southern India. Here, we report the characterization of the Leishmania species involved in the human CL infections. Furthermore, studies are in progress on the transmission dynamics of the CL infections in the region. The region is thickly forested and does not have transport accessibility. About 1500 tribal people live in 28 hamlets deep in the forest in this region. Most of these people dwell in thatched huts constructed with mud, peeled stem of bamboo, palm trees or sticks. Electricity supplies to the region are provided by installing solar cells in each hutment. One or two dogs are domesticated by each tribal family, especially to protect them from attack by the wild animals in the region.

METHODS

Clinical examination. A total of 768 persons residing in 28 hamlets were examined for the clinical manifestations of leishmaniasis during field visits of medical officers to these villages. The persons having suspected active CL lesions were advised to visit as outpatients at the Government Medical College and Gokulam Medical College hospitals, Thrivananthapuram, Kerala. They were clinically examined and required treatment was administered. Ethical clearance for the study was obtained from the Institutional Ethical Committee of Vector Control Research Centre (Indian Council of Medical Research), Puducherry, India.

Histopathology. Histopathological examination of skin biopsy specimens was carried out in the Department of Pathology, Government Medical College, Thrivananthapuram, Kerala, India for 13 patients who visited the hospitals as outpatients. After identifying a relatively new skin lesion, 1% lignocaine was infiltrated into it and by using a surgical blade a linear incision of about 0.5 cm, reaching up to the level of dermis, was performed. Then the blade was turned 90° and the dermal pulp was scraped out using the blunt end of the knife taking care not to induce bleeding. This pulp was smeared onto a glass slide, stained with Giemsa, and observed under the microscope to visualize the granuloma and identify the amastigote stages of Leishmania parasites – LD bodies.

Molecular studies. Aspirates from skin nodules/lesions of all the samples were also subjected to PCR-based analysis for Leishmania infection at the Vector Control Research Centre Field Station, Kottayam, Kerala, India. Parasite DNA was extracted using a GenElute mini-prep kit (Sigma), following the kit protocol. Briefly, the skin aspirates from all 13 cases were lysed and treated with RNase A. The lysate was loaded and passed through mini-prep columns so that DNA bound to the column silica membranes. The DNA was eluted with de-ionized distilled water and was subjected to diagnostic mini-circle kinetoplast DNA (k-DNA) semi-nested PCR using DNA primers (Aransay et al., 2000). This PCR amplifies k-DNA in eight species of Leishmania parasites and groups them into two categories: (i) major group, comprising L. major and L. aethiopica, which yields a band of about 650 bp; and (ii) infantum group, comprising L. infantum, L. tropica, L. donovani, L. amazonensis, L. mexicana and L. braziliensis, which amplifies a fragment of about 720 bp. Requena et al. (2012) proposed a PCR-RFLP analysis of the UTR region of the heat-shock protein 70 (Hsp70) for species characterization of Leishmania species belonging to both Old World and New World species. Among Old World species, it clearly distinguishes between L. tropica and L. donovani complex by restriction digestion with HaeIII. Hence, we carried out this analysis on the positives. The 750 bp fragment amplified was digested with the HaeIII restriction enzyme and the restriction fragments were resolved in a 3.0% agarose gel to type the species involved. Also, a larger fragment of Hsp70 gene was amplified and sequenced to determine the exact species involved in the infections (Montalvo et al., 2010). The Hsp70 gene fragment amplified was about 1400 bp and could be used to determine the exact species either by RFLP or by DNA sequence analyses. A partial sequence of the 6-phosphogluconate (6-PGDH) gene, a highly variable gene, was also amplified using DNA primers already reported (Ranasinghe et al., 2012). Phylogenetic analyses were carried out with Hsp70 gene sequences as well as partial 6-PGDH gene sequences isolated in this study and those available in GenBank from different countries (Ranasinghe et al., 2012; Zhang et al., 2013), using maximum-likelihood analysis (bootstrapping with 1000 replications) to understand the genetic lineage of the parasite. Thus, four robust genetic markers were used in the study to confirm the species involved in the infections.

Serological studies. The rk39 serological rapid test had been found to be very useful for rapid diagnosis of active VL infections (Badaró et al., 1996). This rapid immunochromatographic test diagnoses VL cases with 97% specificity and 100% sensitivity (NVBDCP, Government of India, http://nvbdcp.gov.in/Doc/Guideline%20on%20use%20of%20rk39_rapid%20diagnostic%20kit.pdf). We carried out this test in all the 13 cases examined in the study, following the national guidelines of the Government of India.

RESULTS

Clinical manifestations

A total of 27 cases were recorded with suspected symptoms of CL. None reported clinical manifestations of VL. Among these, 12 had healed dermal scars of CL lesions. Among 15 cases with active lesions, 13 visited hospitals for follow-up and treatment. The major clinical manifestations recorded among these 13 patients (Table 1) were multiple hypo-pigmented to erythematous papules, with a few showing studded erythematous nodules or ones with erosions, forming ulcers in the upper/lower limbs, head, ear pinna, shoulder, trunk, etc. (Fig. 1).

Histopathology

Skin smears examined under the microscope showed epidermis with different degrees of keratosis to atrophy. Dermis showed granulomas with diffuse infiltration by lymphocytes, and few epithelioid cells and macrophages. Isolates from four patients had LD bodies in the macrophages (Fig. 2).

Molecular studies

Diagnostic PCR analysis (k-DNA) showed five patients to be positive for Leishmania infection. Among these, three
Table 1. PCR and histopathology results of skin nodule aspirates from suspected *Leishmania* cases in tribal villages of Thiruvananthapuram

<table>
<thead>
<tr>
<th>No.</th>
<th>Date of collection</th>
<th>ID no. (OP-MCH)*</th>
<th>Age/sex</th>
<th>Village</th>
<th>Clinical symptoms and provisional diagnosis</th>
<th>Histopathology</th>
<th>PCR (k-DNA) result [Leishmania]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19/10/2012</td>
<td>273400/12</td>
<td>10 F</td>
<td>Thazheamala</td>
<td>Papules on exposed parts of body (limbs and upper chest) for 6 months. Most of the lesions were erythematosus and 5–10 mm in diameter.</td>
<td>No LD bodies; late lesion of CL</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>19/10/2012</td>
<td>273398/12</td>
<td>38 F</td>
<td>Thazheamala</td>
<td>One well-defined erythematos plaque of 1 × 2 cm size on forehead for 6 months.</td>
<td>Macrophages show LD bodies, consistent with CL</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>19/10/2012</td>
<td>273402/12</td>
<td>17 F</td>
<td>Thazheamala</td>
<td>Two erythematos papules on left forearm with 0.5 cm diameter for 6 months.</td>
<td>No significant pathology</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>19/10/2012</td>
<td>273399/12</td>
<td>44 F</td>
<td>Meleamala</td>
<td>Two lesions, hypo-pigmented patches on left ear lobe and right side of forehead. History of diagnosis and treatment for CL 1 year before for the same lesions.</td>
<td>No LD bodies; no features seen to suggest CL</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>19/10/2012</td>
<td>273397/12</td>
<td>75 F</td>
<td>Thazheamala</td>
<td>An erythematos nodule on right forearm 2 × 3 cm in size for 8 months and another lesion on right leg, hypo-pigmented plaque of 2 × 2 cm.</td>
<td>Positive for LD bodies; consistent with CL</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>19/10/2012</td>
<td>273396/12</td>
<td>50 M</td>
<td>Thazheamala</td>
<td>Hypo-pigmented patch on right shoulder 3 × 4 cm. History of treatment for CL 1 year before.</td>
<td>No LD bodies; late lesions of CL</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>30/3/2013</td>
<td>66332/13</td>
<td>13 F</td>
<td>Kombidi</td>
<td>Two well-defined hypo-pigmented nodules, no surface changes on the lateral aspect of right arm. CL.</td>
<td>Macrophages with LD bodies; consistent with CL</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>30/3/2013</td>
<td>66328/13</td>
<td>22 M</td>
<td>Thazheamala</td>
<td>Asymptomatic hypo-pigmented macules and erythematos to skin-coloured small plaques over upper back and forearms for 3 years. Suspected CL &amp; <em>Taenia versicolor</em>.</td>
<td>Macrophages with LD bodies</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
<td>30/3/2013</td>
<td>66330/13</td>
<td>10 M</td>
<td>Thazheamala</td>
<td>Asymptomatic hypo-pigmented to reddish papules over body – 2 years.</td>
<td>No LD bodies</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>26/4/2013</td>
<td>88297/13</td>
<td>28 M</td>
<td>Thazheamala</td>
<td>Erythematos papules on upper limbs and chest of 5–10 mm in size for 6 months.</td>
<td>Epithelioid granuloma with lymphocytic infiltration; no LD bodies</td>
<td>Negative</td>
</tr>
<tr>
<td>11</td>
<td>26/4/2013</td>
<td>88298/13</td>
<td>26 M</td>
<td>Podium</td>
<td>Single well-defined hypo-pigmented plaque on upper limbs of 1 × 2 cm in size for 1 year.</td>
<td>Epithelioid granuloma with lymphocytic infiltration; no LD bodies</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>26/4/2013</td>
<td>88299/13</td>
<td>25 M</td>
<td>Thazheamala</td>
<td>Erythematos papules on upper limbs of 5–10 mm in diameter for 3 months.</td>
<td>Epithelioid granuloma with lymphocytic infiltration; no LD bodies</td>
<td>Negative</td>
</tr>
<tr>
<td>13</td>
<td>26/4/2013</td>
<td>Gokulam Medical College, TVPM</td>
<td>77 F</td>
<td>Kadackal</td>
<td>Nodular skin lesions on both the forearms of 2–3 cm in diameter for last 6 months.</td>
<td>Epithelioid granuloma with lymphocytes; no LD bodies seen</td>
<td>Negative</td>
</tr>
</tbody>
</table>

TVPM, Thiruvananthapuram.

*Outpatient identification number, Government Medical College, Thiruvananthapuram, India.*
were positive for LD bodies on histopathological examination. The size of the k-DNA fragments amplified was about 720 bp, indicating that the species involved belonged to the *L. infantum* group (Aransay et al., 2000). The 3’ UTR regions of the Hsp70 gene of all these positive samples were amplified, which yielded fragments of about 750 bp. On restriction digestion with *Hae*III, a pattern similar to that reported to *L. donovani/L. infantum* was recorded [four fragments of about 450, 170, 125 and 25 bp (very faint)] (Fig. 3). Phylogenetic analysis of the Hsp70 gene sequences of the *Leishmania* parasite isolates indicated the species to be *L. donovani* (Fig. 4). On further analysis of DNA sequences it was found that all five had a cytosine molecule at the 434 position of the Hsp70 gene sequence, indicating the specimens belonged to the species *L. donovani*. *L. infantum* isolates have a thymine molecule in the position 434 of Hsp70, making that a restriction site of the restriction enzyme *Mlu*I (Montalvo et al., 2010). The GenBank accession numbers of the Hsp70 sequences of our isolates are KC884001, KF562068–KF562071. GenBank BLAST analysis showed 100.0% identity for these gene sequences with *L. donovani* isolated from VL cases in India (JQ990221), China (JX021428) and African countries (FN395027 and FN669773). Phylogenetic analysis carried out with the 6-PGDH gene amplified for two positive samples in the study showed their genetic lineage to be closely related to Sri Lankan strains of *L. donovani* (Fig. 5). The Kimura 2 parameter (K2P) genetic distance calculated among the ‘Kerala/Sri Lanka group’ of CL strains was found to be zero. The 737 bp partial DNA sequences of the 6-PGDH genes (KJ461872 and KJ461873) exactly matched with the gene sequences of the Sri Lankan isolates. The K2P genetic distance between this and the ‘north Indian/Bangladesh’ group (AJ888899 – AJ888901) was found to be 0.003. Also, a unique transition event in the 976th nucleotide position (A→G) resulting in a non-synonymous mutation ‘N326D’ was recorded in ‘Kerala/Sri Lanka’ group of isolates compared to the ‘north Indian/Bangladesh’ isolates (VL). Isolates from China and Kenya included in the analysis belonged to the same genetic lineage as the ‘Kerala/Sri Lanka’ group. However, the former isolates were from VL cases. None of the 13 cases were found to be positive with rk39 kits, clearly confirming the cases included did not have detectable levels of antibodies in their blood.

**DISCUSSION**

Agasthyamala Biosphere Reserve (area 3500.36 km²) spanning both Kerala and Tamil Nadu states of India is located in the Western Ghats, the second largest mountainous belt in India, and is a UNESCO (United Nations Educational Scientific and Cultural Organization) World Heritage site (UNESCO, 2012). It is the abode of one of the oldest surviving ancient tribal populations (the...
Kanikarans) in the world. It is also designated one of the world’s eight hotspots of biodiversity (Myers et al., 2000). The current investigation on the 28 tribal settlements of this biosphere reserve indicated endemic transmission of *L. donovani* among the Kanikaran tribal population, causing CL. The first investigation of the prevalence of this disease in this region was carried out by Simi et al. (2010). The investigators made known the active transmission of CL by clinical and histopathological investigations in two tribal villages of Thiruvananthapuram District, never recorded previously in the literature. The region is inaccessible to modern developmental activities and clinical facilities, is located deep in the evergreen forests of Western Ghats and hence has remained isolated for decades. These hamlets were never in the records of the state Department of Health services, Kerala state, for any past activity of vector-control operations. Also, the region belongs to one of the protected bio-reserves of international interest, with very restricted human interventions from outside. Thus, it could be ascertained that the transmission of CL in this region would have remained unnoticed for many decades.

*L. donovani*, the dominant parasite of VL in other parts of the country, brings about cutaneous manifestations in the tribal population of this undisturbed ecosystem, which is intriguing. None of the inhabitants clinically surveyed in these villages (*n* = 768) exhibited the typical symptoms of VL. All 13 cases included in the investigations tested negative with rk39 rapid tests for VL. The nucleotide sequences of both Hsp70 and 6-PGDH genes of *L. donovani* isolates from Kerala exactly matched with those of isolates from neighbouring Sri Lanka. The zymodeme classification of the strain recorded in tribal villages of Kerala may be ‘MON37’. The unique mutation recorded in the 976th position of the 6-PGDH gene (responsible for the change from the uncharged asparagine to negatively charged aspartic acid, which affects the mobility of the 6-PGDH iso-enzyme) recorded among these *L. donovani* isolates was ascertained to belong to zymodeme ‘MON37’, which causes CL in Sri Lanka (Siriwardana et al., 2007).

P. argentipes, the established vector species of *L. donovani* (Tiwary et al., 2012), was found to be abundant in the

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**Fig. 3.** Restriction digest of the 3’ UTR region of the Hsp70 gene of *Leishmania* species with *HaeIII* (the 25 bp fragment is very faint in the agarose gel photograph). The depicted pattern indicates the species to be either *L. donovani* or *L. infantum* (*infantum* group).

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**Fig. 4.** Phylogenetic analysis of the Hsp70 gene of the *Leishmania* isolates from Kerala.
study area. A similar situation had been recorded for Sri Lanka also (Siriwardana et al., 2007). *P. argentipes* was found to be the vector species prevalent in this region (Gajapathy et al., 2013).

Terrestrial links between ‘montane’ regions of Sri Lanka and Western Ghats existed during the Pleistocene era (Rohling et al., 1998). These two areas (about 400 km apart and currently separated by the Palk Strait) are grouped as a single biodiversity hotspot by the International Union for Conservation of Nature (IUCN), as they form a community of species that fits together as a ‘single bio-geographic unit’ (Myers et al., 2000). A few studies have already analysed the genetic similarity of different species of organisms from both regions and concluded that ‘the overall limited biotic interchange has left both the areas with an unexpectedly large number of endemics’ (Bossuyt et al., 2004; CEPF, 2007). The genetic similarity of many organisms recorded between the wet zone of Sri Lanka and the moist forests of the Western Ghats (Conservation International, 2008) may be due to the inability of rainforest organisms to disperse across intervening dry lowlands (Bossuyt et al., 2004). Genetic drift between the organisms living in these conserved rainforest montane regions owing to the geographical isolation may be negligible since these remain isolated as virgin forests without intervention from developmental activities. The villages included in this study are located deep inside the protected forests with an approximately 10 km inaccessible thick forested mountainous region barrier, also a natural reserve of wild animals. The genetic relatedness of *L. donovani* isolates from the Western Ghats region with Sri Lankan isolates recorded in the present study could be cited as another instance of local endemism of a parasite species in this single bio-geographical unit. The Kanikaran tribes, from whom these parasite species were isolated, live exclusively in the evergreen rain-forested region of Western Ghats and are reluctant to migrate to the non-forested regions of the state, in spite of many government schemes to rehabilitate them.

The present study evinces active transmission of CL in the tribal settlements of Western Ghats caused by *L. donovani*. The genetic lineage of the parasite was found to be closely associated with the isolates from the adjacent country Sri Lanka, which forms a single bio-geographical unit along with the Western Ghats. The vector species involved may be *P. argentipes*, as this established vector species for *L. donovani* was found to be abundant in the region. Further studies to confirm this, and to determine whether any reservoir hosts are involved in the maintenance of transmission in the region, are in progress. Chinese and African strains of *L. donovani* were found to be closely related genetically to the isolates from Kerala/Sri Lanka. Nevertheless, they cause visceral manifestations. To prevent the remote possibility of the spread of *L. donovani* from its isolation in the deep forests, to the thickly populated lowlands of Kerala state, urgent intervention measures to contain and eradicate these foci of leishmaniasis transmission are of utmost importance.

**ACKNOWLEDGEMENTS**

The authors are grateful to Dr. T. Dileep Kumar, Directorate of Health Services, Government of Kerala, India, for his suggestions, and to Shri. P. M. Ajithlal, Vector Control Research Centre (VCRC) Field station, Kottayam, and Shri. B. Edwin, VCRC, Puducherry, for the technical assistance rendered.

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