Leishmania donovani zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka

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Although the strain causing cutaneous leishmaniasis (CL) in Sri Lanka was first identified in 2003, the strain causing visceral leishmaniasis (VL) has not yet been identified. We report the first isoenzyme typing of a strain causing VL in Sri Lanka at an early stage of emergence of VL in the country. The parasite was isolated from a 57-year-old civil soldier who had been in the jungle in the Vavuniya district in the Northern Province of Sri Lanka for a period of nearly 6 months immediately before the onset of symptoms. Multilocus enzyme electrophoresis (MLEE) revealed that the strain is *Leishmania donovani* zymodeme MON-37, the zymodeme which was previously identified from the CL patients in the country. The MLEE analysis was confirmed by sequencing the gene encoding the 6-phosphogluconate dehydrogenase isoenzyme. This is an instance of the same *Leishmania* zymodeme associated with both dermatotropism and viscerotropism in the same geographic region. Further investigations into the genetic structure and identification of virulence factors in the parasite and immune factors in the host are required to understand the factors responsible for different tropism shown by the same zymodeme MON-37 *L. donovani* from Sri Lanka.

Keywords: Visceral leishmaniasis, *Leishmania donovani*, Isoenzyme analysis, Sri Lanka

Introduction

Cutaneous leishmaniasis has been considered an important public health problem in Sri Lanka for more than a decade. Although the first case of autochthonous cutaneous leishmaniasis (CL) was reported in 1992 from Southern Sri Lanka,¹ there have now been many more cases reported throughout the country since 2000.² The strain responsible for CL was initially identified by multilocus enzyme electrophoresis (MLEE) in 2003 as *Leishmania donovani* zymodeme MON-37.³ These findings were subsequently confirmed by sequencing the 6-phosphogluconate dehydrogenase (6PGDH) gene from 11 CL isolates from Sri Lanka which showed a non-synonymous single-nucleotide polymorphism at position 976 when compared to the MON-2 strain from India.⁴

In contrast to CL, visceral leishmaniasis (VL) is much more rare and the first autochthonous case of VL was reported in 2007 from the Anuradhapura district of Sri Lanka⁵ (Fig. 1). This is the district that currently reports the highest annual incidence of CL patients in the country.⁷ In the present study, we describe the first isolation and isoenzyme typing of an autochthonous VL isolate derived from the 3rd diagnosed VL patient in Sri Lanka. Isoenzyme typing of this novel VL isolate represents an important advance in understanding and controlling the potential spread of VL in Sri Lanka.

Materials and Methods

An *in vitro* culture of this VL isolate was established in October 2010 during a diagnostic bone marrow aspiration biopsy in a 57-year-old civil soldier who had no history of overseas travel but had lived in the jungle in the Northern district of Vavuniya (Fig. 1) immediately before the onset of symptoms. The promastigotes were cultured in Medium 199 supplemented with

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Figure 1 District map of Sri Lanka: 1, Anuradhapura district; 2, Vavuniya district; N, North. ●CL cases reported districts: + + + +, the highest annual passive CL case report incidence;⁷ + + +, the second highest annual passive CL case report incidence;⁷ + +, the third highest annual passive CL case report incidence.⁷ ▲Confirmed endogenous VL case reports to date (Anuradhapura district VL cases $n=2$, Vavunia district patient described here $n=1$).

10% FBS, 200 mM glutamine, 25 mM adenosine, 10 mg/ml folic acid and 100 μ l/ml penicillin/streptomycin.⁸

The patient had intermittent fever for nearly 6 months, was pale and had moderate hepatosplenomegaly. The investigations revealed an increased erythrocyte sedimentation rate of 110 mm/h, haemoglobin concentration of 5.2 g/dl, white blood cell count of 3000/mm³ and a platelet count of 82×10^9 /l reflecting severe pancytopenia. His albumin and globulin concentrations were 2.8 and 5.8 g/dl, respectively, reflecting the reversal of the albumin globulin ratio. Serum creatinine, blood urea, SGOT and SGPT values were within the normal range. His bone marrow biopsy showed numerous amastigotes. Following the bone marrow biopsy and *in vitro* culture confirmatory diagnosis, the patient was treated with intravenous sodium stibogluconate 20 mg/kg/day in divided doses for 28 days. The patient responded to treatment. The fever settled 3 weeks after the commencement of treatment and the size of the spleen reducing simultaneously. He

was discharged after the completion of treatment. Two follow-up visits were made and there was no evidence of resistance to sodium stibogluconate or recurrence 3 months after completion of treatment.

The first culture inoculate was sub-cultured after 3 days to eliminate inhibitory factors in the bone marrow and to eliminate potential contamination. A sample from the first subculture at a parasite concentration of 7×10^4 /ml was submitted to the Laboratoire de Parasitologie-Mycologie, CHRU de Montpellier, Centre National de Référence des *Leishmania*, Montpellier, France; for *Leishmania* strain isoenzymatic identification and sent to McGill University, Canada, for DNA sequencing of the 6PGDH gene.

For isoenzymatic typing; starch gel electrophoresis was performed according to a method previously described,⁹ using the following 15 enzymes: malate dehydrogenase, MDH, EC 1.1.1.37; malic enzyme, ME, EC 1.1.1.40; isocitrate dehydrogenase, ICD, EC 1.1.1.42; 6-phosphogluconate dehydrogenase, PGD, EC 1.1.1.44; glucose-6-phosphate dehydrogenase, G6PD, EC 1.1.1.49; glutamate dehydrogenase, GLUD, EC 1.4.1.3; NADH diaphorase, DIA, EC 1.6.2.2; purine nucleoside phosphorylase, NP₁, EC 2.4.2.1; purine nucleoside phosphorylase, NP₂, EC 2.4.2.*; glutamate-oxaloacetate transaminases, GOT₁ and GOT₂, EC 2.6.1.1; phosphoglucomutase, PGM, EC 5.4.2.2; fumarate hydratase, FH, EC 4.2.1.2; mannose phosphate isomerase, MPI, EC 5.3.1.8; glucose phosphate isomerase, GPI, EC 5.3.1.9.

Two zymodeme reference strains were used: MHOM/IN/00/DEVI *L. donovani* MON-2 and IMAR/KE/62/LRC-L57 *L. donovani* MON-37.

To confirm the isoenzyme typing, the 6PGDH gene was PCR amplified with primers (6PGDH-F: AATCGAGCAGCTCAAGGAAG and 6PGDH-R: GAGCTTGCGAGAATCTGAC) as previously described.^{4,10} The resulting 997 bp 6PGDH PCR product was cloned into the TA cloning Vector (Invitrogen) and subjected to Sanger DNA sequencing at the McGill University Genome Quebec Innovation Centre.

Results

The first isolate of an autochthonous VL strain from Sri Lanka VL as detailed in methods was designated MHOM/LK/2010/OVN3. Initially, MLEE was performed and based on the migration of the 6PGDH enzyme, it was concluded that the VL-derived strain was a member of zymodeme MON-37.

To verify the MLEE analysis, we sequenced the 6PGDH gene to confirm the presence of the polymorphism in position 976 (A/G for India and Sri Lanka, respectively) encoding amino acid at position 326, Asparagine, AAC (India), or Aspartic

Ld(India) 278	VPAPSLSMAVISRQMTMCKEERIANCKAFPNFPRGPSAEARDKSPNSPNAKQLYHAVSLC	337
Ld(SrLan)	-----D-----	
LdSL-VL	-----D-----	
L.inf	-----D-----	
L.tro	-----N-----I-----E--T-----E-----	
L.maj	-----N-----Y-G-----E--T-----E--K-----	
L.mex	-----N-----Y-A--V--S-----H--C--CEK-T-----E--F-----S	
L.bra	-----N-----D--AE-F-----T--CSVREQVK-----A-EV-----S	

Figure 2 Partial amino-acid sequence alignments of the 6-phosphogluconate dehydrogenase (6PGDH) isoenzyme from different *Leishmania* species. *L. donovani* 6PGDH partial amino acid sequences from amino acids 278 to 337 are used for alignments. The main *L. donovani* India (Ld-India) zymodeme (MON-2) sequence is given on top. The previously reported⁴ *L. donovani* Sri Lanka (Ld-SrLan) zymodeme (MON-37) is shown next. LdSL-VL is the visceral disease causing Sri Lanka *L. donovani* that was isolated in this study. L.inf, *L. infantum*; L. tro, *L. tropica*; L. maj, *L. major*; L. mex, *L. mexicana*; L. bra, *L. braziliensis* are shown for comparison to other *Leishmania* species. Note: the single amino-acid difference at amino acid 326 distinguishes the Indian strain (N, asparagine) from the Sri Lanka cutaneous strain (D, aspartic acid) and that the new LdSL-VL isolate causing visceral leishmaniasis described in this paper with D at amino acid 326. The GenBank accession number is JX481773 for the 6PGDH gene for the VL isolate from Sri Lanka.

Acid, GAC (Sri Lanka). As shown in the Fig. 2, similar to the previously reported CL isolate from Sri Lanka,⁴ the VL isolate from this study has the same 6PGDH gene sequence as the previously reported *L. donovani* zymodeme MON-37. The VL MON-37 isolated from Sri Lanka differed from the India *L. donovani* zymodeme MON-2 by a single amino acid at position 326 as previously reported.⁴ The GenBank accession number for the 6PGDH gene from this VL isolate is JX481773.

For further comparison of the 6PGDH gene, we also included the known corresponding sequences from *L. infantum*, *L. tropica*, *L. major*, *L. mexicana*, and *L. braziliensis* demonstrating the close relationship between the *L. donovani* zymodemes from Sri Lanka and India. Note that in this stretch of amino acids, the sequence of the *L. infantum* 6PGDH protein is the same as the sequence of the VL *L. donovani* isolate from Sri Lanka. It is important to appreciate that this analysis does not, however, rule out the possibility that there are other genetic differences between the Sri Lanka VL and CL zymodeme MON-37 strains that could govern different pathologies caused by these isolates.

Discussion

Several cases of VL have now been reported in Sri Lanka^{5,6,11,12} and the present study represents the first isolation and identification of an autochthonous VL strain from Sri Lanka. Isoenzyme typing represents the gold standard for classifying *Leishmania* species and this study therefore represents an important advance in understanding and controlling the potential spread of VL in Sri Lanka. The *L. donovani* zymodeme MON-37 is known to cause human VL in other parts of the world, including India^{13,14}, Kenya,¹⁴ and Cyprus.¹⁵ Sri Lanka was the first to report *L. donovani* zymodeme MON-37 causing human CL in the world.³ *L. donovani* has also been reported to be occasionally associated with

CL in Asian and East African foci.¹⁶ Recent studies using microsatellite typing revealed that the MON-37 strains of Kenyan, Indian and Sri Lankan are genetically closer to each other than other zymodemes of the same geographic region.^{15,17} It has also been shown that the genotype of *Leishmania* parasite is known to strongly correlate with the geographic origin but not with the clinical outcome.¹⁸ Furthermore, studies have shown the presence of genes required for visceralization of *L. donovani*¹⁹ and it will be interesting to compare some of these genes in the CL and VL isolates from Sri Lanka. Sequencing of the genomes of the MON-37 CL- and VL-derived isolates from Sri Lanka is necessary to detect potential genetic differences responsible for the different tissue tropisms observed with these isolates. Investigation into host immunity would be equally important in understanding the different pathologies associated with *L. donovani* MON-37 strain infections in Sri Lanka.

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