First Case of Indigenous Visceral Leishmaniasis from Central India

Ayan Dey, Umakant Sharma, and Sarman Singh*

Division of Clinical Microbiology, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India

Abstract. Visceral leishmaniasis is endemic in the eastern states of India, but central India remains free of leishmaniasis. This report describes the first indigenous case of visceral leishmaniasis in a seven-year-old girl from central India. The child presented with fever for 10 days and was diagnosed by bone marrow examination, serology using rKE16 and rK39 antigens, and a polymerase chain reaction specific for the kinesin gene. Sequencing of the immunodominant region of the kinesin gene of the parasite showed four tandem repeats, each 117 basepairs. The first tandem repeat of this strain had 97% homology with the corresponding first tandem repeat of the Leishmania donovani KE16 strain and 92% homology with the L. chagasi BA-2 strain. The second, third, and fourth tandem repeats had 97%, 98%, and 99% homology, respectively, with the L. donovani KE16 strain, and 89%, 96%, and 92% homology, respectively, with the L. chagasi BA-2 strain. This case shows that more than one genetic variant of L. donovani is circulating in various parts of India.

INTRODUCTION

Leishmaniasis is a major public health problem causing significant morbidity and mortality in Africa, Asia, and Latin America. It has three clinical forms, of which visceral leishmaniasis (VL) (kala-azar) is the most severe. Recent outbreaks of VL in India and the epidemic of human immunodeficiency virus (HIV) make VL a re-emerging problem in India. Visceral leishmaniasis spreads from person to person through sand fly bites. An anthropopotic VL is endemic in the tropical and sub-tropical regions of Africa, Asia, southern Europe, and South and Central America. However, distribution of anthropopotic VL in these areas is patchy and often associated with areas of drought, famine, and densely populated villages with little or no sanitation. Outbreaks and epidemics of leishmaniasis have been associated with urban development, deforestation, environmental changes, and population migrations. Risk factors for this disease include HIV and malnutrition, and genetic factors are also responsible for the epidemiologic diversity of leishmaniasis.

India accounts for half of the 500,000 VL infections that are reported annually worldwide. Most cases in India are reported from the northeastern states of Bihar, eastern Uttar Pradesh, West Bengal, and Assam. Sporadic cases have also been reported from Gujarat (western India), Tamil Nadu and Kerala (southern India), and sub-Himalayan parts of northern India including Uttar Pradesh, Himachal Pradesh, Jammu, and Kashmir. To the best of our knowledge and available literature, VL has not been reported from Madhya Pradesh (central India), although a sand fly vector (Phlebotomus argentipes) has been reported from this area. This case report is unique and significant because possible spread of VL in this region may affect the health of local inhabitants as well as tourism and the economy of this state.

CASE REPORT

A seven-year-old girl from Morena (26°30’N, 78°04’E) in Madhya Pradesh in central India was referred to our institute. She had a high-grade fever (105°F) with chills and rigors for three weeks and did not respond to standard treatment. Initially, for her febrile illness, she was tested locally for malaria (peripheral blood smear examination and Plasmodium falciparum and P. vivax antigen detection) and typhoid fever (Widal and TyphiDot test) but results were negative. Results of routine serum biochemical and hematologic investigations were also negative. When no conclusive diagnosis could be made and she did not respond to treatment with anti-malarial drugs and third-generation cephalosporine antibiotics, she was transferred from Madhya Pradesh to a nursing home in Delhi. She was again tested for malaria and other causes of fever of unknown origin by blood culture (BacTec 460; Becton Dickinson, Heidelberg, Germany), chest radiograph, and abdominal ultrasonography. Results of blood cultures and chest radiographs were negative. However, ultrasound showed moderate hepatosplenomegaly, with both the liver and spleen palpable below costal margins. Results of routine and culture examinations of urine were normal. Hematologic examination showed mild normocytic hypochromic anemia with hemoglobin levels of 11 g%, a total leukocyte count of 8,000/mm³ with slight lymphocytosis (40%). Her liver enzyme levels were within normal limits, and she had a moderately low total serum protein level (7 g%) and increased globulin levels (5 g%).

Her past and family history showed that she was a full-term normal delivery through the vaginal route. She and her family members had never traveled to any areas endemic for kala-azar, including the endemic districts of eastern Uttar Pradesh and Bihar located approximately 620 km from Morena. She did not have any prolonged illness and had not received any blood transfusions.

Her bone marrow, other clinical samples, and all clinical and laboratory details were referred to us for the expert opinion of the senior author of this report (SS). Her bone marrow smears were examined by staining with Giemsa and showed numerous plasma cells and an overall reactive pattern. The smear also showed Leishman-Donovan body-like structures. Her serum samples were tested with the anti-r-K39 dipstick test (Insure™; Inbios, Seattle, WA) anti-rKE16 spot test (Signal KA™; Span Diagnostics Ltd., Surat, India), and the rKE16 latex agglutination test (up to a dilution of 1:16). All three tests showed strongly positive results. The novel recombinant antigen (rKE16) has recently been prepared in our laboratory from KE16 (MHOM/IN/1998/KE16) isolate of

* Address correspondence to Sarman Singh, Division of Clinical Microbiology, All India Institute of Medical Sciences, PO Box 4398, Ansari Nagar, New Delhi 110029, India: E-mail: sarman_singh@yahoo.com
Leishmania donovani. This strain was isolated from a kala-azar patient from Bihar in eastern India. The details of this recombinant antigen and its preparation have been recently reported. Using this antigen, we titrated serum anti-rKE16 antibodies from our patient. The end point titration results showed strong antibody response with titers of 1:102,400. However, all the family members including her father, mother, and sister were negative for anti-rK39 and anti-rKE16 antibodies.

For genotyping, the Morena strain and bone marrow and blood samples of the patient were subjected to a polymerase chain reaction (PCR) specific for an immunodominant region of the kinesin gene as described by Burns and others for L. chagasi. However, after elucidating the complete kinesin gene sequence of L. donovani (AY615886) in our laboratory, a set of L. donovani-specific primers (forward primer: 5'-AGCAGCAGCTTCGTACTCC-3', reverse primer: 5'-CGTGGCCCTCGTTCTGC-3') were used, as previously reported.

DNA was isolated and purified from the patient’s blood buffy coat and bone marrow samples by a standard protocol. The amplification conditions included initial denaturation at 95°C for 10 minutes, followed by 35 amplification cycles of denaturation at 95°C for one minute, annealing at 53°C for one minute, and extension at 72°C for one minute. A final extension was conducted for 10 minutes. The PCR was repeated three times with appropriate positive and negative controls. The PCR products were resolved by electrophoresis on a 2% agarose gel and were positive in all experiments. An amplified product of approximately 468 bases was sequenced for each tandem repeat (each 117 bases) and was considered positive for L. donovani.

The amplified product was extracted and purified from the gel using the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and cloned in the pGEM-T vector (Promega, Madison, WI). Three sets of positive clones were selected. Using T7 and SP-6 promoters, we sequenced both strands of these fragments (Microsynth; Balgach, Switzerland). The sequence data was aligned using Clustal W multiple sequence alignment software, with the kinesin gene sequences of two Leishmania species (L. donovani KE16 strain MHOM/IN/1998/KE16 and L. chagasi MHOM/BR/82/BA-2,CI) available in the GenBank database (accession nos. AY615886 and L07879, respectively).

Results for the cloned fragment showed clear and consistent identification of 464-basepair product and 94% homology with the L. donovani KE-16 strain and 90% homology with the L. chagasi BA-2 strain. There were also inter-tandem genetic differences among the four tandem repeats in the amplified product. The first tandem repeat had 97% homology with the second tandem repeat, 92% homology with the third tandem repeat, and 98% homology with the fourth tandem repeat. The second tandem repeat had 89% homology with the third tandem repeat and 99% homology with the fourth tandem repeat. The third tandem repeat had maximum inter-tandem heterogeneity that was evident from the fact that it had only 90% homology with the fourth tandem repeat.

Comparison of the kinesin genes of other Leishmania species showed that the first tandem repeat of the Morena strain had 97% homology with the corresponding first tandem repeat of the L. donovani KE16 strain. Similarly, the second, third, and fourth tandem repeats of the Morena strain had 97%, 98%, and 99% homology with the corresponding second, third, and fourth tandem repeats of the L. donovani KE16 strain. As expected, the first tandem repeat of the Morena strain had only 92% homology with the L. chagasi BA-2 strain and the second, third, and fourth tandem repeats had 89%, 96%, and 92% homology, respectively, with the corresponding tandem repeats of the L. chagasi BA-2 strain (Figure 1). Gene sequence data of the new strain has been submitted to GenBank (accession no. DQ648599).

Immediately after the serological and PCR-based diagnosis, anti-leishmanial treatment with a full course of ambisome (liposomal amphotericin B, 2 mg/kg/day) was given intravenously for 10 days. The child began to improve within 48 hours and was clinically and parasitologically cured after the full course of treatment. She was last followed-up after 10 months and was completely asymptomatic and healthy. At this time, her anti-rKE16 antibody titers had decreased to 1:400.

**DISCUSSION**

The visceral form of leishmaniasis is endemic in eastern states of India particularly in Bihar, West Bengal, eastern Uttar Pradesh, and Assam. However, in last quarter of the 20th century, western migration of the disease was observed and new foci of this disease were reported from previously non-endemic regions. The best of our knowledge and available literature, no case of VL has ever been reported from Madhya Pradesh in central India, although a sand fly vector (Ph. argentipes) has been reported from this region. It is possible that because of a lack of awareness and diagnostic facilities for this infection in non-endemic areas, several cases might have been misdiagnosed by the local physicians.

This case provides an opportunity to investigate whether the parasite has been established in this part of India or our patient was an accidental case. Our patient may have been infected by her schoolmates or other neighbors who visited areas endemic for kala-azar and became recently infected with L. donovani. Another source of infection could have been infected persons who migrated from these areas to her town during the latent period of their infections, and a vector could have transmitted the infection to her from these persons. However, it is not clear why no other case has been reported after our case from this area. It is also possible that this strain is already circulating in some wild animal reservoirs in the area and the patient had contact with these animals or was exposed to the infected insect vector bites. However, the child and her family had never traveled to any areas endemic for kala-azar, which are more than 600 km from Morena.

Madhya Pradesh in central India is free of kala-azar and has rich and diverse forest resources. There are four important forest types in this state: tropical moist forests, tropical dry forests, tropical thorn forests, and subtropical broad-leaved hill forests. It also contains great river basins and the watershed of the Chambal, Godavari, Mahanadi, Narmada and Tapti Rivers. Catchments of these rivers are also located in this region. Morena is situated in the Chambal River valley and surrounded by tropical forest. The soil in this district is alluvial, sandy, and sandy-loam, which are suitable for breeding of sand flies. Thus, developmental projects leading to deforestation and settlement might have
Figure 1. Nucleotide sequence homology of the immunodominant region of the kinesin gene of a Leishmania donovani strain from Morena, central India (GenBank accession no. DQ48599) with L. donovani from Bihar, eastern India (MHOM/IN/1998/KE16, GenBank accession no. AY615886), and L. chagasi (MHOM/BR/82/BA-2, C1, GenBank accession no. L07879). = sequence identity.
lead to environmental degradation that caused sand flies to migrate from forests to human habitats. We are conducting entomologic surveillance in this area to validate this hypothesis. The present case report highlights geographic changes leading to ecologic disturbance with a possible spread of leishmaniasis in previously non-endemic regions of India.

The gene sequence data of the Morena strain clearly shows that the causative agent was *L. donovani* closely related to the *L. donovani KE16* strain (MHOM/IN/1998/KE16), a clinical isolate from Bihar in eastern India. Interestingly, genetic differences were mainly in the third tandem repeats. Conversely, the Morena strain showed significant genetic distance from the *L. chagasi* MHOM/BR/82/BA-2,C1 strain isolated from a VL patient in Brazil.

We used the kinesin gene for genetic analysis of the Morena strain. The kinesin gene and its diagnostic importance were first described by Burns and others using the *L. chagasi* MHOM/BR/82/BA-2,C1 strain isolated from a VL patient in Brazil. The sequence of this gene, which has 4–7 tandem repeats, was submitted to GenBank (accession no. L07879). Following the availability of the kinesin gene sequence, we sequenced the kinesin gene from Indian *L. donovani* and subsequently developed our own PCR primers and protocols. The same protocol was used for this case. The kinesin protein plays an important role in cell division and intracellular transport of vesicles, organelles, large protein complexes, and cytoskeletal filaments. It is highly conserved in all kinetoplastids. The immunodominant repeat region is part of a large kinesin-related protein expressed predominantly during the amastigote stage of *Leishmania* species. Accordingly, recombinant proteins from *L. chagasi* (Lc-rK39) and *L. donovani* (Ld-rKE16) have been prepared and are being marketed for rapid and accurate diagnosis of VL.

Our case emphasizes the need for careful examination of clinical material and consideration of non-endemic diseases in the differential diagnoses of fever with moderate-to-severe hepatosplenomegaly. Because non-invasive, rapid, and cost effective tests for kala-azar are available, patients with pyrexia of unknown origin who do not respond to treatment with anti-malarial drugs and broad-spectrum antibiotics should also be investigated for VL, even when the disease is not endemic in a specific region.

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Authors’ address: Ayan Dey, Umakant Sharma, and Sarman Singh, Division of Clinical Microbiology, All India Institute of Medical Sciences, PO Box 4398, Ansari Nagar, New Delhi 110029, India, Telephone: 91-11-2658-8484, Fax: 91-11-2658-8663 and 91-11-2658-8641, E-mail: sarman_singh@yahoo.com.

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