DNA Polymorphism Assay Distinguishes Isolates of *Leishmania donovani* That Cause Kala-Azar from Those That Cause Post-Kala-Azar Dermal Leishmaniasis in Humans

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***Leishmania donovani* in India causes visceral infection (kala-azar) and dermal infection (post-kala-azar dermal leishmaniasis). We report here the identification of polymorphism in a well-defined genetic locus among the *Leishmania* parasites causing the visceral and dermal manifestations, in a comparison of 15 post-kala-azar dermal leishmaniasis and 12 kala-azar patient isolates.**

Visceral leishmaniasis (VL), or kala-azar (KA), caused by *Leishmania donovani*, is responsible for severe mortality and morbidity with 500,000 new cases every year (2). In India the disease is endemic in eastern parts of the country, primarily in the state of Bihar. Post-KA dermal leishmaniasis (PKDL) is an unusual dermatosis that develops as a sequel of KA in 10 to 20% of VL cases in India and in 60% of VL cases in Sudan (11, 19). KA transmission in India is thought to be anthroponotic, and PKDL patients are believed to be the sole source of the parasites (17). Studies of parasite biology in PKDL are few, primarily because of the difficulty of culturing the parasite. Intrinsic differences are reported to exist between the KA and PKDL isolates (1).

One of the hallmarks of these pathogenic protozoa is the diversity of tropism and disease resulting from infection. It is hypothesized that reactivation of persistent infections underlies some of the most severe forms of leishmaniasis, including PKDL. Little is known about parasite factors required for persistent infections (16). Moreover, humoral immune responses in PKDL patients are distinct from those in KA patients (12). Therefore, we sought to identify molecular differences between parasites isolated from KA and PKDL patients that may underlie the diversity in clinical manifestations of the disease.

The nuclear gene probe LdP13 allowed discrimination between *L. donovani* and *L. infantum* and between geographical isolates of *L. donovani* (9, 10). We utilized this probe to examine the genetic variation in parasites isolated from Indian KA and PKDL patients.

Parasite isolates. Parasite isolates from PKDL and KA patients were prepared from clinical samples as described previously (13, 14). The KA and PKDL patients came from different regions within Bihar, categorized as being of high, moderate, and low endemicity. An immunofluorescence assay with monoclonal antibodies D2, T-1, and T-10 (kind gifts from Tropical

Disease Research, World Health Organization) specific to *L. donovani*, *L. major*, and *L. tropica*, respectively, and analysis of isoenzyme profiles with a panel of five enzymes (6-phosphogluconic dehydrogenase, nucleoside hydrolase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, and malate esterase) identified all the isolates as *L. donovani* (5, 6).

Description of the probe and Southern blot analyses. The DNA fragment LdP13 has been cloned previously and mapped to a chromosome of 0.8 Mb in size (10). The nucleotide sequence of the LdP13 probe was found to be identical to that of the ζ and ε subunit regions of the 28S rRNA gene of *L. donovani* MHOM/SD/00/Khartoum (18). The 774-bp probe used spanned the region from bp 674 to 1448, reported in the GenBank database (accession no. AF115465).

This probe (LdP13) was used to investigate intraspecific DNA polymorphisms among 15 isolates of *L. donovani* obtained from skin lesions of PKDL patients and 12 isolates from bone marrow of KA patients and identified clear molecular variations between Indian KA and PKDL patient isolates. Southern blots were hybridized with ^{32}P-labeled LdP13 probe under standard conditions (9).

Based on the pattern obtained with MvaI, NaeI, NcoI, and PstI initially on a subset of isolates, subsequent analysis was done with MvaI. MvaI restriction followed by hybridization with LdP13 revealed three discrete restriction patterns in the 27 isolates. Figure 1 shows the three different restriction patterns. A majority of the KA patient isolates and the reference isolate of Indian origin (Ld AG83) gave an identical pattern (termed K), yielding fragments of 14.0, 10.0, 6.5, 5.5, and 3.0 kb (Fig. 1). In contrast, a majority of the PKDL patient isolates gave an identical pattern (termed P) that showed fragments of 12.0, 10.0, 6.5, 5.5, and 3.0 kb. The remaining PKDL and KA patient isolates could be clustered into a third group (termed M for mixed) that showed both the 14.0- and the 12.0-kb fragments, in addition to the smaller common fragments. Taken together, these data suggested that the PKDL patient isolates in the first group have gained an MvaI site that the KA patient isolates lack. The third group, giving both the 14.0- and 12.0-kb

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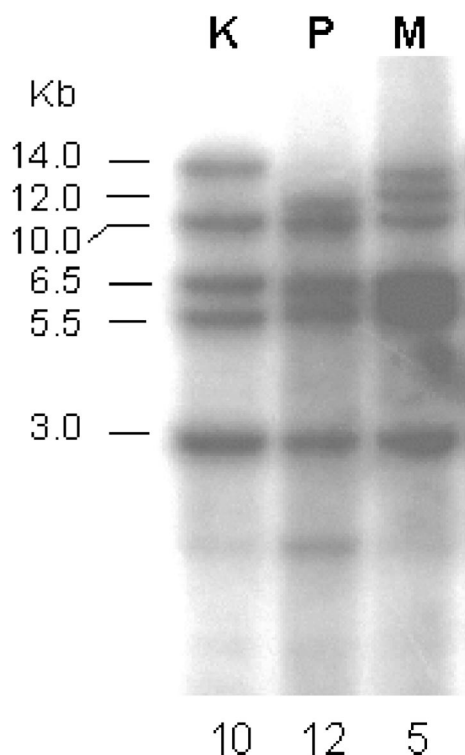
FIG. 1. Southern blot analysis of representative isolates from the three groups. Genomic DNA (5 μg) of *L. donovani* from groups K, P, and M was digested for 16 h at 37°C with MvaI (4 U/μg of DNA; MBI Fermentas) and resolved on a 1% agarose gel, and blots were probed with ^{32}P-labeled LdP13 and visualized by autoradiography. The numbers below the lanes indicate the numbers of isolates falling in the respective categories.

fragments, could represent a transition stage between the two groups. The intensity of the 14.0-kb fragment in the mixed group was approximately half of that in the first group, suggesting that only one of the alleles might have gained the MvaI site. Interestingly, the percentage of KA patient isolates falling in the mixed group (16.7%) was quite comparable to the percentage of KA patients who go on to develop PKDL. Further studies are necessary to examine if the observed differences are responsible for the phenotypic differences.

No correlation was found among the groups K, P, and M with the geographic origin with respect to KA endemicity, as all groups contained samples from low-, medium-, and high-endemicity areas within the state of Bihar (Table 1). Within PKDL patient isolates, a majority of the P group sample patients had a history of KA in excess of 4 years while all members in patient group M had a comparatively short history (Table 2).

Molecular techniques such as PCR-restriction fragment length polymorphism, random amplified polymorphic DNA, and single-strand conformation polymorphism analyses have been used to demonstrate the genetic variability within and between different *Leishmania* species including *L. donovani* complex (4, 8, 15). Earlier studies to differentiate between VL and PKDL patient isolates in Sudan did not reveal any correlation between sequence polymorphisms and clinical manifestations of human disease (3, 7). No such studies have been

TABLE 1. Geographic origins of the KA and PKDL isolates in relation to KA endemicity

Isolate[a]	Region of origin	Level of KA endemicity in region[b]
K group		
K1	Vaishali	M
K2	Bhagalpur	M
K3	Munger	L
K4	Siwan	L
K5	Saaran	H
K6	Madhubani	M
K7	Darbhanga	H
K8	Saaran	H
K9	Katihar	H
K10	Saaran	H
P group		
P1	Siwan	L
P2	Siwan	L
P3	Gopalganj	L
P4	Darbhanga	H
P5	Vaishali	M
P6	Saharsa	H
P7	Begusarai	M
P8	Muzaffarpur	H
P9	Araria	H
P10	Saaran	H
P11	Khagaria	H
P12	Saharsa	H
M group		
K11	Saharsa	H
K12	Gopalganj	L
P13	Madhubani	M
P14	Gopalganj	L
P15	Madhubani	M

[a] Groups K, P, and M refer to KA, PKDL, and mixed groups, respectively, as defined in the text. K1 to K12 and P1 to P15 are isolates from 12 KA and 15 PKDL patients, respectively.

[b] L, M, and H refer to low, medium, and high endemicity, respectively. Information on endemicity is taken from reference 8a.

reported with isolates of *L. donovani* from Indian populations. Despite overall similarities there are many important differences between Indian and African forms of KA and PKDL, warranting investigations in this direction (19). Our approach using a probe from an expressed region reported to show intraspecific differences has led to the identification of a correlation between genetic variability among and clinical manifestations of Indian isolates. Whether similar differences occur in Sudanese VL and PKDL patient isolates in the LdP13 locus remains to be investigated.

TABLE 2. Profile of PKDL patients belonging to the PKDL (P) and mixed (M) groups

Group	No. of patients with:					
	History of disease (yr)				Clinical presentation	
	KA		PKDL			
	<4	4–12	<1	1–6	Polymorphic	Macular
P	4	8	3	9	8	4
M	3	0	3	0	3	0

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