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Kinetoplast and Nuclear DNA Polymorphism of Visceral *Leishmania* Isolates from Northeastern India

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ABSTRACT

The recent epidemic outbreaks of visceral leishmaniasis have created a major health problem in India. Whether these outbreaks and the drug-resistance seen in about a quarter of these cases are genetically based remains unknown. To facilitate a better understanding of these possibilities, we have begun to analyse kinetoplast (k) and nuclear (n) DNA heterogeneity of *Leishmania* isolates from Bihar (DD8, RMRI and SS) and West Bengal (AG and UR6). Schizodeme and Southern blot analyses of their kDNA separated these isolates into three groups, i.e. DD8, RMRI and SS; AG and UR6. This grouping is consistent with the results of restriction fragment length polymorphisms seen at the gp63 and β-tubulin loci by Southern blot analysis of nDNA. The results obtained better define the genetic heterogeneity of Indian visceral *Leishmania* isolates for further characterization of their relationships to virulence and drug-resistance.

INTRODUCTION

The causative agent of kala-azar or visceral leishmaniasis (VL) was first discovered and named *Leishmania donovani* in eastern India in 1903 (Fig. 1, shaded area). Since then, extensive studies have shown that the Indian kala-azar is anthroponotic and transmitted chiefly by 1 or 2 sandfly species (Sanyal 1985). The epidemiology is thus less complicated than that of VL in other endemic areas where multiple reservoirs and vectors are often involved. Consequently, homogeneity of Indian *L. donovani* isolates may be expected. Surprisingly, analyses of their isoenzymes, serologic properties of the excretory factors and kDNA have revealed considerable heterogeneity among Indian isolates (Schnur et al. 1981; Barker 1989; Dasgupta et al. 1991). We report here our initial effort to further define the genetic heterogeneity of 5 Indian isolates from Bihar and West Bengal on the basis of their chromosomal

as well as kinetoplast DNA polymorphisms.

MATERIALS AND METHODS

Five clinical isolates originally from northeastern India (Fig. 1; Table 1) were kindly provided by Dr. R. C. Mahajan, Post Graduate Institute of Medical Education and Research, Chandigarh (DD8, RMRI and SS from Bihar) and Dr. M. K. Basu, Indian Institute of Chemical Biology, Calcutta (AG and UR6 from Calcutta, West Bengal). All parasites were initially obtained by bone marrow punctures from kala-azar patients and isolated in 3N medium. They were subsequently adapted to grow in Medium 199 containing 10% heat inactivated fetal bovine serum and 25 mM HEPES, pH 7.4. All isolates were previously cloned by the donors, except SS. This isolate was therefore cloned by plating on agar medium and one clone was randomly selected for the present study.

Kinetoplast and nuclear DNAs (kDNA and nDNA) were isolated as described earlier (Lu et al. 1994). Briefly, 1-5x10^9 promastigotes were lysed at 60° C with 100 µg proteinase K/ml and 1.5% sarkosyl in NET buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA) for 3-4 hrs. The kDNA networks were sedimented by centrifugation of the lysates at 50,000 *g* for 1 hr. The kDNA pellets were resuspended in Tris-EDTA (TE) buffer and subjected to several cycles of phenolchloroform extraction with vigorous shaking followed by resedimentation and ethanol precipitation. The original supernatants left after

Figure 1. Map of India showing endemic areas of kala-azar (shaded areas) and the origin of the five isolates used for the present study.

Table 1. Differentiation of Indian *Leishmania donovani* isolates into three genotypes

Isolate	kDNA type	nDNA type	Geographic origin	Disease
HOM/IN/80/DD8	I	I	Bihar	VL
HOM/IN/68/RMRI	I	I	Bihar	VL
HOM/IN/88/SS	I	I	Bihar	VL
HOM/IN/83/AG	II	II	West Bengal	VL
HOM/IN/78/UR66	III	III	West Bengal	VL

the first cycle of kDNA sedimentation were further digested overnight at 60° C for isolation of nDNAs. The subsequent procedures were essentially as described for kDNAs, except that care was taken to avoid shearing nDNAs. The kDNAs were digested with AluI, HaeIII , and MspI followed by electrophoresis in 1.5-1.8% agarose gels. The nDNAs were digested with PstI or PvuII and electrophoresed in 0.75% agarose. Standard procedures were followed for DNA blotting as recommended for Zetabind) (Cuno, Inc., Meriden, CT). The kDNAs from individual isolates were used as probes. The probes used for nDNA blots included a 1.79 kb gp63 gene cloned from *L. amazonensis* and a 1.7 Kb β-tubulin gene from the same species. All probes were labeled with [^{32}P]-dCTP using random hexanucleotide primers (United States Biochem. Corp., Cleveland, Ohio). Prehybridization and hybridization were carried out under stringent conditions in presence of 150 μg/ml calf thymus DNA (Sambrook et al. 1989). Filters were washed 4 times at 65° C with 0.1xSSC and 0.1% SDS. Blots were stripped off the [^{32}P] label with 0.4 N NaOH and reprobed after prehybridization.

RESULTS

The 5 Indian isolates were separated into 3 groups according to their kDNA minicircle heterogeneity. Digestion of these DNAs with all the endonucleases used yielded restriction fragments, all being smaller than 1 kb in size, indicative of their origin largely from kDNA minicircles (e. g. Fig. 2A). Three restriction patterns seen grouped DD8, RMRI and SS as type I (Lanes 1-3), and AG (Lane 4) and UR6 (Lane 5) as types II and III, respectively (Table 1). This grouping was further substantiated by Southern hybridization of HaeIII digested samples with representative kDNAs (Fig. 2B-D). Thus, the kDNA from each of the group I isolates hybridized only with other members of the same group (Fig. 2B, Lanes 1-3), but not with either group II (AG) (Lane 4) or group III (UR6) (Lane 5). Similarly, kDNAs from AG and UR6 hybridized significantly only with homologous samples (Fig. 2C, Lane 4; 2D, Lane 5), but not with each other (Fig. 2C, Lane 5; Fig. 2D, Lane 4) or with any member of group I (Figs. 2C-

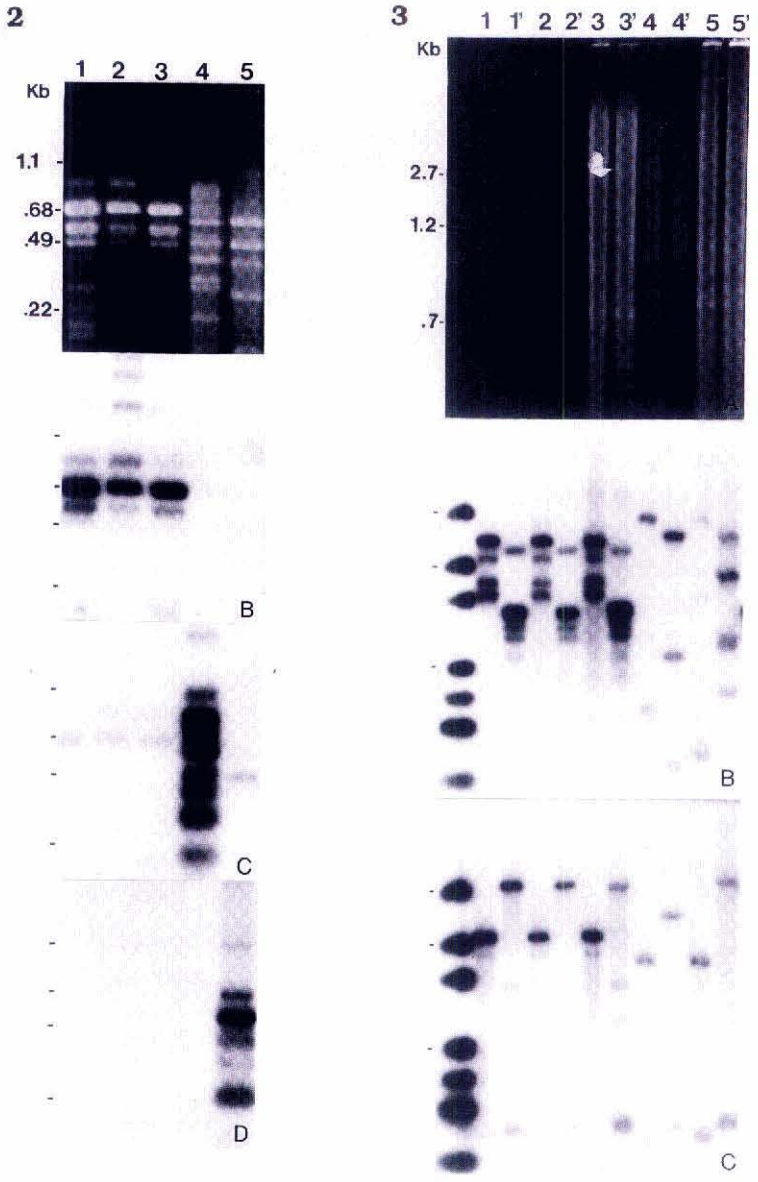

Figure 2. Kinetoplast DNA heterogeneity of 5 Indian *L. donovani* isolates. kDNAs were digested with HaeIII and electrophoresed on 1.5% agarose. A, EtBr staining; B-D, The gel from A was blotted and probed with kDNAs from DD8, AG and UR6 successively after removing the previous probe. MW markers, pGEM fragments; Lane 1: DD8, Lane 2: RMRI, Lane 3: SS, Lane 4: AG, Lane 5: UR6.

Figure 3. Heterogeneity of 5 Indian *L. donovani* isolates at gp63 and β-tubulin chromosomal loci. The nDNAs were digested with Pst I (Lanes 1-5) and Pvu II (Lanesl'-5'), electrophoresed on 0.75% agarose and Southern transferred. A, EtBr staining; B and C, Gel from A was blotted and probed successively with gp63 gene and β-tubulin gene after removing the previous probe.

D, Lanes 1-3).

The grouping of Indian isolates based on their kDNA heterogeneity is further supported by their nDNA polymorphisms at the chromosomal loci of 2 multigene families. The total nDNAs were cut to completion by either PstI (Lanes 1-5) or PvuII (Lanes 1'-5') (Fig. 3A). Southern hybridization of these digested nDNAs with gp63 and β-tubulin genes (Figs. 3B-C) revealed mutiple bands of different intensity at both loci typical of multicopy gene families. Three banding patterns emerged from the 5 isolates which corresponded exactly to the 3 kDNA types, i. e. DD8, RMRI and SS as type I (Figs. 3B-C, Lanes 1-3 and 1'-3'), AG as type II (Lanes 4 and 4') and UR6 as type III (Lanes 5 and 5').

DISCUSSION

In the present study, 5 *Leishmania donovani* isolates from northeastern India were grouped into 3 genotypes. Previously, heterogeneity of Indian *Leishmania* isolates has been recognized on the basis of different criteria, e. g. kDNA minicircle sequence heterogeneity alone (Barker 1989; Dasgupta et al. 1991), isoenzyme mobility and serologic differences of excretory factors (Schnur et al. 1981). Our grouping is based not only on the hybridization of their kDNA minicircles but also on the restriction fragment length polymorphism of 2 nuclear gene families. Identical conclusions derived from the study of both kDNA and nDNA lend credence to this grouping. Although the genomic sites examined are limited, they all contain genes of functional significance and thought to contribute directly or indirectly to virulence (Chang et al. 1990). The kDNA minicircles produce transcripts crucial for RNA editing and may undergo profound changes or "transkineto-plastidy" under certain selective conditions (Lee et al. 1992). It awaits further study of additional Indian isolates to determine if we can relate the differ-ences among the 3 genotypes at these sites to their clinical or epidemiologic phenotypes. Of particular interest is UR6, which is pentamidine-resistant in vitro (unpublished observations) and resembles isolates from PKDL (Dasgupta et al. 1991).

The three type I isolates (DD8, RMRI and SS) were all from patients native to central Bihar, while type II and III were from the Calcutta metropolitan area of West Bengal (Table 1). Homogeneity of *Leishmania* isolates from Bihar vs. heterogeneity of those from the West Bengal has been noticed previously (Schnur et al. 1981). Possibly, the presence of genetically heterogeneous isolates in the latter case might have resulted from their introduction into the metropolitan Calcutta from the adjacent areas, especially Bangladesh.

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