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Cloning, sequencing and BLAST analysis of kDNA minicircles from *Leishmania donovani* AG83.

KEYWORDS: kDNA minicircle, Leishmania donovani, infectious clone, gRNA, sequence variation.

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ABSTRACT *kDNA in kinetoplastid microbes are unique due to presence of few 30kb maxicircle mitochondrial DNA inter-catenated with 10,000 of ~0.8-1.4kb minicircle plasmids. kDNA minicircles from Leishmania donovani AG83 was digested with PstI restriction enzyme and was cloned into pBluescriptSK+ plasmid vector. The cloned minicircle DNA was sequenced by di-deoxy method giving 837bp circular sequence. BLAST analysis suggested the infectious clone minicircle was very similar conserved UMS sequence involved in DNA replication but it had very canonical TATA-box and gRNA sequences involved in ND2/ND3 gene editing. Major sequence variations with respect to UR6 were found between nucleotides 31-41 and 434-444. We are first to show kDNA minicircle sequence of any infectious clone of Leishmania of Indian origin.*

Introduction: Human leishmaniasis is caused by *Leishmania donovani* or related species of single cell parasites containing unusual mitochondrial DNA (kDNA) containing thousands of ~0.85kb minicircles and few ~30kb maxicircles hold by extensive catenation and such DNA structure was not found in any eukaryotes (Jensen & Englund, 2012; Chen et al., 1995). The *Leishmania* present as flagellates promastigote form (fig 1, panel A, upper) inside the sand fly vector but inside human it stays as non-flagellate amastigote form (fig 1, panel A, lower) (Chakraborty, 1990). The unusual structure of kDNA needs novel DNA topoisomerases (Chakraborty et al., 1994; Bodley et al., 2003; Tang & Shapiro, 2010) that decatenate kDNA (fig 1, panel B) into free circles (fig 1, panel C) and after replication the progeny circles were back catenated by DNA catenating enzymes (Chakraborty AK & Majumder HK 1987, 1991, 1999). The similar kinetoplastid parasite, *Trypanosoma brucei* or related species also causes fatal diseases in human and animal promoting an extensive research on those unicellular microbes. The trypanosomes contain few chromosome pairs (~30, ~2000kb) but more than 100 mini-chromosomes (50-500kb) were detected (Van der Pleogi et al., 1994). Those mini-chromosomes were involved in recombination creating antigenic variation of surface glycoproteins (Hom D, 2014). Polycistronic mRNA was transpliced (Sadhukhan et al., 1997) and also edited extensively by minicircle-coded guide-RNA creating novel proteins, not coded directly by DNA itself (Avila & Simpson, 1995, Shaw et al., 1988; Kim et al. 1994; Clement et al., 2004). So we are interested to characterize the minicircle DNA of *Leishmania donovani* AG83 infectious parasites hoping there could be difference with that of non-infectious clone, UR6.

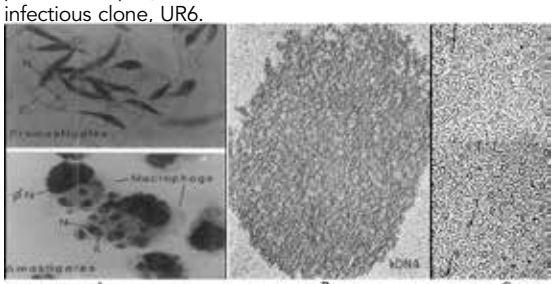

Fig. 1 Structures of *Leishmania doovani* and its kDNA. Panel A, upper is *L. donovani* promastigotes that grow at 26°C *in vitro* or in sand fly vector and panel A, lower is *L. donovani* amastigotes in macrophages that grow at 37°C. Panel B is a electron microscope picture of kDNA network (mitochondrial DNA complex of *Leishmania*, 15000x) and panel C is electron microscopy of decatenated kDNA by DNA topoisomerase II showing minicircles at 30,000x [Chakraborty, 1990; Chakraborty & Majumder, 1999].

Material and Methods: kDNA was kindly provided by Dr H. K. Majumder and *L. donovani* parasite was kindly provided by Dr S. Roy (CSIR-IICB, Kolkata). Agarose, Ethidium bromide, PstI restriction enzyme, T4 DNA ligase were purchased from SRL. Bactro-Tryptone, Yeast extract and Bactro-agar from HiMedia. The very motile parasites were grown in Brain-Heart Infusion medium at 26°C for three days before isolation of DNA. About 2µg kDNA was digested with 10 unit of Pst I at 37°C for 4 hrs. The digested DNA was loded onto 1% agarose gel containing 0.5µg/ml EtBr and ran gel in 1xTAE buffer at 50V for 1.5hrs. The visible 0.85kb band was excised from gel and purified by gel extraction kit ( ). The ligation reaction contained (20µl) 5µl kDNA minicircle, 5µl CIP+PstI treated plasmid, 2µl 10x Ligation buffer, 1 unit T4 DNA ligase and reaction continued at 16°C for over night. *Escherechia coli* DH5α cells were grown to 0.4 OD_{600}nm and washed with 0.1M $CaCl_2$ to make Ca^{+2} –cells (Chakraborty et al., 1993). The 30µl competent cells were incubated with 5µl ligated DNAs and heat shocked at 42°C for 1 min. and 300µl SOC media was added and shaked for 1 hr at 37°C. 100µl of the transformed bacteria was then plated onto 1.5% LB-Agar plate containing 100µg/ml ampicillin and was incubated for over night at 37°C. The individual colonies were picked up into 3 ml LB medium containing drug and miniprep DNA was isolated by alkaline lysis method (Chakraborty, 2015).

Result: kDNA minicircles were heterogeneous in sequence as EcoRI (lane 2), PstI (lane 3) and SalI (lane 4) had linearized varying degree of minicircles (fig 2, panel A). kDNA minicircles with SalI cut sequence had minimum in *L. donovani* AG83 (fig. 1, panel A, lane 4). Also none of the enzyme could digest all catenated minicircles in free form as reported earlier (Singh et al., 1995). When recombinant plasmids were digested with PstI enzyme, all the four recombinant gave a 0.86kb minicircle band

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(fig1, panel 2, lanes 1-4). The sequence of the clone pLdkP3 was indicated that it was 837bp and contained conserved UMS sequence (GGGGTTGGTGTA, underlined in fig. 3; Ray, 1989) and canonical TATA box (TATATAAT, boxed in fig. 3) and also gRNA sequences (Clement et al., 2004) at nucleotides 464-534 (fig. 3 and was shown by arrow). It has unique NdeI site (ca/tatg) at nt 510, EcoR1 site (g/aattc) at nt 655, ClaI site (at/cgat) at nt 772, PstI site (ctgca/g) at nt 828 and two Hae III sites (gg/cc) at 538 and 800 nucleotides (fig 2, Panel A, lane 5). BLAST analysis suggested it has 94% similarity with the minicircle clone from *Leishmania donovani* UR6 (fig 3). However, it was 70%-90% similarity to the minicircles DNA from other *Leishmania* species. As for example, the minicircles sequence similarities among the different species were: 87% similarity to *L. major* (AN:U51718), 77-83% to *L. infantum* (AN:AF1683357, AJ275334, KC536649, EU437406); 85% to *L. aethiopica* (AN:U77892), 83% to *L. mexicana* (AN:Z11549); 85% to *L. tarentolae;* 80% to *L. chagasi* (AF103739) and 83% to *L. amazonesis* minicircle DNA (AN:M94089) (data not shown).

Discussion. We have undertaken to clone the different populations of kDNA minicircles from *L. donovani* AG83 on the basis of hexa-cutter restriction enzymes like PstI, BamHI, SalI, HindIII, and NdeI and more, those have specific sequence preference and uniquely cut on the minicircle DNA only once. The approach was to get a pool of different kDNA minicircles sequences that might contain different functions involving gRNA, recombination and promoters (Srividya et al., 2012). This was our first attempt to clone and analyze the PstI digested minicirles (fig 2, 3). Such cloning from non-infectious clone (UR6) using different restriction enzymes have been reported in India (Bhattacharya et al., 1993). We compared the AG83 minicircle sequence with other species of *Leishmania*. Such studies may also give clues for species and strain specific primers for identification of clinical isolates in human blood and may through some highlights into virulence patterns in minicircle sequences particularly creating gRNA for surface glycoproteins in *Leishmania* which appears more complex than *Trypanosoma brucei* or *Trypanosoma cruzi (*Pandey et al., 2014; Vincent & Barrett, 2015). Very high copy number and heterogeneity in sequences also have been attributed to minicircle insertion in human blood cells during drug resistance leishmaniasis and certainly demands a careful study of all minicircles sequences in *Leishmania* and *Trypanosoma* (Romano et al., 2014; Gaunt et al., 2003).

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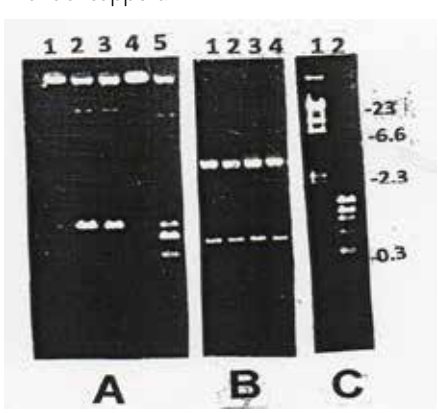

Fig. 2. Restriction analysis of kDNA network and minicircle clones. Panel A, kDNA (lane 1); plus EcoR1 (lane 2); plus PstI (lane 3); plus SalI (lane 4); plus HaeIII (lane 5). Panel B pLDkP1,2,3,4 clones digeted with PstI (lanes 1-4). Unique linearized 2.9kb vector and 0.85 kb minicircle DNA were shown. Panel C, Lane 1, λHindII marker DNA (23130, 9416, 6557, 4361, 2322, 2027, 564, 125 bp DNA) and lane 2, ϕx174+HaeIII marker DNA (1.4, 0,87, 0.6, 0.31, 0.28, 0.23 kb DNA) ran in a similar 1% agarose gel containing 0.5μg/ml EtBr and 1xTAE buffer.

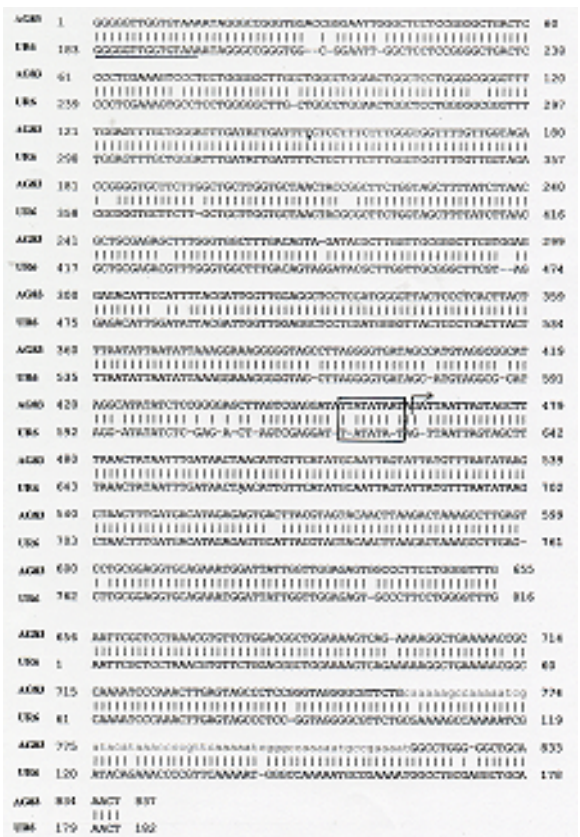

Fig.3. Sequence of pLDkmP3 minicircle DNA and BLAST sequence analysis. The plasmid DNA was purified from 10ml ON culture in LB media and purified by alkaline lysis method (Chakraborty et al., 1993). The DNA was further purified by Qiagen column. The di-deoxy sequencing was performed using T7 forward primer (5′-GTA ATA CGA CTC ACT ATA GGG CG-3′) and M13 reverse primer (5′-GGA AAG AGC TAT GAC CAT GA-3′) as described previously (Chakraborty, 2015). BLAST analysis was performed using NCBI database (www.ncbi.nlm.nih.gov/blast**).**

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REFERENCE

[1] Jensen RE & Englund PT (2012) Network news: the replication of kinetoplast DNA. Annu. Rev. Microbiol. 66:473-491. | [2] Chen J, Rauch, CA, White JH, Englund PT & Cozzarelli NR (1995) The topology of the kinetoplast DNA network. Cell 80:61–69. | [3] Chakraborty AK (1990) Molecular biological studies on kinetplastid hemoflagellate Leishmania donovani with reference to enzymes involved in nucleic acid metabolism. Ph. D. Thesis, Calcutta University. | [4] Chakraborty AK, Majumder HK & Hodgson CP (1994) DNA topoisomerase inhibitors: potential uses in molecular medicine. Exp. Opini. Ther. Patents. 4(6), 655-668. | [5] Bodley A., Chakraborty AK, Xie S, Burri C, Shapiro TA (2003) An unusual type IB topoisomerase from African trypanosomes. Proc. Natl. Acad. Sci. U.S.A. 100:7539-7544. | [6] Tang SC & Shapiro TA (2010) Newly identified antibacterial compounds are topoisomerase poisons in African trypanosomes. Antimicrob. Agents Chemother. 54:620–626. | [7] Chakraborty AK & Majumder HK (1987) Decatenation of kinetoplast DNA by an ATP-dependent DNA topoisomerase from the kinetoplast hemoflagellate Leishmania donovani. Mol. Biochem. Parasitol. 26:215–224. | [8] Chakraborty AK & Majumder HK (1991) An ATP-independent catenating enzyme from the kinetoplast hemoflagellate Leishmania donovani. Biochem. Biophys. Res. Commun. 180: 279–285. | [9] Chakraborty AK & Majumder HK (1999) Molecular biology of Leishmania: kinetoplaat DNA and DNA topoisomerases as novel therapeutic targets. Current Science 76(11):1462-1472. | [10] Chakraborty AK (2015) High mode contamination of multi-drug resistant bacteria in Kolkata: mechanism of gene activation and remedy by heterogenous phyto-antibiotics. Indian J. Biotechnol. 15:00 (in press). | [11] Chakraborty AK, Zink MA, Boman BM & Hodgson CP (1993) Synthetic retroposon vector for gene therapy. FASEB J. 7:971-977. | [12] Van der Ploegi LHT, Cornelissen AWA, Barry JD & Borst P(1984) Chromosomes of kinetoplastida, The EMBO J. 3 (13):3109-3115. | [13] Hom D (2014) Antigenic variation in African trypanosomes. Mol. Biochem. Parasitol. 195(2):123-129. | [14] Sadhukhan PK, Chakraborty AK, Dasgupta A & Majumder HK (1997) Chromatographic separation of DNA dependent RNA polymerases and molecular properties of RNA polymerase II from Leishmania Spp. Mol. Cellu. Biochem. 171:105-114. | [15] Ray DS (1989) Conserved sequence blocks in kinetoplast minicircles from diverse species of trypanosomes. Mol. Cell Biol. 9(3):1365-1367. | [16] Singh R, Siddiqui KA, Valenzuela MS, Majumder HK (1995) Kinetoplast DNA minicircle binding proteins in a Leishmania Spp: interference of protein interaction by berenil. Indian J. Biochem. Biophys. 32(6):437-441. | [17] Bhattacharya R, Singh R, Hazra TK, Majumder HK (1993) Application of polymerase chain reaction with specific and arbitrary primers to identification and differentiation of Leishmania parasites. FEMS Microbiol. Lett. 114(1):99-104. | [18] Avila HA, Simpson L (1995) Organization and complexity of minicircle-encoded guide RNAs in Trypanosoma cruzi. RNA 1(9):939-947. | [19] Shaw JM, Feagin JE, Stuart K & Simpson L (1988) Editing of kinetoplastid mitochondrial mRNAs by uridine addition and deletion generates conserved amino acid sequences and AUG initiation codons. Cell 53(3):401-411. | [20] Kim KS, Teixeira SM, Kirchhoff LV, Donelson JE (1994) Transcription and editing of cytochrome oxidase II RNAs in Trypanosoma cruzi. J. Biol . Chem. 269(2):1206-1211. | [21] Clement SL, Mingler MK & Koslowsky DJ (2004) An intragenic guide RNA location suggests a complex mechanism for mitochondrial gene expression in Trypanosoma brucei. Eukaryotic Cell 3(4):862-869. | [22] Srividya G, Kulshrestha, Singh R & Salotra P (2012) Diagnosis of visceral leishmaniasis: developments over the last decade. Parasitol Res. 110(3):1065-1078. | [23] Pandey K, Pandey BD, Mallik AK, Acharya J, Kato K et al. (2014) A new molecular surveillance system for leishmaniasis. Am. J. Trop. Med. Hyg. 90(6):1082-1086. | [24] Vincent IM & Barrett MP (2015) Metabolomic-Based strategies for anti-parasitic drug discovery. J. Biomol. Screen. 20(1):44-55. | [25] Romano A, Inbar E, Debrabant A, Charmoy M, Lawyer P et al. (2014) Cross-species genetic exchange between visceral and cutaneous strains of Leishmania in the sandfly vector. Proc. Natl. Acad. Sci. U S A. 47:16808-16813. | [26] Gaunt MW, Yeo M, Frame IA, Stothard JR, Carrasco HJ et al. (2003) Mechanism of genetic exchange in American trypanosomes. Nature 421(6926):936-939. |