Arch. Inst. RAZI (1998) 48-49

Sequence Heterogeneity in Kinetoplast Minicircle DNA of Leishmania Major

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Summary

A partial DNA library of kinetoplast DNA (kDNA) from *leishmania major* (MRHO/IR./64/Nadim-1) was prepared in Bluescript plasmid. Using Dot blot and Southern blot analysis against three different *leishmania* strains, *L. infantum, L. major* and *L. tropica*, one of them (IRI-m1), showed to be specific for *L. major* (MRHO/IR./64/Nadim-1) and the other, IRI-m2 hybridized with *L. infantum* as well as *L. major*. Both of these DNA fragments were sequenced. It was shown that these two fragments possess only 55% sequence homology. These finding denote that heterogeneity of minicircle DNA exist in a single species.

Key words: *Leishmania*, Kinetoplast DNA, Minicircle, DNA probe, Riboprobe.

Introduction

Leishmania are protozoan parasites of mammals that have two distinct life stages. They exist as extracellular promastigotes within the gut of the sandfly vector and are introduced into the mammalian host when the infected insect take a blood meal. Subsequently, promastigotes are engulfed by mononuclear phagocytic cells, such as macrophages, and then the promastigotes convert into their intracellular amastigote form (Read and Chandler, 1961). These protozoan parasites are responsible for cutaneous and visceral Leishmaniasis in man (WHO, 1990). Cutaneous Leishmaniasis has caused serious health problems, fatalities and economic losses world wide. It is endemic in some countries such as Iran, Afghanistan, Brazil, India and many South American and African zones. Cutaneous Leishmaniasis is subdivided into two major categories: 1) anthroponotic cutaneous Leishmaniasis and 2) zoonotic cutaneous Leishmaniasis.

Differentiation of cutaneous *Leishmania* species depends on different factors such as ecology, biology, biochemistry and immunology of the parasite (Wilson 1995) and it is important for prognosis and correct chemotherapeutic agents as well as the epidemiological studies of the disease (Lawire 1985) So far no standard method for species differentiation for the genus *Leishmania* existed. For the last decade and a half, DNA based techniques have been used to differentiate the *Leishmania* species using kinetoplast DNA as the template (Lopes *et al.* 1984, Sheline and Ray 1989, Kessler 1992).

Leishmania mitochondrial DNA, unlike other eukaryotic cells is organized in specific topological DNA molecule named kinetoplast DNA (kDNA)(Simpson *et al.* 1990). Kinetoplast consists two distinct DNA molecules. They are both circular DNA encircled together, individually or in combination. Large circles or maxicircle DNA of 40-50 kb in size and 30-50 in number encode for mitochondrial enzymes (Simpson, *et al.* 1980, Cruzie *et al.* 1984, Simpson 1987). Minicircle DNA consists of smaller circles of 0.5-2.9kb (2.9kb minicircles are known in Crithidia) and there are approximately 10,000 per kinetoplast molecule (Simpson *et al.* 1994) which confirm a network (Cheng and Simpson 1978, Englund 1979). Coding for small guide RNA (gRNA) is their only known biological function. gRNA, in turn is involved in mitochondrial RNA editing phenomena (Sturm and Simpson 1991, Benne 1993, (Simpson *et al.* 1994, Theimann *et al.* 1994, Kulesikov 1994, Gutrerres-Solar *et al.* 1995). Minicircle DNA sequences are conserved in minicircle sequences in both promastigote and amastigote forms of the parasite (Simpson 1987, Simpson *et al.* 1994).

Due to rather high numbers and the conserve sequences of minicircles, they have been selected as good targets for differentiation studies of *Leishmania* species using molecular biology techniques (Lawire *et al.* 1985, Bozza *et al.* 1995).

Minicircle sequences vary between parasite species and between minicircles of same kinetoplast molecule (Simpson *et al.* 1980, Simpson 1987). In this study we have shown that there are at least two different kDNA with 55% homology.

Materials & Methods

Parasite: The *Leishmania* strains used in this study are *Leishmania major* (MRHO/IR /64/Nadim-1), *Leishmania infantum* (MHOM/IR/74/Edrissian) and *Leishmania tropica* (MHOM/SO/58 OD strain).

Medium: N.N.N medium was used for parasite maintenance and cultured subsequently in RPMI 1640 (Rosewell Park Memorial Institute) medium supplemented with 10% heat inactivated fetal calf serum and 100U penicillin per ml (Osland *et al.* 1992).

kDNA extraction: Barker's method was used for lysate preparation and kDNA extraction (Barker *et al.* 1985). Briefly, 10^9 promastigotes were harvested, transferred to cortex tubes and spun at 8500×g for 1 min. Supernatant was decanted and pellet of 10^8 promastigotes was suspended in 1 ml of NET 100 buffer (10mM Tris pH 8, 10mM NaCl and 100 mM EDTA pH 8). This stage was repeated and the pellet was suspended in 300μl of 10% sarkosyl and 700μl of NET 100 buffer (for the whole of 10^9 promastigotes). Lysate was incubated at 60°C for 1hr followed by adding 20μg/ml Proteinase K and incubating for 30min at 55°C. This was spined at 16000×g for 1 hr kDNA was extracted by phenol chloroform isoamyl alcohol (P.C.I) and precipitated by ethanol.

Elution of DNA fragment from agarose gel: For elution of DNA fragments from agarose gel, Tautz and Renz method was used (Tautz and Renz 1993). Briefly, DNA fragments were electrophoretically separated, stained by ethidium bromide (2 μg/ml) and DNA visualized with UV light (366 or 254 nm). Precipitated bands were cut out and intact gel slices were diluted in 2 volume of TE buffer (10mM Tris pH 8, 1mM EDTA pH 8) and incubated in 68°C water bath for 5min then followed by P.C.I extraction and ethanol precipitation.

kDNA digestion and palsmid DNA dephosphorylation: 10μg each of kDNA and pBluescript were digested using BamHI restriction enzmye for 2hrs at 37°C. Digested plasmid was dephosphorylated using alkaline phosphatase (Sambrook *et al.* 1989) and was used in a cloning along with *L. major* kDNA using T4 DNA ligase (Dugaiozyk *et al.* 1975, Gastra and Hansen 1984).

Transformation and colony screening: *E. coli* XL1Blue [F (pro AB+lacz ΔM15 Tn 10 (tet^r) (Brown 1994). Competent cell was prepared by Hanaham method (Hanaham 1983), then transformed with 50 ng of ligated DNA and grown on LB agar plate containing X-gal and IPTG (Sambrook *et al.* 1989). Ten randomly selected white colonies [containing recombinant plasmid] were grown in 5 ml of LB broth containing ampiciline and tetracycline for over night.

Plasmid DNA preparation: Plasmid DNA was extracted using Alkaline method (Sambrook *et al.* 1989). The extracted DNA was screened by agarose gel electrophoresis for quality and cut by BamHI.

labeling: The inserts from restriction enzyme reaction were separated on 1.5% agarose gel (Tautz and Renz 1993). DNA was recovered and used for DNA labeling method according to manufacturer guide (Boehringer Mannheim Biochemica 1994). Briefly, 1μg of DNA was incubated at 37°C over night in 20μl reaction mixture

containing 2μl of hexanucleotide, 2μl of dNTP Digoxygenin labeling mixture and 1μl of klenow enzyme. Probe was ethanol precipitated and quantitated using dot blot on Pall Biodyne nylon membrane and compared to control labeled DNA. RNA probes were prepared by Run off Synthesis method (Sturzl and Rothman 1990). One μg of linearized recombinant plasmid was incubated at 37°C for 2hrs in 20μl reaction mixture containing 2μl of NTP Digoxygenin labeling mixture 2μl of interested RNA polymerase and 1μl of Rnasine. Probe was ethanol precipitated and quantitated using dot blot on Pall Biodyne nylon membrane and compared to control labeled RNA. T7 and T3 promoters are located one on either side of pBluescript multiple cloning site (MCS), when each of them become activated by suitable RNA polymerase, RNA will be transcribed from DNA that had been cloned in plasmid multiple coloning site (Sambrook *et al.* 1989, Barton *et al.* 1991).

Hybridization: Both RNA and DNA probes were used in Dot blot and Southern blot hybridization (Southern 1975) studies by spotting kDNA extracted from *L. major, L. infantum* and *L. tropica* on the nylon membrane (Pall *et al.* 1989). The nylon membrane was given 120,000 mj in UV cross linker and placed in a plastic bag (Darling and Briekell 1994) and treated with 2 ml/100 cm^2 of prehybridization buffer (Barker *et al.* 1985). Plastic bags were sealed and incubated for 1 hr at 42°C for DNA probe and 50°C for RNA probe. The required amount of boiled DNA probe or RNA probe (approximately 20-50 ng) were added to hybridization buffer (Barker *et al.* 1985) and replaced immediately with prehybridization buffer and incubated at 42°C for DNA probe and 50°C for RNA probe over night. The membranes were washed and followed by color development according to manufacturer's Digoxygenin guide line (Sturzl and Kurt Rothman 1990).

Sequencing: Sanger chain terminating inhibitors method was used (Sanger *et al.* 1977).

Results

kDNA library: Partial DNA library from *L. major* was prepared using BamHl restriction enzyme. All digested kDNA were cloned in pBluescript. The cloned samples were screened against kDNA from different *Leishmania* species, *L. tropica, L. major* and *L. infantum* and two clones were obtained, IRI-m1 and IRIm-2 were selected in respect to their low cross reactivity with other strains. IRI-m1 with 800 bp and IRI-m2 with 850 bp size is shown in Fig. 1. Since the size of a complete minicircle DNA in *L. major* is between 800 to 1300 bp, IRI-m1 and IRI-m2 can cover most of minicircle in this species. *L. tropica* and *L. infantum* were digested with EcoRl restriction enzyme and cloned into pUC18 then used for control. The third lane in Fig. 1 is one possessing a 250 bp size from *L. tropica.*

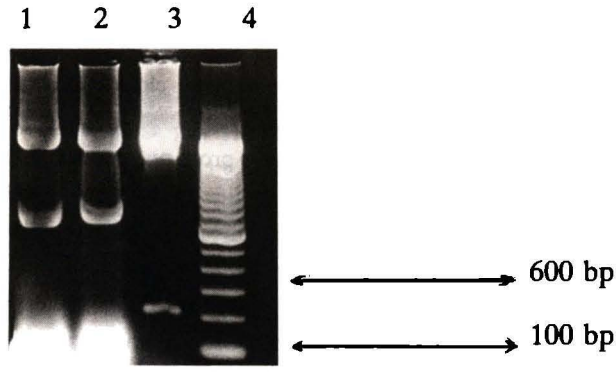

Fig. 1: 1% agarose gel electrophoresis of recombinant plasmids (after digestion by BamHI restriction enzyme). Lane 1,IRI-m1 showing an insert of 800 bp in size; Lane 2, IRIm-2 showing an insert of 850 bp in size; Lane 3, a 250 bp clone of *L. tropica* kDNA. lane 4, 100 bp ladder.

Hybridization : The IRI-m1 hybridized strongly with pBluescript possessing the 800 bp kDNA and showed a positive control capacity for hybridization experiment. IRI-m1 bonded strongly with 2 and 20ng while hybridized weakly with 200pg kDNA template (Fig. 2 lane 9). This probe also hybridized at a lower density with IRI-m2 at 20ng (Fig. 2 lane 1) as well as with kDNA template derived from *L. tropica*(Fig. 2 lane 3).Using different kDNA template, the best hybridization results were obtained with kDNA from *L. major* (Fig. 2 line 9). Thus this probe (IRI-m1) seems to be more specific.

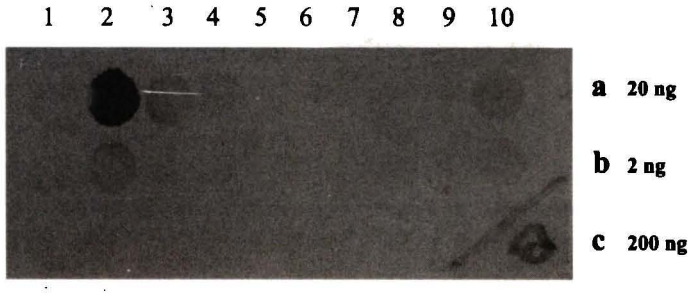

Fig. 2: kDNA dot blot hybridization with IRI-m1 probe

Lane 1,A pBluescript clone possissing a fragment of *L. major* kDNA (IRI-m2).

Lane 2, A pBluescript clone possissing a fragment of *L. major* kDNA (IRI-m1).

Lane 3, A pUC18 clone possessing a fragment of *L. tropica* kDNA.

Lane 4, A pUC18 clone possessing a fragment of *L. tropica* kDNA.

Lane 5, A pUC18 clone possessing a fragment of *L. infantum* kDNA.

Lane 6, A pUC18 clone possessing a fragment of *L. infantum* kDNA.

Lane 7, A pUC18 clone possessing a fragment of *L. infantum* kDNA.

Lane 8, *L. tropica* kDNA.

Lane 9, *L. major* kDNA.

Lane 10, *L. infantum* kDNA.

The experiment was confirmed by using Southern blot hybridization technique (Fig. 3). Three different kDNA templates from *L. infantum, L. major* and *L. tropica* was subjected for hybridization with IRI-m1 probe. The resulted band represents a specific banding capability of this probe with *L. major* only (Fig. 3).

Fig. 3: kDNA Southern blot hybridization by IRIm-1 Riboprobe.
Lane 1, *Leishmania infantum* kDNA.
Lane 2, *Leishmania major* kDNA.
Lane 3, *Leishmania tropica* kDNA.

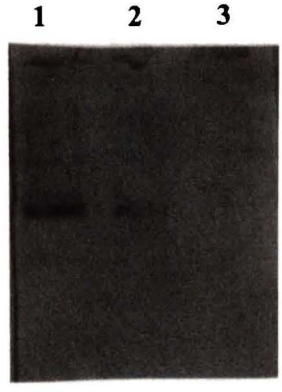

Fig. 4: kDNA Southern blot hybridization by IRIm-2 Riboprobe.
Lane 1, *Leishmania infantum* kDNA.
Lane 2, *Leishmania major* kDNA.
Lane 3, *Leishmania tropica* kDNA.

IRI-m2, is hybridized not only with *L. major* but also with *L. infantum* with less specificity (Fig. 4). Three kDNA templates from *L. infantum, L. major* and *L. tropica* were subjected to hybridization. The probe hybridized with kDNA from *L. infantum* or either its origin, *L. major*. Indeed it represents that there are at least some common minicircles among different species of *Leishmania*. Meanwhile, this probe didn't hybridized with *L. tropica* (Fig. 4 line 3). This finding could be due to similarity between kDNA sequences among different species.

Both IRI-m1 and IRI-m2 were sequenced. The sequencing data are shown in Fig. 5 and Fig. 6. A total of 659 bp of IRI-m1 probe was sequenced and compared with a Genebank data. The maximum similarity, 51% was observed with a clone of *L. tarentolae* minicircle (Kidan *et al.* 1994). The GC residues were 51.9% with no open reading frame (ORF).

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1   GGATCCCCCA GATATATAAT ATATTCTATT ATCTAACTCC ATCCCCATAG
51  CTATGGGTTA GGAGGATACC CAGGCCAGGG CAATCAACAC AGGTCTACAG
101 CCATGATCGT ATAGGACCAA GGAGCTGAA  ACCCCACAAG CCAAAGAATC
151 AAAGCGAGCA AGGATCGGTA GGCCAACCGG GCACCAGAAT CAAACCTCCG
201 GGCCCACAAT CACGATACCT AGCCGACCTC CGGCCTGATC GTCCGGATAT
251 TCCTCCGGCG ATCTGGAACG ATATATAATA TATCTATTAT CTAACTCCAT
301 CCCCATAGCT ATGGGTCTAG AGGATACCCA GGCCAGGGCT ATGAACACAG
351 GTCTACAGCC ATGATCGTAT AGGACCAAGG AGCGTGAAAC CCCACAAGCC
401 AAAGAATCAA ATGCGAGCAA GGATCGGTAG GCCCAGGCCA GGGCTATCAA
451 CACAGGTCTA GCCATGATCG TATAGGACCA AGGAGCGTGA AACCCCACGA
501 AGCCAAAGAA TCAAAGCGAG CAAGGATCGG TAGGCCAACC AGGGTCATCC
551 AGAATCAAAC CTCCATGGCC CAAGGATCGG TAGGCCAACC AGGGTCATCC
601 CCAACACAAG GGTTCCCTCA CGACAGGGTT AGAGCGATCA GGTTGTGTTG
651 GCGGCGGGG
```

Fig. 5: IRI-m1 sequence

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1   GATCCAATAA GTCCTATGCA TAAGAGCCTA GGCGCAACAG ACTAGGTATA
51  CATCAAGCTC AGACGCCAGT CAAAGACCCT AGAACTCGAG AGCCTAGAGC
101 CTACCNNCCC GGGGATAAGT CCAGTAACTA AGAGCCAAGG TCCGCACAAC
151 AAAGACTAGG TATAACTCCA ACCTAAGCCT ACAGAGCCCA GTCAAAAGAC
201 CGCTAGACCA TGCGAGCCTC ACCAGAGTAC AGACGCCTAG CAGACACGGA
251 AGCACGAAGA CGTGACTGAC GACCGCATAC TGTTAGTCCC ATCATGACGT
301 AACCGAAGCC TGAACGGCCC GACAACAAGA AGCATAGGTA AACCGAAGCC
351 AGGCCTCCAG CAGCCAGTCC TAGAAAGCCA CCTAGACCCA TCGAGCCAGC
401 CTCAACCCAG AGTCAGCCAC AAGCTCAGAG TCACCAGAGA CCGCACAGCA
451 GCAGCCAGTC CAAAAAGCCA CGCTAGACCC AAGTTCCCCT GAGTCCAGCC
501 CTCGAAACCC AGAGTCAAGC CCAAAAAGAC CTCAGAAGTC CCACCAAGAG
551 CCAGCAACCC GGTTCCAGAC TGGTCCCAGA TGGAACCAGT TCTAGGCAGA
601 GCCACGGAGA TCCGATCAGG ACACCCATAT TCCACCGTTG AGCTGGCATG
651 TTGAGATTAG GTTGTAGTAT GAGTTGTATG ATCATGATTC TAG
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Fig. 6: IRI-m2 sequence

From the second probe, IRI-m2, 693 bp was sequenced and compared as above with Genebank. The maximum homology was 52% with a clone of *L. tarentolae* minicircle . (Kidan *et al.* 1994). The percentage of the GC residues in this sequence was 53.2%. There was no ORF in this sequence, too. Regarding with this finding both sequences show that in fact minicircles are not encoding for any proteins. The comparison of the two sequences showed 55% homology. This is the highest homology found with *L. major* presented here and upto date published data. These findings reflect that there are 45% heterogeneity among minicircles of *L. major*.

Discussion

For differentiation of *Leishmania* species, kDNA RFLP (Restriction Fragment Length Polymorphism) pattern (schizodeme analysis) is a more accurate method in comparison with mixed isoenzymatic pattern (used for *Leishmania* characterization). Since changes occur in the characters of the axenic cultures during long term maintenance (Simpson and Silva 1971, Solari *et al.* 1992) schizodeme analysis alone or in combination with other techniques of DNA analysis (Van Eys *et al.* 1991) has been shown to be very useful in the characterization of several trypanosomatids including *Leishmania*. Lopes and his workers in 1984 reported some variations using endonuclease Msp I, in the profiles among the strains which had been typed as being identical by enzyme electrophoresis.

In this paper we have shown at least two classes of minicircle in *leishmania major* a) IRI-m1 specifically found in *L. major* and b) IRI-m2 is common between *L. major* and *L. infantum*. These findings represent a heterogeneity among minicircle kDNA sequence of *L. major* as shown previously by Pacheco *et al.* (1990), they showed the heterogeneity among clone of New World *Leishmania* species by schizodeme analysis. While Simpson *et al.* (1980) and Kidan *et al.* (1994) showed the heterogeneity in *L. tarentolae* minicircle sequence.

Dot blot hybridization of IRI-m1 with three *Leishmania* kDNA shows that this probe is hybridized strongly with total kDNA of *L. major* and hybridized with lower density with total kDNA of other *Leishmania*, this is due to sequence homology between some of minicircles kDNA (Wirth and McMahon Pratt 1982, Lawire *et al.* 1985, Sheline and Ray 1989) and it could possibly also be a technical problems either the dot blot is not specific or due to the GC rich sequence.

Southern blot hybridization shows specificity for *L. major* with IRI-m1 and does not

cross hybridize with other *Leishmania* kDNA. This minicircle in *L. major* is comparable with that of *L. donovani* reported by Gupta previously (Gupta *et al.* 1991).

The IRI-m2 probe hybridized strongly with *L. infantum* kDNA compared to *L. major* kDNA; it is possiblly due to more numbers of this minicircle class in *L. infantum* kDNA than of *L. major* kDNA. Some of minicircle kDNA are unique for species (like IRI-m1) and some others are homologous to other *Leishmania* kDNA (like IRIm-2) (William and Wirth 1987). In this study we showed different hybridization patterns by probes as it was done by Lawire *et al.* (1985), William and Wirth (1987). They constructed a DNA library of the enzyme digested *L. mexicana amazonensis* kDNA with three subfragments sufficient to different taxonomic subspecies and species specific.

Lu Hong-gang and Hu Xiao-su (1990), reported 59-79% sequence homology between whole minicircle of *L. donovani*. While, previously Jackson *et al.* (1984) had demonstrated homology between kDNA sequence by hybridization with *L. major*, *L. tropica*, *L. chagasi*, and *L. infantum* as we found in IRI-m2 which is common between *L. major* and *L. infantum*. Eichelman *et al.* (1988) compared several lizard *Leishmania* species in term of minicircle kDNA sequence. They reported several different minicircle sequence classes within a single strain yielding fragment. Borst and Fowler (1980) had shown sequence heterogeneity of the minicircle of *Trypanosoma brucei* by digestion of kDNA with a number of restriction endonucleases.

Our experiments show that Riboprobe is more specific than DNA probe because DNA probes are prepared by random prime of hexanucleotides and cause labeled DNA with unequal different sizes (Boehringer Mannheim Biochemica 1994). But Riboprobe synthesized the same template DNA size (Kessler 1992).

In summary, according to DNA sequencing and hybridization analysis it was shown that there are at least two minicircle classes in *Leishmania major* kinetoplast DNA and 55% homology existing between these two sequences. One is specific for *L. major* and the other is found in *L. infantum*.

Acknowledgements

Thanks to the personnel of Biotechnology Dept. at Pasteur Institute of Iran in particular Mr. R. Golbari, Dr. R. Tehrani, Mr. A. Ghadiri, Miss. S. Dilmaghani, and Mrs. Z. Moghaddam for their support.

References

Barker DC, Butcher G, Gibson LJ, Williams RH. (1985). Characterization of *Leishmania* by DNA hybridization prbes. A Laboratory manual W H. O/TDR.

Barton PJR, Valis WJ, Lyons GE, Brand NG, Yancoo BM. (1991). RNA detection methods in basic cardiac research. In Genetic manipulation, edited by Grange JM, Fox.A and Morgan, L. pp:265-272.

Benne R. (1993). RNA editing, an overview. In RNA editing, the alteration of protein coding sequences of RNA. Edited by banne. R. pp:13-24.

Boehringer Mannheim Biochemica (1994). The DIG system user's guide for filter hybridization. pp:31-46.

Borst P, Fowler. (1980). Variation in maxicircle and minicircle sequence in kDNAs from different *Trypanosoma brucei* strain. Biochemica et Biophysica Acta 610:197-210.

Bozza M, Fernandes, O Degrave WM, Lopes UG. (1995) Characterization of old world *Leishmania* species. Using amplified minicircle variable regions as molecular probe. Transactions of the Royal Society of Tropical Medicine and Hygiene. 89:1-2.

Brown TA. (1994). DNA sequencing. IRL Press. pp:7-25.

Cheng D, Simpson L. (1978). Isolation and characterization of kDNA and kRNA of phytomonas davidi. Plasmid 1:297-313.

Cruz DVF, Neckelman N, Simpson L. (1984). Sequences of six genes and sveral ORF in the kinetoplast minicircle DNA of *Leishmania tarentolae*. The Journal of Biologicl Chemistry. 259:15136-47.

Darling DC, Biekell PM. (1994). Nucleic Acid Blotting, The basics. IRL Press pp:37-38.

Dugaiozyk A, Boyer HW, Goodman HM. (1975). Ligation of EcoRl endonuclease-generated DNA fragments in to linear and circular structures. The Journal of Molecular Biology. 96:171-184.

Eichelman MCG, Holz D, Simpson A, Simpson L. (1988). Comparison of several lizard *Leishmania* and strains in terms of kinetoplast minicircle and maxicircle DNA sequences, nuclear chromosomes and membrane lipids. Molecular and Biochemical Parasitology. 27:143-158.

Englund PT. (1979). Free minicircle of kinetoplast DNA in *Crithidia fasciculata*. The journal of Biological Chemistry. 254(11):4895-4900.

Gastra W, Hansen K. (1984). Ligation of DNA with T4 DNA ligase. In Methods in molecular biology vol, 2. Nucleic acids. Edited by John M. Walker pp:225-230.

Gupta ASD, Ghosh DK, Majemder HK. (1991). A cloned kinetoplast DNA minicircle fragment from a *Leishmania* spp. specific for Post-Kala-Azar-Dermal Leishmaniasis strains.Parasitology. 102:187-191.

Gutierres-Solar B, Smyth AJ, Alvar J, Barker DC. (1995). *Leishmania infantum* : Sequence homology within minicircle classes regardless of geographical distance. Experimental Parasitology. 81:416-419.

Hanaham D. (1983). Studies on transformation on *E. coli* with plasmids. The Journal of Molecular Biology. 166:557-580.

Jackson PR, Wohleiter JA, Jackson JE, Sayles P, Diggs CL, Hockmeyer WT. (1984). Restriction endonuclease analysis of *Leishmania* kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. American Journal Tropical Medicine and Hygiene. 33(5):808-819.

Kessler C. (1992). Non-radioactive labeling methods for nucleic acids.In non isotopic DNA probe techniques. Edited by Krieka LJ. pp:30-92.

Kidan G, Hughes D, Simpson L. (1994). Sequence heterogeneity and anomalous electrophoresis mobility of kDNA from *Leishmania tarentolae*. Gene 27:265-277.

Kulesnikov AA. (1994). *Leishmania gymenodactyli* and *Leishmania infantum* minicircles contain the same gRNA genes as do minicircle of *Leishmania tarentolae*. Molecular and Biochemical Parasitology 67(1):171-174.

Lawire JM, Jackson PR, Stiteler M, Hockmeyer WT. (1985). Identification of pathogenic *Leishmania promastigote* by DNA hybridization with kDNA cloned into *E. coli* plasmid. American Journal of Tropical Medicine and Hygiene 34(2):257-265.

Lopes UG, Moomen H, Grimaldi GJR, Morzochi MCA, Pacheco RS, More CM. (1984). Schizodeme and zymodeme characterization of *Leishmania* in the investigation of foci of visceral and cutaneous Leishmaniasis. J. Parasitology 70(1):89-98.

Lu Hong-gong and Hu Xiao-su. (1990). Identification of *Leishmania* by kinetoplast DNA minicircle and cloning of minicircle. Chinees Medical Journal 102:187-191.

Osland A, Beyene D, Shenafi S, Beetsma A. (1992). Identification and characterization of recombinant antigens from *Leishmania etiopica* that react with human antibodies. Infection and immunity. 60(4):1364-1374.

Pacheco RS, Grinmaldi G, Momen H, Morel CM. (1990). Population heterogeneity among clones of New World *Leishmania* species. Parasitology 100:393-398.

Pall Biosuport. Protocols for DNA and RNA transfer, DNA electrophoresis and protein transfer to BioDyne nylon membranes.

Read CP, Chandler AC. (1961). An introduction to Parasitology, Toppn Company pp:111-130.

Sambrook J, Fritch T, Maniatis T. (1989). Molecular cloning, A laboratory manual, 2nd edition cold Spring Harbor.

Sanger F, Nicklen S, Coulson AR. (1977). DNA sequencing with chain-terminating inibitors. Proc. Natl. Acad. Sci. USA. 74: 5463-5467.

Sheline C, Ray DS. (1989). Specific discontinuities in *Leishmania tarentolae* minicircles map within universally conserved sequence blocks. Molecular and Biochemical Parasitology. 37:151-158.

Simpson L. (1987). The mitochondria genome of kinetoplastid protozoa, genomic organization, transcription, replication and evolution. Annual Review Microbiology 41:363-382.

Simpson L, Silva AD. (1971). Isolation and characterization of kinetoplast DNA from *Leishmania tarentolae*. The Journal of Molecular Biology 56:443-473.

Simpson L, Simpson A, Kidan G, Livingstone L, Spithill T. (1980).The kDNA of hemoflagellated protozoa. American Journal Tropical Medicine and Hygiene. (supplement) 29(5):1053-1063.

Simpson L, Simpson A, Blum B. (1995). RNA editing in mitochondria. in RNA processing. Edited by Higgin and Hame. pp:69-105.

Solari A, Monuz S, Vengas J, Wallace A, Aguilera X, Apt. W, Brenier SF, and Tibayrene M. (1992). Characterization of Chilean, Bolivian and Argentinean *Trypanosoma cruzi* populations by Restriction Endonuclease and Isoenzyme analysis. Experimental Parasitology. 75:167-195.

Southern E. (1975). Detection of specific sequences among DNA fragment by gel electrophoresis.The Journal of Molecular Biology 98:503-517.

Sturm NR, Simpson L. (1991). *Leishmania tarentolae* minicircles of different sequences encode single gRNAs located in the variable region approximately 150 bp from the conserved region. Nucleic Acids Research. 19(22):6277-6281.

Sturzl M and Kurt Rothman (1990). Run off synthesis and application of defined single-stranded DNA hybridization probe. Analytical Biochemistry. 185:164-169.

Tautz D, Renz M. (1993). An optimized freeze squeeze method for the recovery of DNA fragments from agarose gels. Analytical Biochemistry. 132:14-19. Theimann OH, Maslov DA, Simpson L. (1994). Disruption of RNA editing in *Leishmania tarentolae* by loss of minicircle-encoded gRNA gene. EMBO Journal. 13(23):5689-5700.

William RO, Wirth FD. (1987). Kinetoplast DNA minicircle: Region of extensive sequence divergence. Proc. Natl. Acad. Sci. USA. 84: 565-569.

Wilson SM. (1995). DNA -based methods in the detection of *Leishmania* parasites: field application and practicalities. Annual Tropical Medicine and Parasitology 89 (supplement no.1):95-100.

Wirth DF, McMahon Pratt D. (1982). Rapid identification of *Leishmania* species hybridization of kinetoplast DNA in cutaneous lesion Proc. Natl. Acad. Sci. USA.79:6999-7003.

Word Health Organization, control of Leishmaniasis. (1990). Technical report series no 719.

Van Eys GJJ, Mm, Guizani T, Ligthart GS, Dellagi K. (1991). A nuclear DNA probe for the identification of strains within the *Leishmania donovani* complex. Experimental Parasitoloy. 75:167-195.