GENERATION OF SPECIFIC DNA PROBES FOR ETHIOPIAN ISOLATES OF LEISHMANIA DONOVANI

By

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4. Instituto Oswaldo Cruz, Brazil;
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ABSTRACT

In this study the generation of kinetoplast DNA (kDNA) probes specific for Ethiopian *Leishmania donovani* are described. kDNA was isolated from cultured *L. donovani* promastigotes and digested with restriction endonuclease. The restriction fragments were cloned into pUC18 plasmid vector and propagated in *Escherichia coli JM109* competent strains. Recombinant clones were identified by selecting for plasmid born ampicillin resistance followed by colony hybridization screening using radiolabelled *L. donovani* total kDNA probe. The generated kDNA library was further analyzed by differential hybridizations to total kDNA probes from *L. aethiopica* and *L. major.* Further screening was conducted by hybridizing radiolabelled plasmid DNA from the *L. donovani* kDNA sequence bearing clones with dot blots of *L. donovani*, *L. major*, *L. aethiopica* and *L. tropica* promoastigotes. Six clones that hybridize only to the parent *L. donovani* were selected and analyzed by the same procedure using several *L. donovani* and other isolates. Five clones which hybridize with all Ethiopian *L. donovani* isolates but not to any of the cutaneous parasites tested, were identified. These *L. donovani* specific cloned minicircle kDNA sequences showed various specificities and sensitivities within the *L. donovani* complex. One of these sequences -Ld 14, specifically detected Ethiopian and other East African visceral *Leishmania* parasites. A polymerase chain reaction (PCR) assay was designed for the sensitive detection of visceral *Leishmania.* The kDNA probes and PCR described in this study may be useful in the detection of *L. donovani* parasites in Ethiopia.

INTRODUCTION

Leishmaniases: Parasites and the Diseases

Leishmania which are members of the protozoan order Kinetoplastida, are the etiologic agents of human leishmaniases. *Leishmania* are dimorphic parasites, living as flagellated (promastigote) forms in the gut of their sandfly vectors (phlebotomidae) and as ex-flagellated rounded (amastigote) forms in the macrophage cells of Mammals (Molyneux and Ashford,1983). Transmission to man is by the bite of an infected sandfly vector. The majority of infections are zoonotic in origin, though there are important circumstances where man to man transmission is recognized (Molyneux and Killick-Kendrick,1987).

Human leishmaniasis is a world wide public health problem with more than 400,000 reported new cases per year (WHO, 1990). As a result of their major impact on public health, leishmaniases were included in the UNDP/WORLD BANK/WHO Special Program for Research and Training in Tropical Diseases (TDR) as one of the six major communicable diseases (Garnaham, 1987).

The *Leishmania* species infecting man are very similar morphologically, but produce three strikingly different diseases and numerous varieties (Table 1 and Fig.1). A spectrum of response is seen in man from complete refractivity to actual fatal susceptibility. The actual outcome of a given infection depends on the species or strain of parasite, on the immune status of the host,and on its immunological competence (Molyneux and Ashford, 1983).

The three major clinical forms of leishmaniases are: (1) Cutaneous leishmaniasis where infection is restricted to the skin, (2) Muco-cutaneous leishmaniasis where infection is restricted in the mucous membranes and (3) visceral leishmaniasis where infection is predominantly visceral.

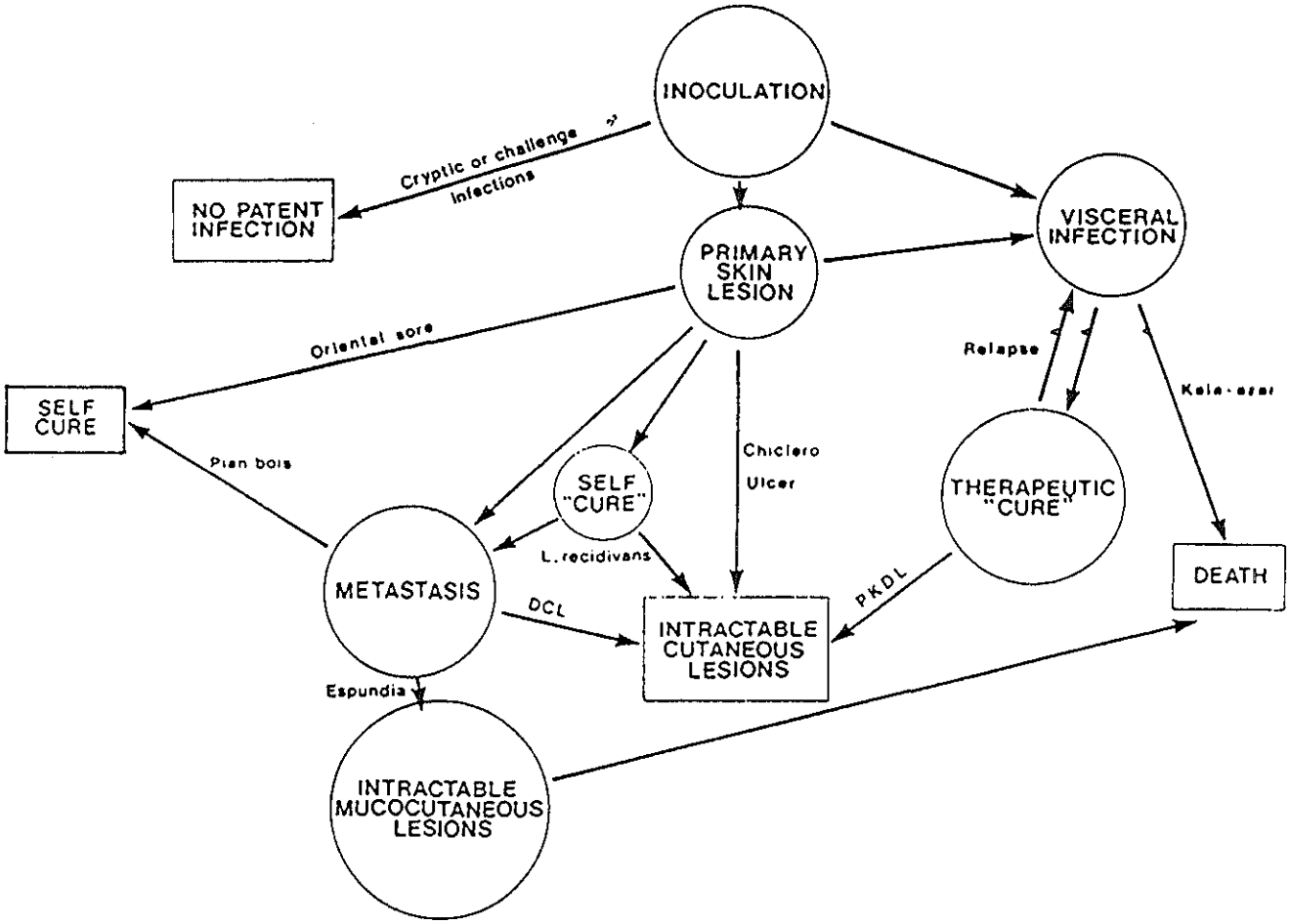


Figure 1. The Leishmania spectrum. Potential sequence of events following natural inoculation with Leishmania parasites. The diseases named are only examples(Molyneux and Ashford,1983).

Table.1 Major *Leishmania* Species Causing Human Disease.

Type of Disease	L. Species	Primary Locations
Cutaneous Leishmaniasis (CL):		
a)Simple CL	*L.mexicana*	Central America
	L.amazonensis	Brazil
	L.venezuelensis	Venezuela
	L.pifanoi	Venezuela
	L.tropica	Mediterranean countries
	L.major	Asia, FarEast, Africa
	L.aethiopica	Ethiopia
	L.guyanensis	Brazil
	L.peruviana	Peru, Argentina Panama, Costarica,
	L.panamenensis	Colombia
b) Diffused CL	*L.aethiopica*	Ethiopia
	L.amazonensis	Brazil
	L.pifanoi	Venezuela
Muco-Cutaneous Leishmaniasis (MCL)	*L.braziliensis*	Brazil
	L.aethiopica	Ethiopia
Visceral Leishmaniasis (VL)	*L.donovani*	India
	L.infantum	Mediterranean Countries
	L.chagasi	South and Central America
	L.archibaldi	Sub-Sahara Africa

Among the clinical forms, visceral leishmaniasis is a priority of concern due to its impact on public health. Human visceral leishmaniasis (Kala-azar) is primarily caused by parasites of the *L. donovani* complex. In general,infections belonging to the *L.donovani* complex are systemic. They affect spleen, liver, bone marrow and untreated disease is usually fatal. Visceral leishmaniasis is a serious disease with a death rate exceeding 90% within 6 months if treatment is not instituted early (Hommel, 1978; Ayele,*et al.* 1988). It is a major public health problem in the tropics and sub-tropics, probably with more than 100,000 new cases each year (WHO, 1990). The disease is reported in several foci in Ethiopia (Ashford, *et al.* 1973; Gemetchu and Fuller, 1976; Gemetchu, *et al.* 1976; Fuller, *et al.*, 1979; Maru, 1979; Lindtjorn, 1980; Ayele, 1982; Habte-Gaber, 1982; Lindtjorn and Olafsson, 1983; Ayele and Ali, 1984; Ayele, *et al*, 1986; WHO, 1990).

The presence of possible insect vectors (Gemetchu *et al.*1976; Gemetchu and Fuller, 1976; Gemetchu, 1983; Gebre-Michael *et al.*. 1986) probably indicates the distribution of *L. donovani* since the invertebrate host is the more active limiting factor for the prevalence of the disease in the Old World (Garnaham, 1987; Ashford and Bettini, 1987).

In Ethiopia, human infection by *L. donovani* is reported to be low- 173 cases during 1981-1987 (WHO, 1990). This is partly because the lowland areas where visceral leishmaniasis is endemic are sparsely populated and the inhabitants are often nomadic in their life style (Ashford & Bettini, 1987).

However, fundamental structural changes are taking place in the development strategy of Ethiopia, giving a significant attention to the low land and valley areas of the country in recent years. These areas of the country are parts of the East African macrofocus of visceral leishmaniasis (Fig.2). The development projects in these areas

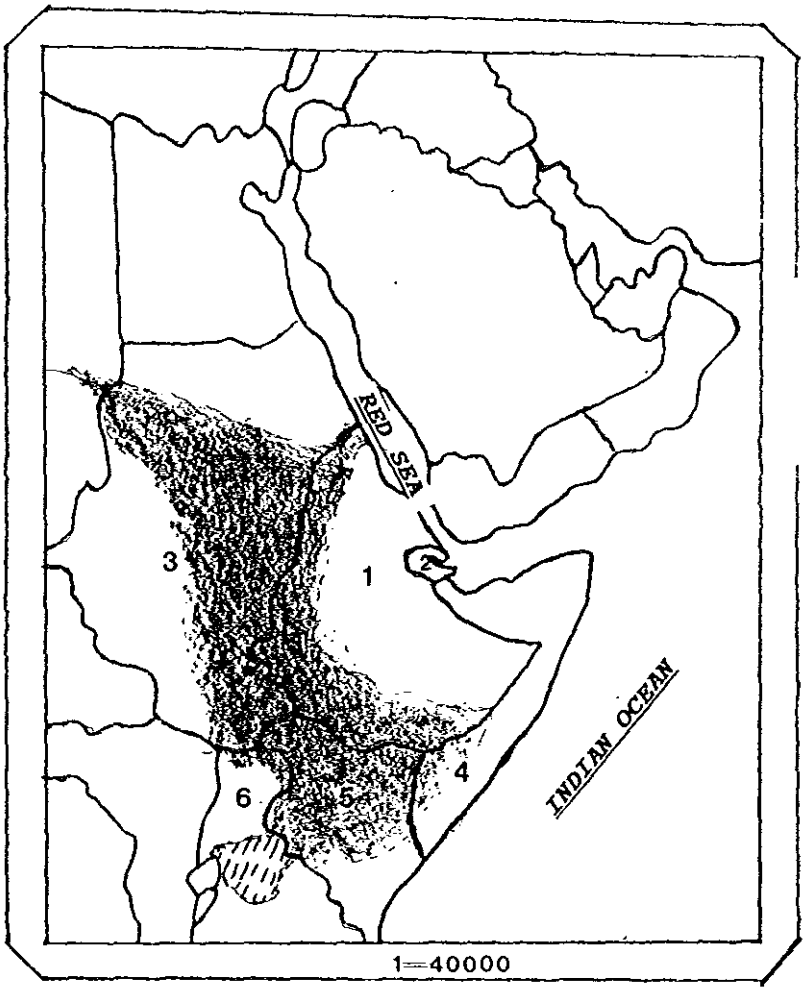


Figure 2.

The East African foci of visceral leishmaniasis. The shaded area is an approximation of the foci based on available evidences (Molyneux and Ashford,1983; Lainson and Shaw,1987; WHO,1990).

include, hydroelectricity, mining, agriculture, resettlement and villagization. The overall effect of these development projects and the impact of drought and famine in the Ethiopian highland regions would lead to the migration of more people to the areas of high vector-reservoir concentration of visceral leishmaniasis which might increase the likelihood of epidemic visceral leishmaniasis.

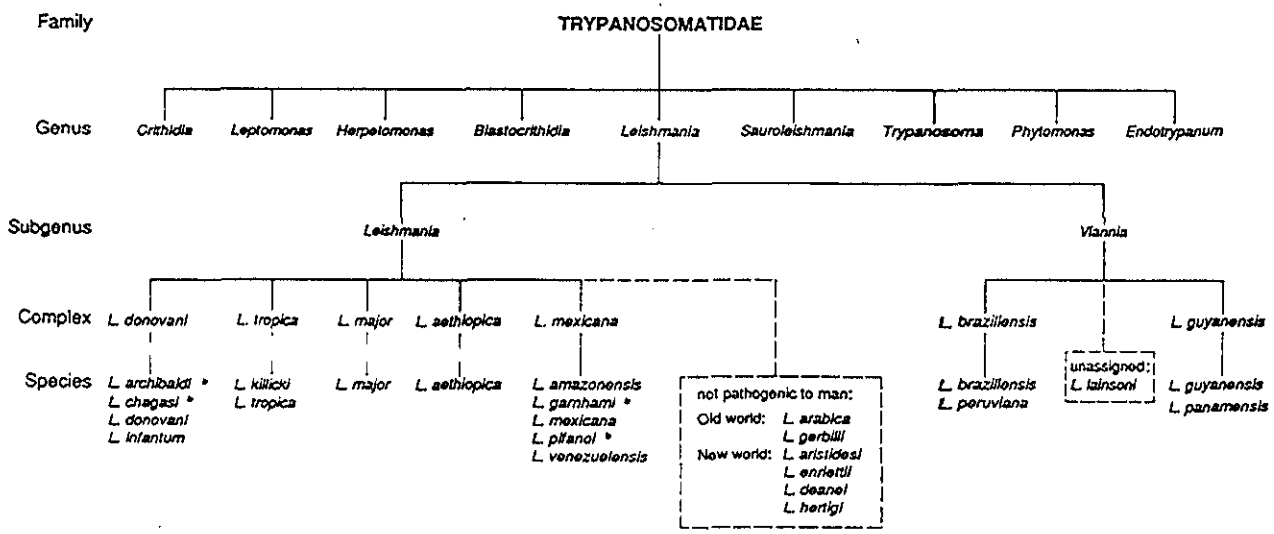
Classification and Identification of *Leishmania* parasites:

Different methods have been used for the classification of the genus *Leishmania* (Gardner, 1977; Lainson and Shaw, 1987; WHO, 1990) generating different taxonomic categories in this parasite complex. One of the recent taxonomic classifications of *Leishmania* is shown in Fig. 3. The summary of the characteristics and techniques used in the identification and classification of *Leishmania* species and sub-species is given in Table 2.

Some authorities divide the *L. donovani* complex into four distinct species (WHO 1990). That is, *L. d. donovani* (India), *L. d. archibaldi* (East Africa), *L. d. infantum* (Mediterranean basin, USSR, China), and *L. d. chagasi* (South and Central America).

There is a discordance of opinion as to the taxonomic status of the *L. donovani* parasites in Ethiopia. *L. donovani nilotica* and *L. d. var.* were used (Hommel, 1978) for parasite in Western and Southern Ethiopia, respectively. The names *L. d. sensu lato* and *L. d. archibaldi* were also used for Ethiopian and other sub-Sahara *L. donovani* species (Lainson and Shaw, 1987, Ashford and Bettini, 1987, Molyneux and Ashford, 1983). Zymodemes intermediary between *L. donovani donovani* and *L. d. infantum* have been also indicated (WHO, 1990) for Ethiopian visceral *Leishmania* parasites.

Until further data is available for *L. donovani* parasites from Ethiopia, Lainson and



*The classification of genera and subgenera is based on extrinsic characters, and that of the complexes mainly on intrinsic characters (isoenzymes).
*Some workers do not consider these to be separate species.

FIG 3. the classification of species of Leishmania (WHO,1990).

Table 2. Methods For Identifying *Leishmania* (WHO 1990).

BIOLOQICAL CHARACTERS
Development in Sand flies
Virulence of clones in Rodents[a]
Characteristic in Culture
IMMUNOLOGICAL CHARACTERS
Excreted Factor Serotyping[a]
Monoclonal Antibodies[b]
In vivo Cross-Immunity Tests
BIOCHEMISTRY
Isoenzyme Characterization[c]
Cell membrane Structure (e.g., Lectin binding)
Fatty acid Analysis[b]
DNA Sequence Analysis
DNA (nuclear and kinetoplast) Buoyant Density
Nucleic Acid Hybridization
RNA (ribosomal RNA genes sequence analysis)
Restriction endonuclease fragment length analysis (RFLP).[b]

[a]. method demonstrates infra specific differences but is of limited use at the species level.

[b]. method limited to a few laboratories; preliminary results suggest it is potentially useful for identification at the species or infra specific level.

[c]. method currently most common for identifying strains at the species or infra species level.

Shaw (1987) suggested the use of "other possible species in the donovani complex". In general the taxonomy of the African Visceral *Leishmania* parasites is not clear.

Although the recognition of the organism responsible for a particular effect is an essential element in biological investigation of an infectious disease, the identification of *Leishmania* parasites is particularly difficult and it has always been and remains as the focal point in the study of *Leishmania.*

Most of the work on determining *Leishmania* species is dependant on the extremely laborious task of identifying the parasites, which involve the isolation of the organisms either directly in culture media or following passage through a susceptible laboratory animal. The major drawbacks of culturing the organisms are, the time it takes to grow the parasite, the laborious work, the number of samples that can be handled at any one time, and more importantly the difficulty to use these methods under field conditions due to the risk of contaminations. Furthermore, it is difficult to grow *Leishmania* in culture and by passage through experimental animals (Evans, 1989).

Immunological methods have been used in the identification of *Leishmania.* However the methods are of limited value because of: (1) the difficulty in identifying current from previous infection, (2) the emphasis to show only sub-specific differences, (3) the difficulties for direct identification of parasites, (4) the skill needed and (5) their limited advantage for large scale epidemiological studies.

Identification of *Leishmania* using monoclonal antibodies is of great value compared to the other immunological methods (Mac Mahon Pratt and David,1981). But this method would have to overcome the problem of using a battery of monoclonal antibodies for the different stages of each parasite to be of any practical use (Le Blancq, *et al.* 1987). Although, the mentioned parameters remain important, the recombinant DNA technology is being explored to develop techniques for unequivocal diagnosis/ identification of

Leishmania.

DNA Diagnostic Techniques

Considerable effort has been made on the cloning and synthesis of specific DNA probes for the detection of pathogenic organisms, for use in clinical diagnosis, in veterinary diagnosis and in food and environmental sciences (Maloin, *et al.* 1988; Moench, 1987; Viscidi and Yolken, 1987).

DNA probes are now being widely used in research and beginning to make an impact on routine settings in medicine, plant breeding, forensic science and others (Matthews and Kricka, 1988). The application of DNA probes to diagnosis has begun for the detection of *Plasmodia, Leishmania* and *Trypanosoma cruzi.* To a lesser extent this approach has been utilized to detect other parasitic diseases such as filariasis, amebiasis and onchocerciasis (Barker, 1990; Wilson,1991).

The ability to use DNA probes in the detection of infectious agents is derived from a few biological properties of DNA molecules (Lewin, 1985; Keller and Manak, 1989).

1. All organisms contain DNA with certain nucleotide sequences which are unique to a particular species.
2. The double stranded DNA molecule can be denatured into single strands and renatured (hybridize) into double stranded state in appropriate conditions.
3. The hybridization of DNA is specific since it depends upon specific nucleotide base pairing on appropriate strands.

These properties suggest the possibility of isolating sequences of DNA which are specific to any given organism so that it will hybridize with the DNA of the species from which the isolation have been made, but not with others.

There are three phases in the development of a DNA probe based diagnostic

procedure (Barker, 1990; Barker, *et al.* 1986): 1. Development of specific DNA probes; 2. Generating an assay system encompassing all steps from sample collection to final read out; and 3. Extensive field trials comparing the new method with the ones already in use.

Inside a cell the DNA helix is surrounded and further coiled by various proteins. For years this made it difficult to study genes. Then in the 1970's enzymes known as restriction endonucleases were discovered: these enzymes snipped strands of DNA into smaller, more identifiable pieces and thereby made it easier to isolate the pieces containing a gene of interest.

Subsequently these DNA pieces have to be propagated in large amounts. To this end, they are cloned in so called vectors. Cloning of DNA is made possible by the use of cloning vectors which can incorporate a foreign DNA molecule into their DNA structure *in vitro.*

Because of their reliability and ease of handling, plasmids have become the work horses of molecular cloning. They work better than any other vectors when the DNA to be cloned is a simple structure of a small size (< 10 kilo base).

Bacterial plasmids are closed circular DNA molecules found in different bacteria species, where they behave as accessory genetic units that replicate and are inherited independently of the bacterial chromosomes. Nevertheless, they rely on enzymes and proteins encoded by the host (Sambrook, *et al.*1989).

Frequently plasmids contain genes coding for enzymes that under certain circumstances in nature are advantageous to the bacterial host. Among the phenotypes conferred by plasmids are resistance to antibiotics, production of colicins, enterotoxins and restriction and modification enzymes.

The following properties make plasmids suitable for cloning and propagating DNA (Ray, 1987; Gomez-Marquez, *et al.* 1987).

1. They can replicate autonomously in *E. coli* (they are replicons in their own right) even when joined covalently to foreign DNA.

2. They can be easily separated from bacterial nucleic acids and purified.

3. They contain regions of DNA that are not essential for propagation in bacteria, foreign DNA inserted in this region is replicated and propagated as if it was a normal component of the vector.

4. They confer a selectable phenotype for their bacterial host (e.g. antibiotic resistance).

In principle, cloning in plasmid vectors is straight forward. The plasmid DNA is cleaved with a restriction enzyme and joined *in vitro* to a foreign DNA. The resulting recombinant plasmids are then used to transform bacteria (Hanahan, 1985).

Transformation of bacteria is done by treating the bacteria with certain salts (e.g. $CaCl_2$) under cold conditions, which makes their membranes temporarily permeable, a state of competence.

After transformation, the bacteria are plated out on a selective agar media and the clones containing recombinant DNA are selected (see materials and methods and Fig.4) to form a DNA library from which the required specificity can be selected. The sensitivity of DNA probes has been exponentially increased by the use of the polymerase chain reaction (PCR) technology (Saiki, *et al.*1988).

DNA Probes in the Identification of *Leishmania:*

Although there are reports on the use of complementary DNA (cDNA) (Van Eys, *et al.* 1989; Howared, *et al.* 1991), and genomic DNA (Van Eyes, *et al.* 1991) probes for the identification of *Leishmania*, most of the work in the development of DNA probes for *Leishmania* have been based on the kinetoplast DNA (kDNA) of these parasites (Table 3).

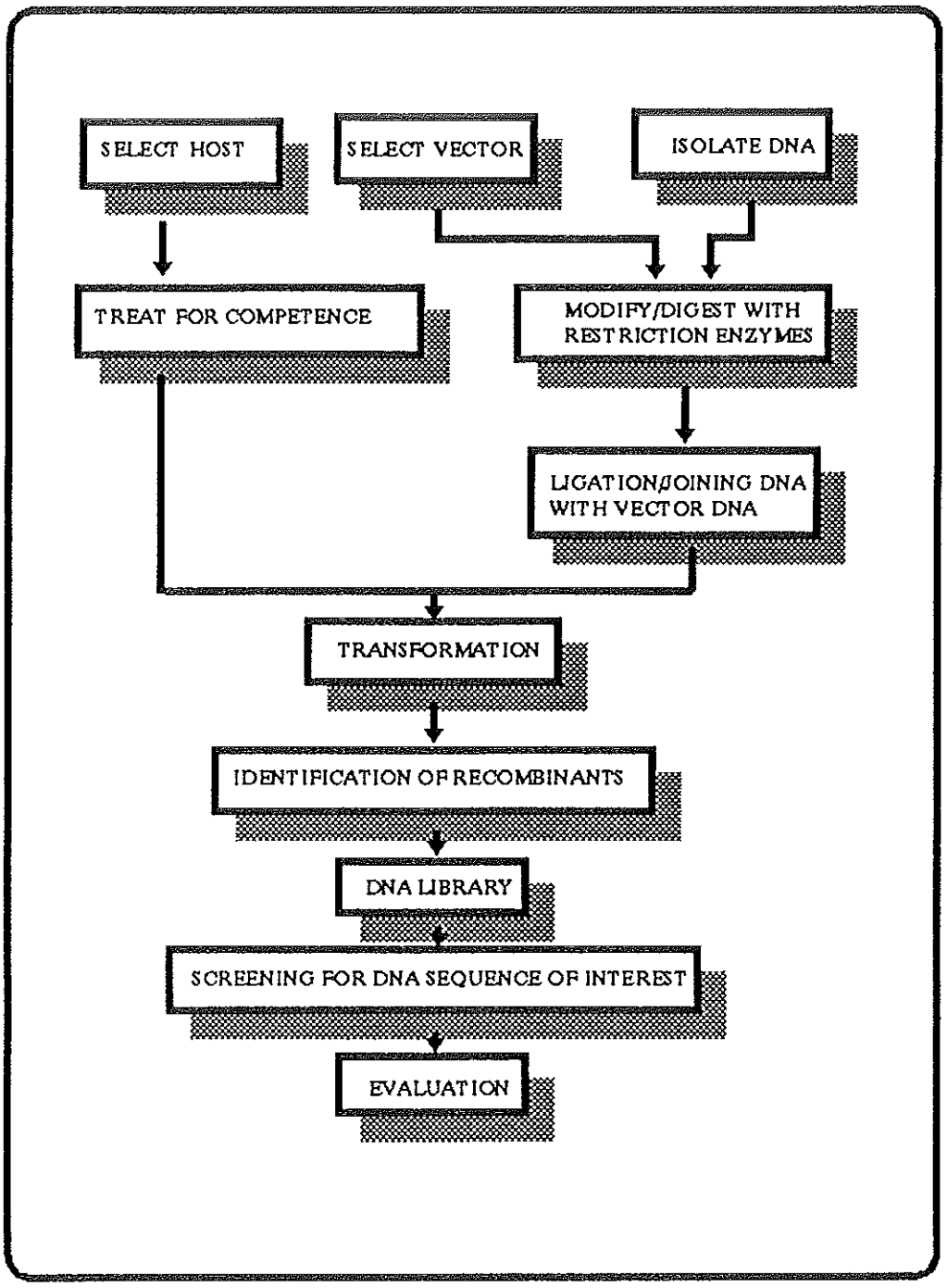


Figure 4. A flow diagram showing the main steps in a typical DNA cloning.

The kinetoplast is the characteristic feature of Kinetoplastid flagellates. It is found as a microscopically visible disc, 1-2 nm in diameter. The kinetoplast is situated at the base of the flagellum within a short distance of the basal body from which the flagellum arises.The membrane capsule surrounding the kinetoplast is continuous with the mitochondrial membrane; this demonstrates that the kinetoplast is a specialized part of the mitochondrion (molyneux and Ashford, 1983). It contains two DNA components: mitochondrial DNA (approximately 10^7 base pairs (bp) and kinetoplast DNA (kDNA) (Janovy, 1987). The kDNA of *Leishmania* lends itself an important feature for the diagnosis and identification purposes regardless of the state of knowledge of its function.

The kDNA consists of two types of circular DNA structures referred to as the maxicircle and the minicircles. The maxicircle is estimated to be 20-40 kilo base (kb) pairs in size and it is present in 10-20 copies per organism. The kDNA minicircles are the smallest mitochondrial DNAs with high copy numbers (Ray, 1987) but they have no known function eventhough recently Sturm and Simpson (1990), have reported guide RNA (gRNA) coding function.

In *Leishmania* there are approximately 10,000 to 20,000 copies of minicircles per organism. Each minicircle is estimated to be about 1kb in size, with 0.56x 10^6 mol. weight. Minicircle kDNA sequence studies have shown that minicircles are composed of conserved and variable regions. The sequence of kDNA minicircle varies considerably in different species and sub-species of *Leishmania* and undergo rapid sequence variation, on an evolutionary scale (Rogers and Wirth, 1987).

Two important requirements for a diagnostic method are sensitivity and specificity. The highly repetitive minicircle kDNA in *Leishmania,* provide the required sensitivity and the minicircle kDNA sequence variation can be used to develop specific DNA probes. Either a broad specificity, a narrow species or geographically distinct isolate specificity

Table 3. Hybridization Specificities of cloned *Leishmania* kinetoplast DNA fragments

L. species	Specificities	Reference
L.tropica	Isolate	Kennedy (1984)
L. aethiopica	"	" "
L. donovani	"	Lawrie,*et al* (1985)
L.infantum	"	" " "
L.donovani	Species	Jackson, *et al.*(1986)
L.aethiopica	"	" " "
L.chagasi	"	" " "
L.pifoni	"	" " "
L.infantum	"	" " "
L.donovani	Complex	Barker, *et al.* (1986)
L.mexicana	"	" " "
L.mexicana	Species	" " "
L.braziliensis	"	" " "
L.donovani	Visceral Complex	Lopes & Wirth (1986)
L.chagasi	Visceral Leishmania	Lopes & Wirth (1986)
L.mexicana	Species	Rogers & Wirth (1987)
L.mexicana	Sub-species	" " "
L.aethiopica	Species	Laskay, *et al.* (1991)
L.major	Species	Smith, *et al.* (1989)
L.major	Species	Chapman, *et al.*(1989)
L.tropica	Species	" " "
L.arabica	Species	" " "

can be selected from the sequences in the minicircle kDNA. The minicircle kDNA is isolated, purified and different regions of the minicircle can be produced by cutting with restriction endonuclease enzymes.The fragments can be cloned and propagated in order to select the desired specificity. The methodology based on kDNA probes for the detection of *Leishmania* provides a direct means of diagnosis and eliminates the need of culturing parasites before specific identification. This direct diagnosis had not been possible with any of the other methods (Wirth, *et al.*, 1986). Amplification of DNA by the polymerase chain reaction (PCR) has revolutionized DNA based diagnostic tests. PCR is a rapid procedure for *invitro* enzymatic amplification of a specific DNA segment (Siaki *et al*,1988). It has been shown that PCR amplification of minicircle kDNA sequences can be used for sensitive detection of *Trypanosoma cruzi* (Avilla,*et al.*1991) and *Leishmania* (Rodgers, *et al.*1990).

The Study:

In order to prevent an outbreak of visceral leishmaniasis, prevention and control strategies should be devised with development projects. Before designing control and prevention strategies for leishmaniasis, extensive base line information concerning the status of human infection, the status of vector and reservoir hosts should be collected.

Medical practitioners should know the precise nature of *Leishmania* infecting their patients, for prognosis greatly depends on which species is infecting the patients. Accurate and rapid diagnosis of *Leishmania* must be achieved before treatment and control measures are started.

The accomplishment of this task largely depends on the accurate identification of the parasites in humans, insect vectors and reservoir hosts. Ideally the method used should be specific sensitive, quick and reproducible.

In addition a method of direct identification of *Leishmania* parasites from clinical samples, sandfly vectors, and mammalian reservoirs is necessary if broader epidemiological studies are to be initiated. Among the several diagnostic methods available, the use of kDNA probes would be a candidate to provide fast, reliable, direct diagnosis and identification of *Leishmania* parasites.

Given the current direction of development and movement of population in Ethiopia, it will be appropriate to develop an identification tool for parasites causing visceral leishmaniasis. The experience of the 1968 agricultural project established in Humera (N-West Ethiopia) with the aid of the World Bank should have to be considered. Large number of peasants were attracted from the highland regions of Ethiopia to Humera. But the laborers suffered miserably from malaria. Some of them were able to be seen at Gonder hospital and treated for malaria. Despite treatment many of them died. Only after sometime it was realized that those dying of 'malaria' despite treatment were in fact dying of visceral leishmaniasis (Molyneux and Ashford, 1983).

The aim of this study, therefore, was to generate specific kDNA probes which would be useful for the identification of Ethiopian *L. donovani* isolates in large scale epidemiological studies and in clinical specimens. kDNA probes proven to be specific for *L.aethiopica* (the principal causative agent of cutaneous leishmaniasis in Ethiopia) have been generated in the Armauer Hansen Research Institute (AHRI), Ethiopia. This study is to supplement this work by developing a specific kDNA probe for the detection of visceral *Leishmania* parasites in Ethiopia, so that the Identification of *Leishmania* parasites in Ethiopia could attain a better status and promote research in leishmaniasis.

In this thesis, the cloning of minicircle kDNA from an Ethiopian *L. donovani* (IPB/399) isolate and the systematic screening to identify *L. donovani* specific clones is presented. The potentials of this study is discussed.

MATERIALS AND METHODS

Reagents and Culture Conditions:

(a) Reagents

Stock solutions were prepared from highest grade reagents/ chemicals using autoclaved double distilled water, sterilized and stored at room temperature, 4°C, -20°C or -70°C, according to the requirements needed for the stability of each preparation (Sambrook, *et al.*, 1989). Commercially available preparations and enzymes (Appendix 4) were used according to the specifications given by the manufacturers.

(b) Culture

Leishmania promastigotes were cultured in RPMI-1640 (Sigma,USA) medium supplemented with 10% heat inactivated foetal calf Serum (FCS) (SERVA, F.R.G.), 200 U/ml Penicillin, 200 μg/ml streptomycin and 200 mM Glutamine. Cultures were maintained by successive transfers in the same medium at room temperature. The *Leishmania* species used in this study are described in Appendix 1.

Escherichia coli JM109 strain (Pharmacia, Sweden), were streaked on YT agar plates (0.8% Tryptone, 0.5% NaCl, 0.5% Yeast extract and 1.5% Bacto agar) and a single colony culture was initiated in YT broth (0.8% Tryptone, 0.5% NaCl and 0.5%Yeast extract, pH 7.2). YT agar plates containing 100 μg/ml ampicillin were used for the selection and maintenance of recombinant *E. coli* JM109 cells. Long term storage was made in 15% sterile glycerol at -70°C. *E. coli* cultures were grown overnight at 37°C. An orbital shaker (Gallen Kamp, England) at a speed of 200 revolution/min was used for aeration.

Preparation of kDNA for cloning:

(a) kDNA Isolation

An Ethiopian isolate of *L. donovani* (IPB/399) was used as a source of kDNA for cloning. 10^{10}-10^{11} *Leishmania* promastigotes (Barker, et al.1985) were washed with NTE buffer (0.1 M NaCl, 10 mM EDTA, and 10 mM Tris-Cl,pH 8.0) and pelleted at 1000g, 4°C for 10 min. The pellet was lysed in the same buffer (NTE) by adding sodium dodocyl sulphate (SDS) to a final concentration of 0.1%. Chromosomal DNA was sheared by passing through a 21 G hypodermic needle. Proteins were then removed from the lysate by incubating with 25 mg/ml of Proteinase K (Boehringer Mannhem, F.R.G.) for 1 hour at 50°C.

kDNA was then pelleted by centrifugation at 10,000g, 21°C for 1 hour in Beckmann LU-8 ultra-centrifuge (Beckmann, USA). The pellet was washed with TE buffer (10 mM Tris-Cl pH 8.0, and 1 mM EDTA) and resuspended in the same buffer.

(b) Phenol-Chloroform extraction

The remaining protein contaminants were removed from the kDNA preparation by phenol chloroform extraction. An equal volume of phenol equilibrated with Tris-Cl, pH 8.0 buffer was added to the kDNA sample, vortexed for 1 min. and centrifuged 5 min. in an eppendorff centrifuge at 12,000g at 4°C. The aqueous layer was transferred into new tubes and re-extracted by an equal volume of phenol: chloroform:iso-amyle alcohol (25:24:1) as above. Traces of phenol were removed by extracting the aqueous phase with an equal volume of chloroform: iso-amyle alcohol (24:1). Then, the aqueous phase was transferred to a fresh tube for ethanol precipitation.

(c) Ethanol precipitation

The phenol-chloroform extracted kDNA was precipitated by adding 2.5 volume of absolute cold ethanol in the presence of 0.1 volume of 3 M sodium acetate (pH 4.8) and by incubation for 1 hour at -70°C. The precipitate was collected by centrifugation at 12,000g, 4°C for 15 min. in an eppendorff centrifuge and rinsed with 70% cold ethanol.The precipitate was then collected as stated above and briefly dried under vacuum.The kDNA was then resuspended in TE buffer, pH 8.0.

(d) Determination of yield and purity

The amount of kDNA was determined by spectrophotometric measurements using Beckmann DU-64 spectrophotometer (Beckmann, USA). The UV absorbance of the kDNA sample was used to determine the amounts of kDNA/ml from the known 1 OD-260 value of DNA (double stranded) which is 50 μg/ml (Keller and Manak, 1989; Sambrook, *et al.*, 1989). The purity of the kDNA sample was verified from the ratio of A-260 to A-280 (absorbance of nucleic acids and proteins respectively). A sample having a ratio between 1.6 to 1.8 was considered pure (Keller and Manak, 1989).

Vector-Host Preparation:

(a) Plasmid vector

The vector used was pUC18 plasmid (Pharmacia, Sweden). pUC18 plasmid is DNA cloning vehicle (Hanahan,1985) with a 2685 base pair size in which a multiple cloning site is incorporated (Fig.5). pUC18 DNA was digested with *Hinc II* restriction endonuclease which cuts the plasmid at one site creating blunt ends in order to prevent recircularization. The linearized plasmid was dephosphorylated by incubation with calf intestine alkaline

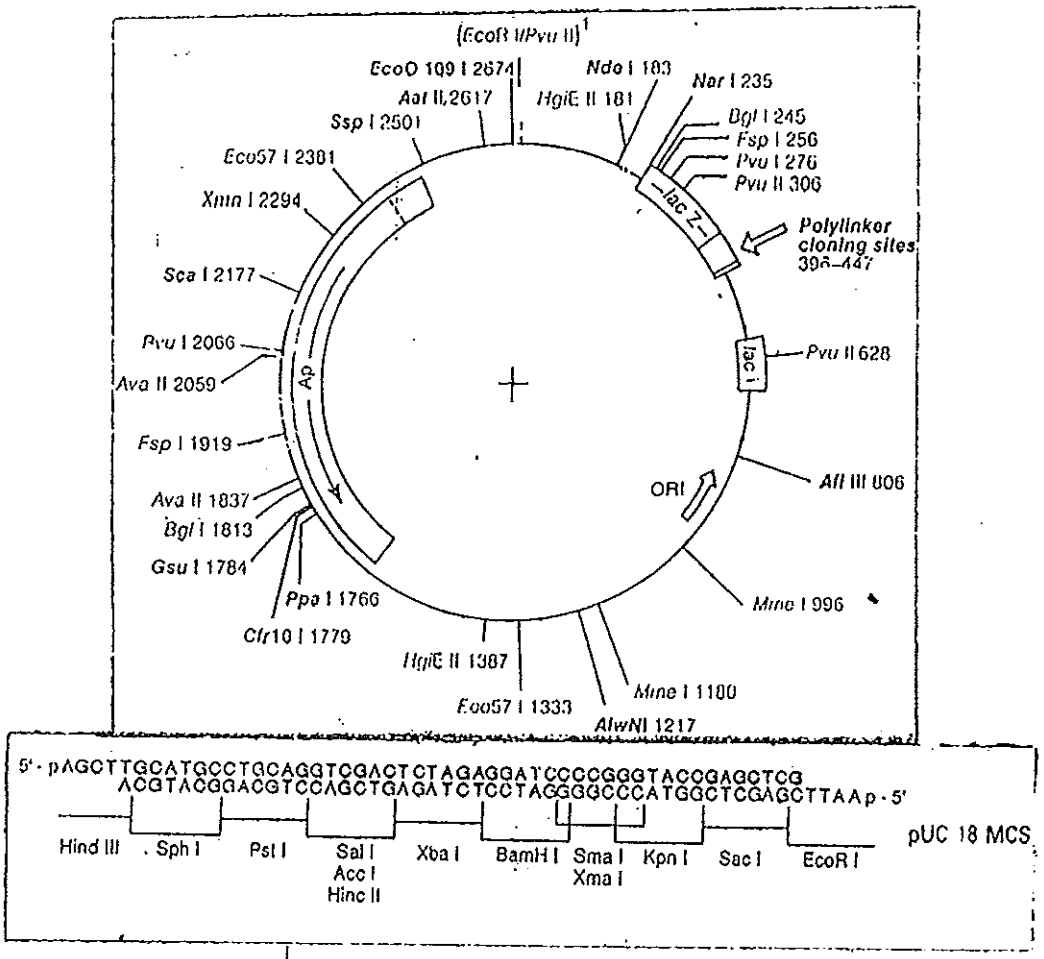


Figure 5.

Map of pUC 18 plasmid DNA. Circular map showing restriction enzyme recognition sites. Enzymes with one recognition site are indicated in bold face. The DNA sequence of the polylinker, multiple cloning site (MCS) is shown below the circular map.

phosphatase. The linearization of pUC18 plasmid was analyzed by electrophoresis on 0.8% agarose gel and photographed after ethidium bromide staining.

The amount of linearized pUC18 plasmid DNA used was estimated by comparing the fluorescent yield between the pUC18 DNA and Lambda *Hind III* cut DNA standard (New England Bio Labs, USA) on 0.8% agarose gel after electrophoresis and ethidium bromide staining using the relation: size of marker DNA/total size of marker X amount of marker DNA loaded on gel = amount of DNA compared.

(b) Bacterial strain

The bacterial host used was *E. coli* JM-109 (genotype = f', traD36, $lacI^q$, Δ(lac Z)M15, $proA^+B^+$/e14($McrA^-$), Δ(lac-proAB), thi, syrA96(Nal^r), endA1, hsdR17($r_k^-m_k^+$), relA1, supE44, recA1, λ^-) strain (Hanahan,1985). These cells were streaked on YT agar plates. A single colony culture was initiated in 5 ml volume over night. The 5 ml culture was expanded into 400 ml culture which was allowed to grow in 2 liter capacity flasks at 37°C with constant shaking by an orbital shaker of 200 revolution/min. Growth was monitored at short time intervals by measuring optical density of the culture.

The late log-phase cells (OD-590=0.5) were placed at 0°C for 30 min. and pelleted at 5,000g at 4°C for 10 min. The pellet was treated for competence by the $CaCl_2$ method (Sambrook, *et al.* 1989). Briefly, the pellet was resuspended in 0.1 M $CaCl_2$, chilled and repelleted under the same conditions as above. Then the pellet was resuspended, again in 0.1 M $CaCl_2$ and 15% glycerol. The cells were stored in 100 μl aliquots at -70°C for use. The transformation efficiency (number of transformed cells per μg of plasmid DNA) of the $CaCl_2$ treated *E. coli* JM109 strains was verified by transforming 100 μl cells with 10 ng of pUC18 DNA.

Cloning of *L.donovani* kDNA:

(a) Restriction endonuclease digestion

Total kDNA was digested with restriction endonuclease enzymes in order to produce fragments of minicircle kDNA for cloning in plasmids. A combination of *Alu I*, *Hae III*, and *Rsa I* restriction endonucleases were used.

Restriction endonuclease digestion of kDNA was done in a 20 μl reaction volume at 37°C for 2 hours. The reaction mix consisted of 125 ng kDNA, 2μl 10X medium salt buffer(10 mM Tris-Cl,pH 7.5;10 mM $MgCl_2$;1 mM dithiothereitol(DTT)) and 1 unit restriction enzyme.

Restriction digests were analyzed by 1.5% agarose gel electrophoresis including phi X-174 *Hae III* and lambda *Hind III* DNA markers. Gels were viewed using ultraviolet light after staining with ethidium bromide.

(b) Agarose gel electrophoresis

1.5% and 0.8% agarose gels were used for the analaysis of kDNA digests and plasmid DNA respectively. The correct amount of NA agarose (Pharmacia, Sweden) was added into a measured amount of TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and heated in a microwave oven until the agarose was completely dissolved. After cooling the solution to 50°C, the agarose was poured into the gel molding cast and allowed to solidify. Then the well forming comb was removed, and the gel was placed into the horizontal electrophoresis tank (Bio Rad, USA). TAE buffer was added so as to just cover the gel.

Samples were mixed with 6x loading buffer (7.5% Ficoll-400, 0.25% xylene cyanol and 0.25% Bromo Phenol Blue) and loaded into the wells. Electrophoresis was carried out at a constant set up of 100 V/100 mA until the tracking dye moved about 2/3 of the gel length. Gel staining was done in TAE buffer containing 0.5 μg/ml ethidium bromide for 30 min. by shaking and destained with TAE buffer to visualize the DNA

clearly. Alternatively, 0.5 μg/ml ethidium bromide was added directly to the gel and the running buffer to stain the DNA during electrophoresis. Gels were visualized by UV transilluminator (Pharmacia, Sweden) and photographed using polaroid film (3000 ASA) and red photographic filter (Polaroid, USA).

(c) Ligation Reaction

Fragments of *L. donovani* kDNA were produced by restriction endonuclease enzymes were cloned into linearized, dephosphorylated pUC18 by a shot gun (random) cloning approach *in vitro* by ligation reaction. Ligations were set up in a final volume of 20μl reaction with a vector to insert molar ratio of 1:25 (1:5 absolute ratio). The reaction setup included 200 ng kDNA insert, 40 ng pUC18 DNA, 2 mM ATP, 6 units *T4* DNA ligase and 2 μl 10X ligation buffer (10 mM $MgCl_2$, 20 mM DTT, 50 μg/ml bovine serum albumin (BSA), and 50 mM Tris-Cl, pH 8.0. The control reactions performed to monitor the efficiency of ligation and the subsequent transformation were: (1) vector cut and dephosphorylated; (2) vector cut; (3) vector uncut; and (4) blank. The reaction mix was incubated at 12°C overnight and there after reaction mix was used to transform competent *E. coli* JM109 bacteria.

(d) Transformation

100 μl of competent *E. coli* JM109 cells were freeze-thawed and the ligation mix was added. The mixture was chilled at 0°C for 30 min.; heat shocked at 42 °C for 2 min. and 1 ml YT broth was added. Then the cells were allowed to recover by incubating at 37°C for 1 hour. The cells were pelleted and resuspended in 100 μl YT broth. The suspension was used to spread on ampicillin agar plates in a volume of 10 μl and 90 μl onto two plates. The plates were incubated at 37°C overnight on which transformed cells

presented growth. The randomly dispersed bacterial colonies were re-plated on ampicillin agar plates in triplicates using grid templates (Fig.6) and used for further analysis.

Screening of *L.donovani* kDNA Library:

Three types of procedures were used in the identification and analysis of *L. donovani* kDNA clones. Colony, Southern and promastigote blots were hybridized with radiolabelled probes.The principle behind these procedures is shown in Fig.7.

(a) Radiolabelling of DNA

kDNA or plasmid DNA's to be used as probes were radiolabelled by the method of nick translation just prior to hybridization reactions. Nick translation reactions were set up according to the following amounts given for 20 μl reaction volume. The reaction mix consisted 0.2 μg kDNA,1.2 nm of 3 dNTPs (dCTP, dTTP, dGTP), 25 μCi α^{32}PdATP, 1 ng DNase and 10X nick-translation buffer (0.5 M Tris-Cl,pH 8.0; 0.05 M $MgCl_2$; 0.01 B-Mercapto ethanol). Reaction volumes were scaled up or down when required.

The reaction mix was incubated for 10 min. at 15^{0}C before *E. coli* DNA polymerase was added and then further incubated at 15^{0}C for 30 min after adding 5 units of DNA polymerase. Alternatively, DNase I and DNA polymerase were added simultaneously and incubation was done for 1 hour at 15^{0}C. Reactions were terminated by stop buffer (0.25% Bromophenol blue, 0.05 M EDTA, pH 8.0) and unincorporated nucleotides were removed by passing through Biogel P-30 (15% w/v) (Biorad, USA) packed column with 0.9ml bed volume or through Sephadex G-50 (Pharmacia, Sweden). Purified probes with greater than 60% incorporation of radiolabel were denatured in a boiling water bath for 5 minutes just prior to use. Alternatively labelling was made by multiprime labelling using Amersham's kit.

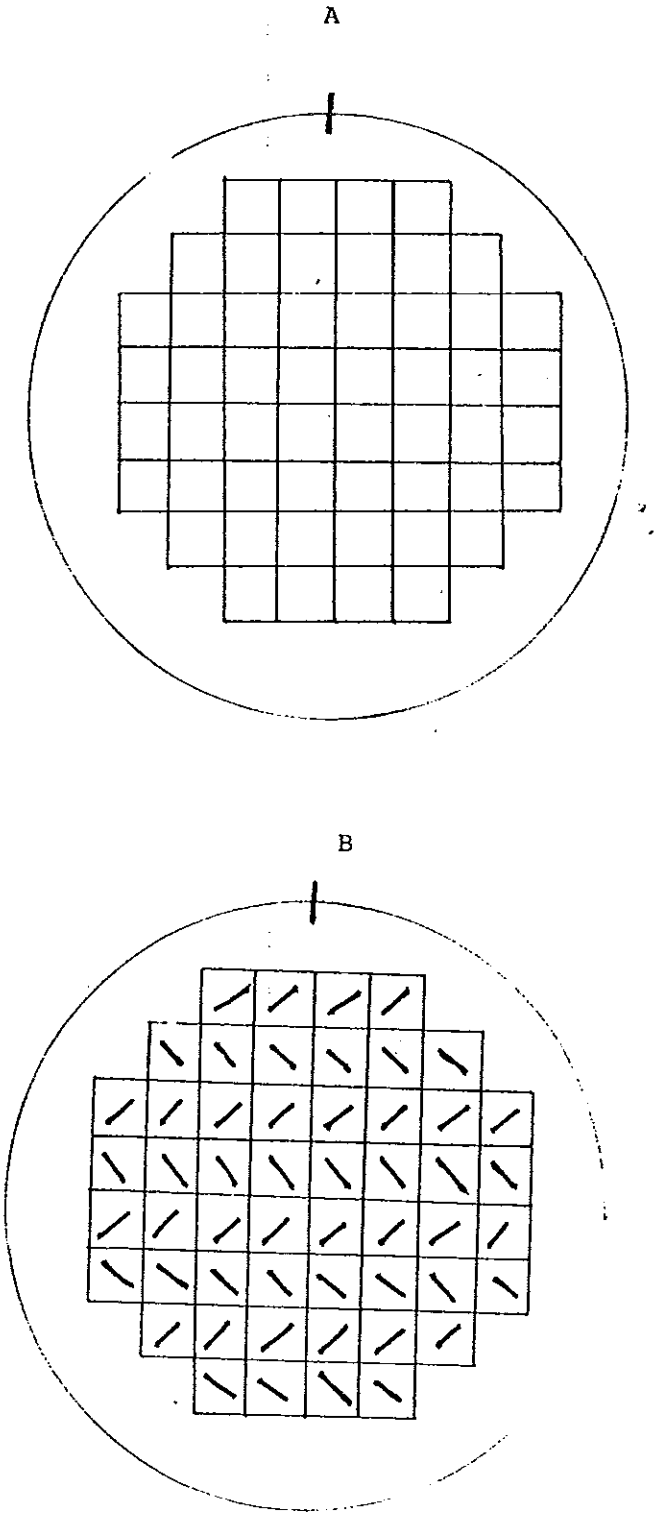


Figure 6. Grid templates used to produce replica plates. A numbered grid template (A) and the pattern of streaking (B) to produce identical bacterial colonies on different plates are shown.

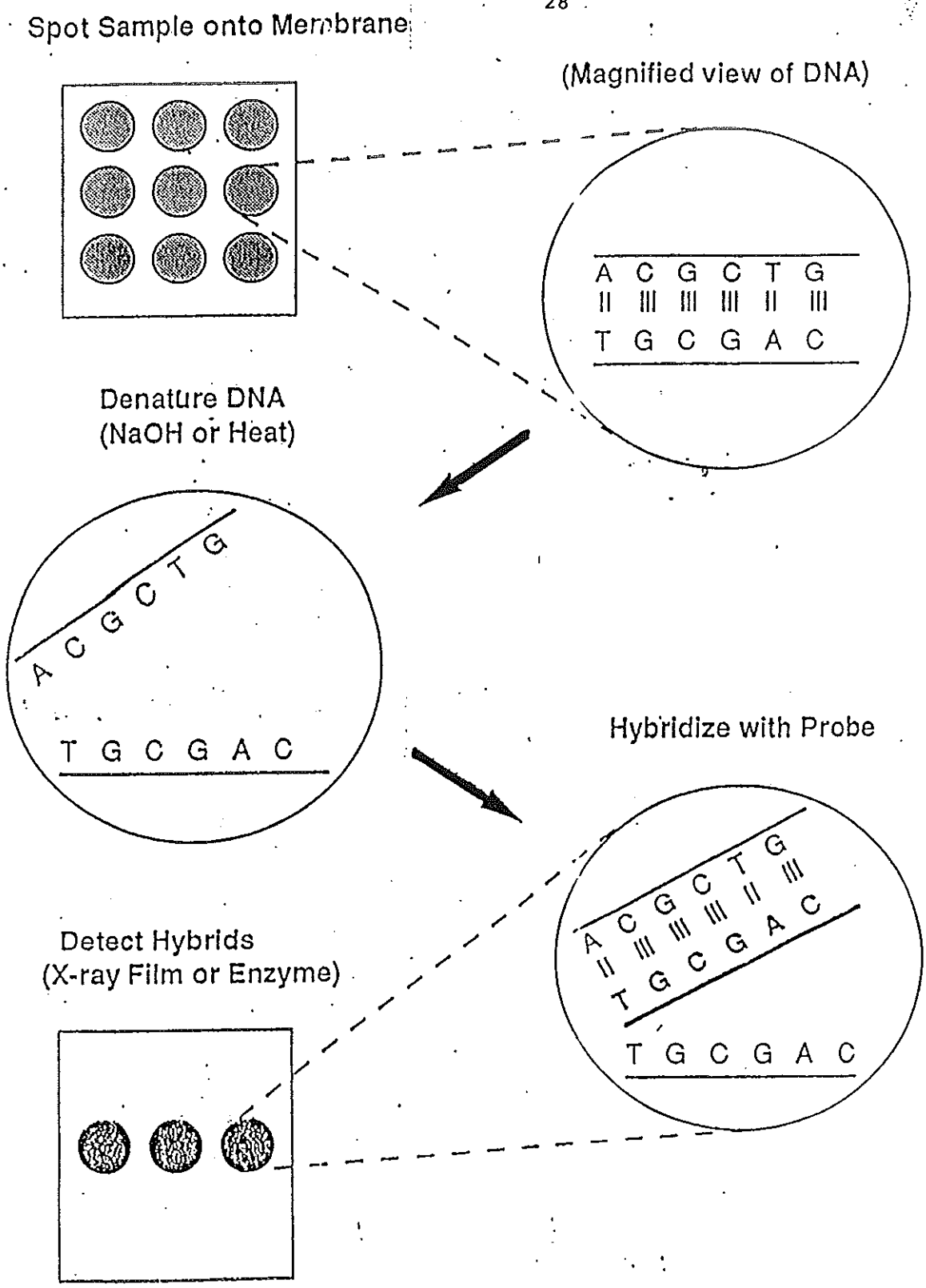


Figure 7. Schemic drawing showing hybridization of DNA bound onto membrane filters. The principle of immobilizing DNA samples on membrane filters enable easy detection of hybrids.

(b) Colony Hybridization:

Bacterial colonies were transferred from ampicillin agar plates on to nitrocellulose (Schleicher and Schuell, F.R.G.) filter and air dried. The filters were floated, colony side up, on a puddle of denaturing solution (0.5M NaOH, 1.5 M NaCl) and on neutralization solution (1M Tris-Cl pH 8.0, 1.5 M NaCl) for 10 min. on each solution. Then filters were air dried, and baked under vacuum at 80°C for 2 hours.

Pre-Hybridization: Filters were incubated in hybridization solution (5xSSc, 50% formamide, 5x Denhardt's solution, 100 μg/ml denatured Salmon sperm DNA and 1% SDS) for at least 2 hours at 42°C with shaking.

Hybridization:α^{32}p dATP labelled probe DNA was denatured by boiling for 5 min. and cooled immediately. Filters were immersed in a fresh hybridization solution in which probe DNA was added. Hybridization was continued for 14-24 hours by gentle shaking.

Washing: Filters were washed three times for 30 min. each time in an excess wash buffer according to manufacturer manual.(0.1xSSC, 0.5%SDS) at 50°C with rocking.

Autoradiography: The filters were air dried, secured on to a backing material and covered with plastic covers. They were exposed to X-ray films (XOMAT-AR5) with an intensifying screen (Lightning plus, Dupont, USA) in an exposure cassette. The exposure was made at - 70°C.The films were processed in Kodak GBX-developer and fixer (Kodak, USA) and hybridization signals were identified.

(C) Southern Hybridization

Southern hybridization involved the preparation of recombinant plasmid DNA; *Leishmania* DNA; agarose gel electrophoresis; capillary transfer of electrophoresed DNA

on to hybridization membranes (Southern blotting) and hybridization of filter with radiolabelled probe.

Plasmid DNA Preparation (Minipreparation): A modified alkaline lysis procedure of Birnboim and Dolly (1979) was used for extracting plasmid DNA from *E.coli* colonies. 1.5 ml of an overnight *E.coli* Jm109 culture was pelleted at 12,000g at room temperature for 1 min. The bacteria were lysed for 5 min in 50mM glucose, 25mM Tris-Cl pH 8.0 and 10 mM EDTA pH 8.0. Then 0.2M NaOH,1% SDS solution was added and kept for 5 min at room temperature. This was followed by the addition of 3M potassium-acetate (pH 4.8) and chilled at 0°C for 5 min. The sample was pelleted at 12,000g/5min/4°C and the supernatant was transferred to fresh tubes. The plasmid DNA was then extracted with phenol-chloroform and ethanol precipitated as described in the previous sections. Finally, the plasmid DNA was briefly dried under vacuum and resuspended in TE buffer. *Leishmania* kDNA was extracted from cultured promastigotes and digested with *Pst I* restriction enzyme.

Agarose-gel Electrophoresis: The plasmid preparations and/ or *Leishmania* kDNA digested with restriction enzyme were electrophoresed on agarose gels as described previously. The gel was soaked in denaturing solution and neutralized for 30 min in each solution.

Southern Blotting (transfer):The capillary transfer of the plasmid DNA from agarose gels to nitrocellulose filters was set up as shown in Fig.8. The transfer buffer used was 20xSSC and transfer was made overnight at room temperature.The southern filters were air dried, baked under vacuum for 1 hour at 80°C and hybridized with the same conditions as in colony hybridizations.

(d) Promastigote blot hybridization

Leishmania promastigotes were counted and adjusted to $5x10^7$/ml and spotted on

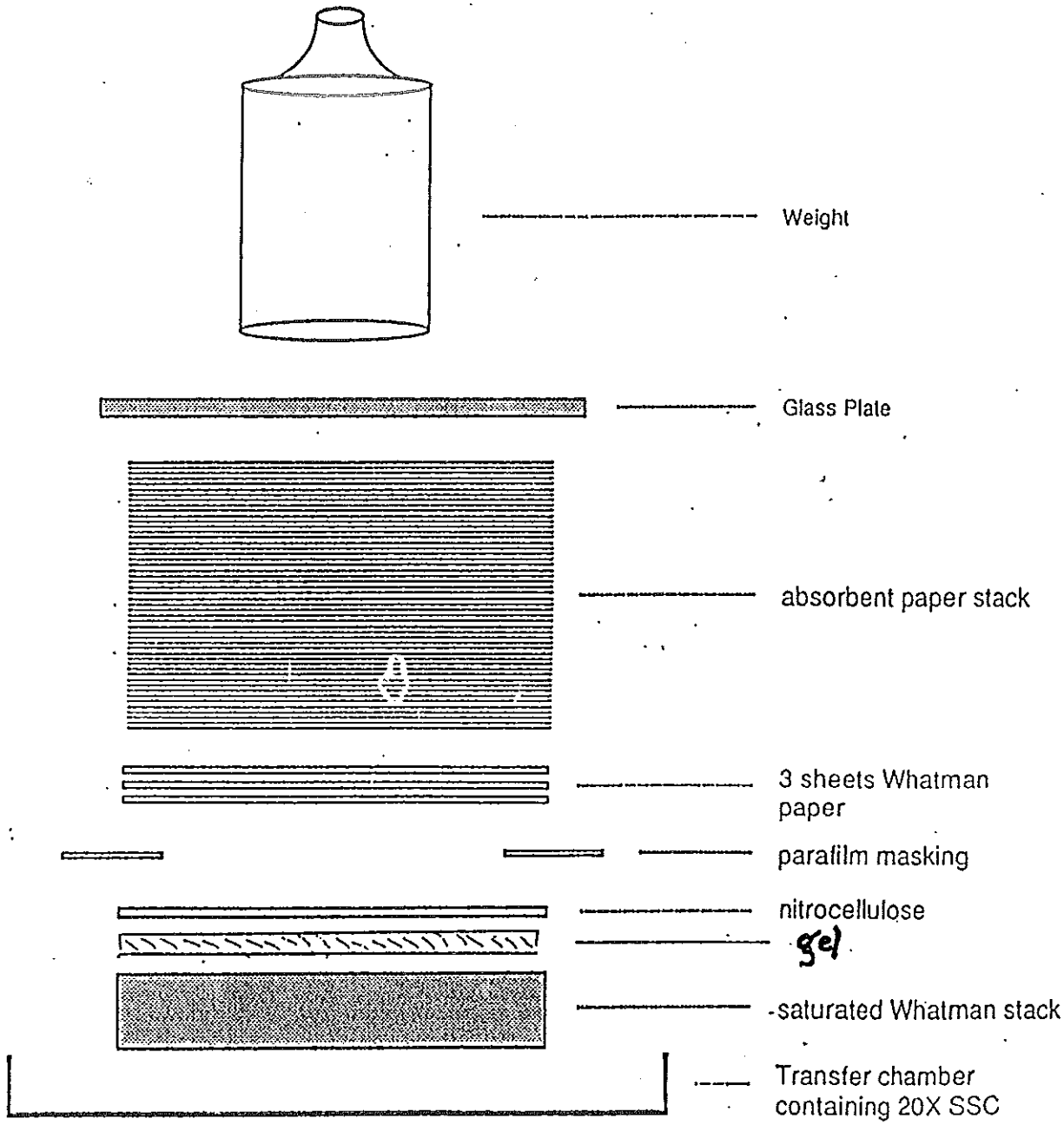


Figure 8. Transfer pyramids for Southern blotting. The capillary transfer of DNA samples, after electrophoresis of sample on agarose gels, was set up as shown.

to hybridization membranes (Nitrocellulose or zeta probe)(BioRad;USA). 10^5 promastigotes in 2 μl volume were applied manually or in 100μl volume using Bio-dot (Bio-Rad, USA) filtration apparatus as described by the manufacturers.

Nitrocellulose filters were processed and hybridized as described previously. Zeta probe membranes were pre-hybridized for at least 15 min at 65^{0}C by rocking in zeta probe hybridization solution (0.5 M $NaHPo_4$ pH 7.2, 7% SDS and 1mM EDTA). Hybridization was conducted for more than 14 hours under the same conditions in a fresh hybridization solution to which ^{32}P-radiolebelled probe was added
after denaturing by boilingfor 5 min.

Zeta probe membranes were washed in 40 mM NaH_2 $P0_4$, pH 7.2/5% SDS/1m M EDTA twice for 30-60min each time at 65^{0}C by rocking. Then filters were washed in 40 mM NaH_2P0_4 pH 7.2/1%SDS/1mM EDTA twice for 30-60 min each time underthe same conditions. Filters were monitored by autoradiography as described in previous sections. Sodium phosphate buffer was prepared according to the Henderson- Hasselbalch equation (Sambrook, *et al.* 1989).

Polymerase chain reaction(PCR):

(a) Sample preparation:

Purified DNA samples were boiled for 2 min. and used for PCR amplification. *Leishmania* promastigotes from culture were boiled for 15 min. and the supernatant was used.Blood samples were collected in 6 M Guanidine/0.2 M EDTA in a 1 to 1 ratio. The lysate was boiled for 30 min. and used for PCR.

(b) PCR primers and reaction:

Oligonucleotide primers-14R and 14F were designed based on the DNA sequence

of clone 14 to direct amplification of an expected 223 bp DNA fragment. The PCR mixture consisted of 10 mM Tris-Cl, pH 8.3.; 50 mM KCl; 25 mM $MgCl_2$; 0.01% gelatin; 0.2mM each of dATP, dTTP, dGTP, dCTP; 2μM of each primer and 2.5 U of *Taq* DNA polymerase in a final volume of 100 μl including 10 μl of sample. 100 μl mineral oil was overlayed to prevent evaporation. The following cycle profile was performed using a DNA thermal cycler. Denaturing at 94^{0}Cfor 45 sec.; annealing at 50^{0}C for 45 sec. and elongation at 72^{0}C for 1 min.; 30 cycles. 10 μl of the reaction was analyzed by electrophoresis on 2% agarose gel.

RESULTS

Preparation and Cloning of *L.donovani* kDNA:

The aim of this study was to clone sub-fragments of the kDNA minicircle in an attempt to develop DNA probes specific for Ethiopian *L.donovani* promastigotes. The yield and purity of the preparation was determined at wave lengths of 220-300 nm(Fig 9).The kDNA concentration was 125 μg/ml and the preparation was considered pure, since the ratio of $OD=_{260}$ to $OD=_{280}$ was 1.8.

Different fragments of kDNA as to their size and number were produced by using the different restriction endonucleases.

The linearized pUC18 DNA forms one band while undigested pUC18 DNA forms two adjacent bands (super coiled and relaxed). The fluorescence of pUC18 DNA was similar with the 3rd band (6557 bp) of the Lambda DNA (Fig 10). Therefore, amount of pUC 18 loaded= 6.557kb x 500ng / 48.514kb = 67.58 ngs. Where,total size of Lambda markers = 48.514 kb; amount of Lambda markers loaded on the gel = 500 ng; size of 3rd Lambda DNA band = 6.557kb. The transformation efficiency of *E. coli JM109* cells was calculatedated to be $4.8x\ 10^4$ transformed cells per microgram of plasmid DNA.

L.donovani kDNA Recombinant Clones:

Transformants for each set of ligation vary in number. A total of 112 transformed cells were obtained. Some of the *E. coli* JM109 colonies that grow in the presence of ampicillin might contain recombinant plasmids and others might contain plasmid DNA that has re circularized during the ligation reaction without insertion of foreign DNA. To discriminate between the two kinds of transformants screening was performed by colony hybridization using *L. donovani* total kDNA as a probe.

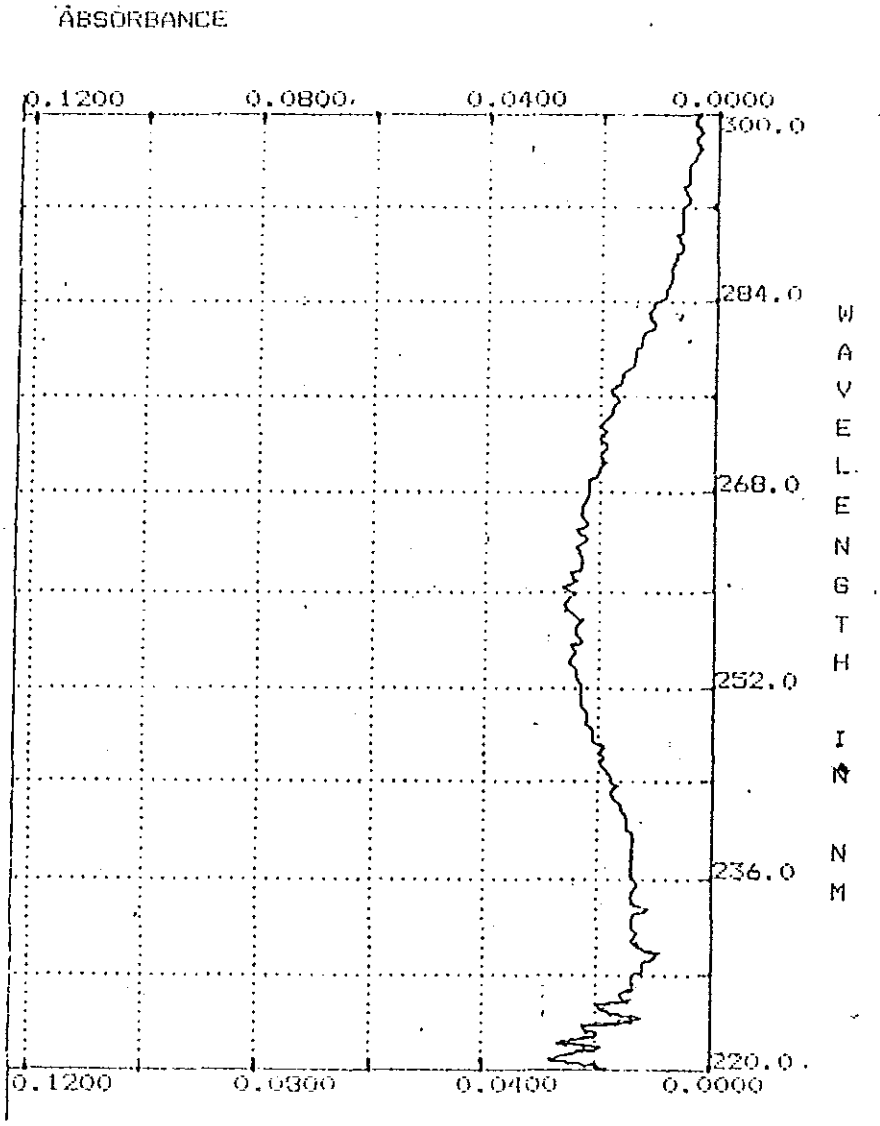


Figure 9. Spectrophotometric quantitation of kDNA. kDNA sample diluted 10^2X was estimated from the graph plotted by the spectrophotometer and the purity was determined as discussed in the materials and methods.

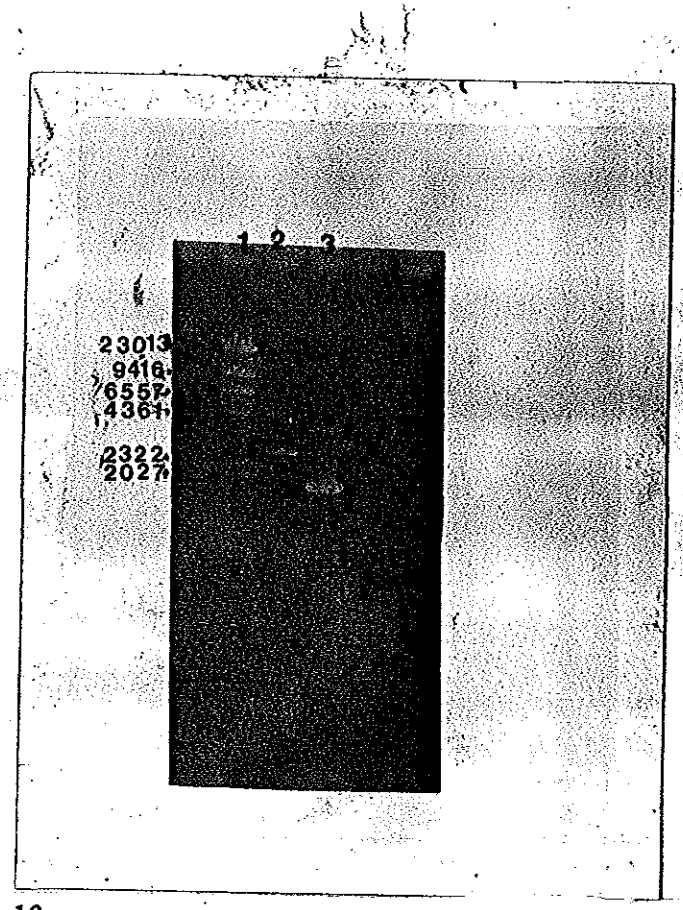


Figure 10.

Linearization of pUC 18 plasmid DNA. pUC 18 was linearized and analyzed by electrophoresis on 1.5% agarose gel. Intact pUC 18 (lane 3) showed supercoiled nature of the plasmid while linearized pUC 18 (lane 2) showed single band. The fluorescence intensity of λ DNA marker (lane 1) fragments were used to estimate the amount of linearized pUC 18.

As a first screening procedure to form *L. donovani* kDNA library, *E. coli* JM 109 colonies growing in the presence of ampicillin (100 μg/ml) were picked and replated using a grid template to enable easy discrimination.

18 colonies showing very strong and reproducible hybridization were selected for further analysis (Fig 11) after confirmation studies by Southern hybridization. The clones were numbered serially from 1 to 18 (Table 4).

Specificity of *L.donovani* kDNA recombinant clones:

As a first differential screening of the 18 *L. donovani* clones, colony hybridization was performed usingα^{32}P labelled whole kDNA probes from *L. donovani, L. aethiopica* and *L. major*. Confirming the previous hybridization results, whole kDNA from *L. donovani* hybridized with all the colonies but not with *E. coli* colonies without kDNA insert. by hybridization of total kDNA from *L. aethiopica* and *L. major* cross hybridzing colonies were idetified.

In another type of screening, 10^5 promastigotes of *Leishmania* were blotted on nitrocellulose or Zeta probe membranes. promastigote blots from *L. donovani*, *L. aethiopica*, *L. major* and *L. tropica* were hybridized with α^{32}-P- labelled plasmid DNA isolated from the *L. donovani* kDNA fragment bearing *E. coli* colonies and with α_{32}P labelled total kDNA isolated from *L. donovani* and *L. aethiopica*. Radiolabelled whole kDNA from *L. donovani* hybridized with all *Leishmania* promastigotes tested, but more strongly with the homologous promastigotes (Fig.12). Radiolabelled total kDNA from *L.aethiopica* also hybridized with all the promastigotes tested.

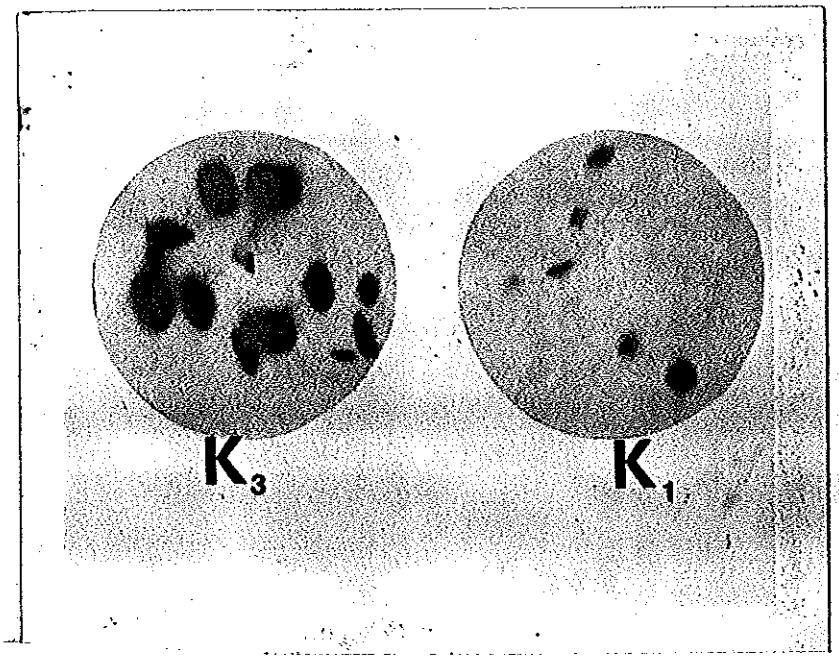


Figure 11.

Identification of recombinant clones by colony hybridization. E.coli colonies growing on ampicillin plates were lifted onto nitrocellulose, denatured, neutralized and baked. Filters were hybridized with radiolabelled L.donovani total kDNA probe. 18 colonies were detected by autoradiography on two filters (K1 and K3).

Table.4. Summary of *L.donovani* kDNA cloning and screening.

Enzymes used	No. of Transformants	No. of colonies with insert	designation.
Hae III	15	6	1 - 6
Rsa I + Alu I	20	–	–
Alu I + Hae III	53	12	7 - 18
Rsa I + Hae III	25	–	–

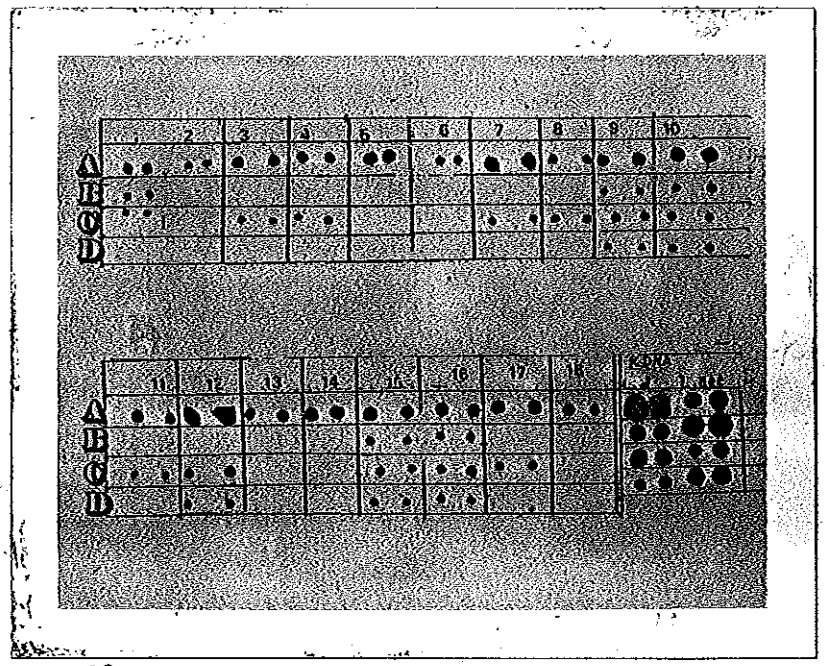


Figure 12.

Promastigote dot blot analysis of L.donovani kDNA clones. 10^5 promastigotes of Leishmania were blotted onto nitrocellulose or zeta probe membrane and hybridized with radiolabelled recombinant plasmid DNA and total kDNA probes.

Numbers 1-18 are clones used as probe. *L.donovani* (A); *L.aethiopica* (B); *L.major* (C), *L.tropica* (D); L.d=*L.donovani*, L.aet=*L.aethiopica*.

Different hybridization patterns were observed for the 18 colonies:

(1) Clones hybridizing with all Old World cutaneotropic *Leishmania* and with *L. donovani* : clones 9, 10,15 and clone 16.

(2) Clones hybridizing with *L. donovani* and any two of the three cutaneous *Leishmania:* In this group there are two clones (clone 1 and clone 12). Clone 1 hybridize with *L. donovani*, *L. aethiopica* and *L. major* but not with *L. tropica* while clone 12 hybridized with *L. donovani*, *L. major* and *L. tropica* but not with *L. aethiopica.*

(3) Clones hybridizing only with *L. donovani* and *L. major.* 6 clones hybridize with *L. donovani* and *L. major* only: These clones (clones- 3,4,7,8,11, and 17) can differentiate between *L. major* and the other two cutaneotropic species. *L. major* hybridized with a significant amount of the *L. donovani* clones (12 clones), unlike *L. aethiopica* and *L. tropica* suggesting a higher relatedness between the kDNA minicircle sequence of *L. donovani* and *L. major.*

(4) *L. donovani* specific clones: Six clones (clones,2, 5, 6, 13, 14, and 18 showed hybridization only with *L. donovani* but not with the other cutaneous *Leishmania* species tested.

These results suggested the presence of minicircle kDNA sequences which differentiate *L. donovani* from cutanotropic *Leishmania.* The overall results from colony and promastigote blot hybridizations using 2 *L. aethiopica*, 2 *L. major* and 1 *L. tropica* isolates (kDNA or promastigotes) showed six clones that were identified to be *L. donovani* specific in their hybridization. These cloned minicircle kDNA sequences apparently contain sequences not found in the minicircle kDNA of cutanotropic *Leishmania* species.

The hybridization intensity of the six clones to *L. donovani* was different. Two of these clones (clones 14 and 18) showed stronger hybridization than the others (clones 2,5,6 and 13). This might be related to the size of the clones. Specificity of *L. donovani* isolate specific clones to other Ethiopian *L. donovani* isolates was studied using the promastigote blot hybridization procedure. Two patterns of hybridization were observed (Fig.13).

(1) Clone hybridizing only with the parent *L. donovani* but not with other *L. donovani* isolates: clone 2.

(2) Five Clones hybridizing with all Ethiopian *L. donovani* isolates. Clones,5,6,13,14 and 18 were hybridizing with all Ethiopian *L. donovani* parasites tested. However the hybridization intensities were variable. When the clones were used as a probe the prefix Ld was used.

The specificity of clone 2 was restricted while the other 5 clones were with broad specificity for Ethiopian *L. donovani* isolates. The hybridization pattern of these 5 clones with *L. donovani* isolates from other geographic regions and with *L. infantum* and *L. chagasi* was not identical (see appendix 1).

The Size of L.donovani Specific Clones:

The size of the *L.donovani* specific clones was estimated in two ways (Table 5).

(1) Size estimation based on migration of restriction cut insert DNA (of the clones) on agarose gels was done.

(2) In a seperate study we have conducted nucleotide sequence studies of these clones (Rinke de Wit and Gessesse, un published) which provide the exact size of the clones as their DNA base sequence was known..

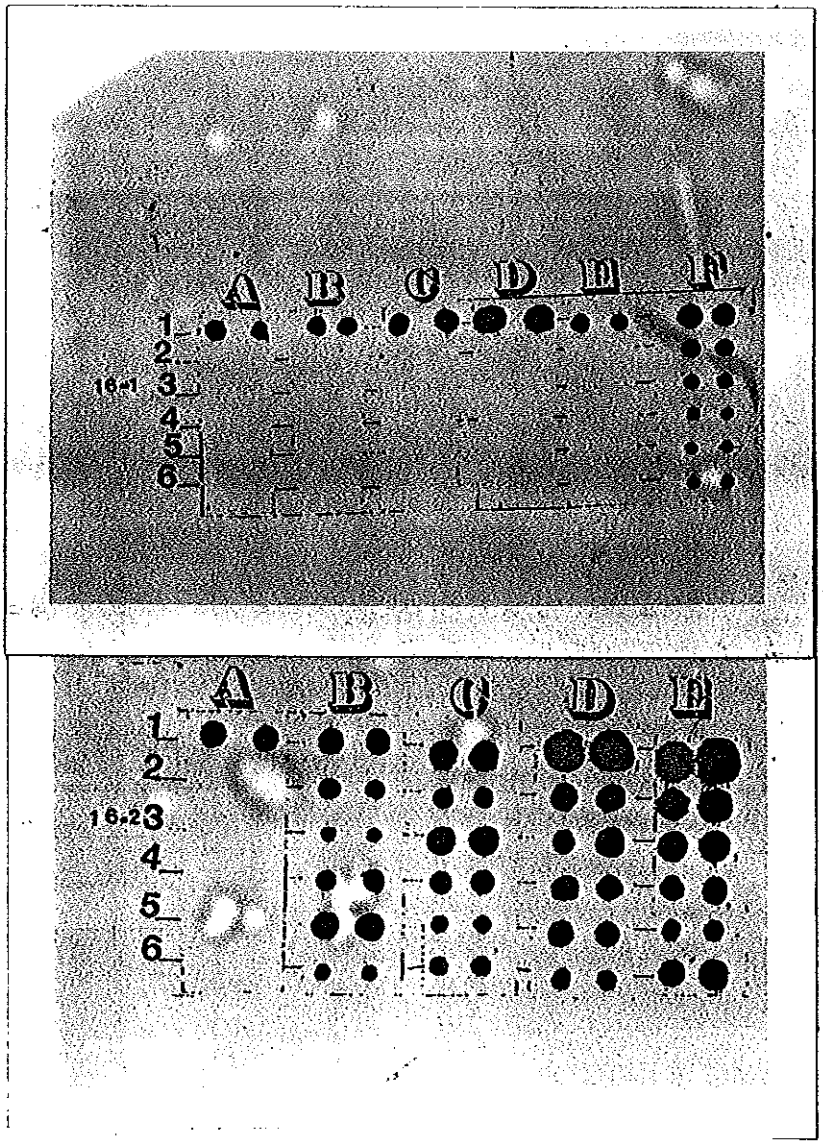


Figure.13. Promastigote dot blot analysis of selected *L.donovani* kDNA clones.
Promastigotes were blotted as described in previous sections and hybridized with radiolabelled recombinant plasmid DNA probes. Probes: clone 2 (A); clone 6 (B); clone 13 (C); clone 14 (D); clone 18 (E) and *L.donovani* total kDNA (F). Isolates= in 16 (a): *L.donovani* (1); *L.infantum* (2); *L.chagasi* (3);*L.aethiopica* (4); *L.tropica* (5) and *L.major* (6). Isolates in 16-2 are Ethiopian *L.donovani.*

Table 5. Size of *L.donovani* specific kDNA cloned fragments.

Clone	Insert size in bp
2	163
5	117
6	117
13	120
14	570
18	490

The DNA sequence of clone 5 and 6 was identical so that these clones have therefore identical inserts. The nucleotide sequence of clone 2,6,13,14 &18 is included in appendix 2. The sequence showed the relationship of these clones and it is in agreement with the hybridization results.

Detection of *L.donovani* parasites.

(a) Using probe Ld-14.

A representative dot blot hybridization(Fig.14) and Southern blot (Fig.15) is presented for clone 14. The sensitivity of this probe was 100 parasites (Fig.14 b). Probe Ld 14 specifically detected visceral *Leishamania* from E.Africa and Yemen but not other visceral parasites excpet one Chinese isolate (Table 6) on dot and Southern blot procedures.

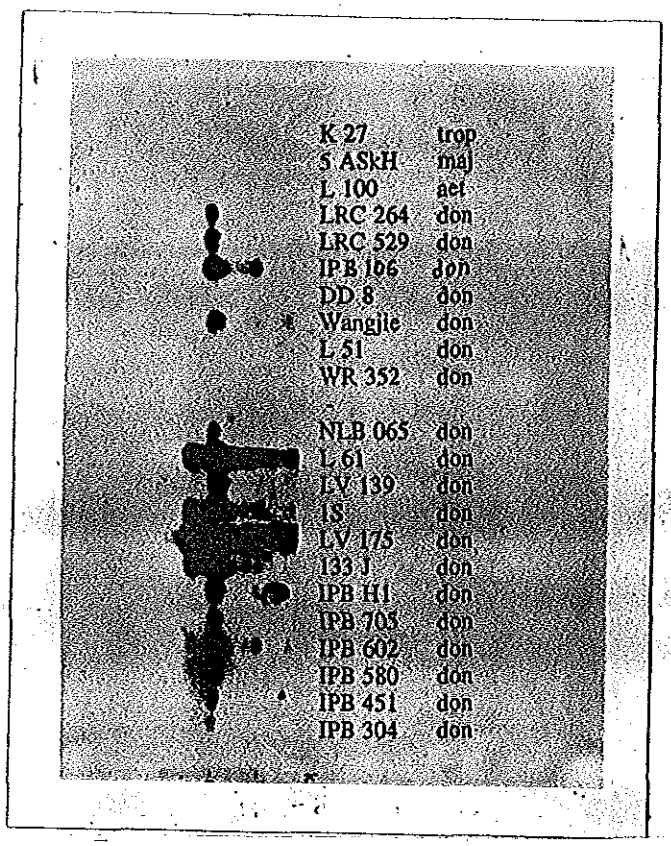


Figure.15. Detection of *Leishmania* on Southern blots. *Leishmania* total DNA (2 μg) was digested with Pst I and Southern blotted. The blot was hybridized with Ld-14 for 16 hours and detected by autoradiography. Probe Ld 14 detected visceral *Leishmania* from Ethiopia;Kenya;and Sudan.

Table 6. *Leishmania donovani* and *L.d.Sl.* species tested:geographial origin.

Probe Ld 14 used on dot and Southern blots.

Country of origin.	Isolates tested	Isolates detected
Ethiopia	22	22
Sudan	12	12
Kenya	2	2
Yemen	2	2
China	2	1*
S.Arabia	1	–
Iraq	1	–
Lebanon	2	–
Italy	1	–
India	4	–

*= Chinese isolate Wanjie I detected.

(b) using PCR.

As one important outcome of the sequence analysis, oligonucleotide primers which can start the amplification of a 223 bp region of the 570 bp clone 14, were prepared and used for PCR (polymerase chain reaction) studies. Amplification of *Leishmania* minicircle DNA using oligoneucleotide primers,14R and 14F, was performed to detect visceral *Leishmania* from cultured promastigotes, cryo-preserved material and from blood samples.

The PCR primers amplified sampels from all visceral *Leishmania* (Fig.16) with the exception of 2 visceral isolates, and 2 *L. aethiopica* isolates out of a total of 9 tested. Amplification from blood samples (10 μl) was achieved (Fig.16 b) with a sensitivity of 10 parasites.

The most widely used method of identification at present, is isoenzyme analysis by electrophoresis and currently this method is the base line of identification. The disadvantage of this method includes, the necessity of culture and the continuous subdivisions caused by the addition of new strains and new enzymes (Le Blancq *et al*,1986; Mebrahtu, *et al.*,1992).

Efforts have been directed toward the molecular biological techniques for the identification of *Leishmania*. Methods developed for this purpose include, chromosomal karyotyping (Bishop and Akinsehinwa, 1989); schizodeme analysis (analysis of kDNA restriction fragments, Lopes, *et al.*1984), DNA and kDNA buoyant density comparisons. However these methods are not simple to use and restricted to specialized laboratories. Ideally, the method should be easy to perform and to interpret, quick, reproducible and of low cost. Furthermore, it should be usable in field conditions.

DNA probe based techniques have been investigated for their potential as tools for identification. As a diagnostic strategy this approach offers significant advantages because all organisms contain DNA sequences which might be utilized in hybridization assays to distinguish among individuals, strains, species or genera. DNA probes have the advantage of being not stage specific and that the selection of species, sub-species and isolate specific probes is possible.

Another advantage of nucleic acid probe based assay is that a parasite infection is always accompanied by the corresponding parasite nucleic acids. Furthermore, by careful selection of unique invariant regions of the parasite genome, such probes can specifically detect reiterated, repetitive or multicopy sequences, thereby enhancing the sensitivity of detection.

Genomic DNA (Van Eys, *et al.*1991) or cDNA probes have been used to distinguish old world *Leishmania* species (Van Eys *et al.*1989; Howard *et al.* 1991) at the

complex level, but only following southern blot analysis and are therefore of little value for the rapid field identification. Further,if characterization is necessary for epidemiological purposes, it should be done by other methods.

More investigations have been done to use *Leishmania* minicircle kDNA because of their two important features. Firstly they are multicopy molecules which is an essential factor for sensitive detection. Secondly there is a gradient of sequence variation of the minicircle kDNA nucleotide sequence which enable the selection of desired specificity for a diagnostic probe.

Previously, total kDNA have been proposed as a diagnostic tool. However undesirable cross-reactions were observed (Lawrie *et al.* 1985; Barker *et al.* 1986). In this study also, it is confirmed that total kDNA of *L. donovani* can cross-hybridize to cutaneotropic species and the reverse is also true. This cross-hybridization of total kDNA hinders its usefulness for identification of various *Leishmania* species & strains.

The problem of cross-hybridization was overcome by the use of recombinant minicircle kDNA probes (Kennedy, 1984,; Barker, *et al.* 1986). Rogers & Wirth (1987) have shown that there are DNA sequences of different taxonomic relevance in a single minicircle kDNA of *Leishmania*. The kDNA minicircles contain conserved regions which are shared between all kinetoplastid parasites. The second category of the minicircle kDNA contain sequences shared between *Leishmania* species and there is a variable region which discriminates between *Leishmania* species.

As a result of the minicircle kDNA sequence diversity and the presence of sequence classes (Rogers, *et al.*1988), kDNA might stress differences between *Leishmania* parasites rather than their similarities (Van Eys, *et al.* 1989) which is a drawback of its use as a taxonomic criterion. Detection and characterization of *Leishmania* parasites of a given focus from mammals and vectors by hybridization with cloned minicircle kDNA

specific probes have the potential of providing extensive and detailed information (Barker *et al*, 1986; Jackson, *et al.*1986; Rogers *et al.*1988; Schoone, *et al.* 1991).

Visceral and cutaneous leishmaniasis are known in Ethiopia (Ayele, 1982; Humber *et al.*1988).Discrimination between the causative agents in humans, reservoir hosts and the insect vectors is an essential condition for the prevention and control of the diseases. Furthermore African visceral leishmaniasis is described (Hommel, 1978) to be associated with skin ulceration which might be confused with cutaneous cases. There is also a great confusion about the species of *Leishmania* responsible for mucocutaneous leishmaniasis, as indicated in recent studies in the Sudan (El-Safi, *et al.*1991). The dynamics of transmission of *L. donovani* in Ethiopia is not known and a proper diagnostic tool is required to elucidate the condition. Previously species specific kDNA probes for *L. aethiopica* have been generated and tested under field conditions (Laskay, *et al.* 1990; Laskay *et al.* 1991).

In this study the cloning of kDNA minicircle sequences specific for Ethiopian *L. donovani* is reported. After cloning *L. donovani* minicircle kDNA in pUC18 plasmids and propagation in *E. coli* Jm109, differential screening with total kDNA probes revealed six clones to be non reactive to cutanious *Leishmania*. Further analysis of these clones showed that two clones, clone 5 and 6 were identical and one,clone 2, was hybridzing only to *L. donovani* (IPB/399) and one Sudanise isolate.

Clones 6,13,14 and 18 were hybridizing with all Ethiopian *L. donovani* from S.W. and N.W. Ethiopia. Clone Ld 14 can be used for the specific detection of E. African visceral *Leishmania* by dot blot,touch blot and Southern blot procedures directily and/ or using PCR as non specific target amplification procedure for subsequent detection by specific probes. The cross hybridization of Ld 14 to one Chinese isolate should have to be verefied by further testing of the isolate for its identity. The aim of the PCR assay in

this study was to indicate the use of minicircle kDNA as PCR targetes for very highly sensitive detection of *Leishmania*.

In the use of these clones as a probe for *Leishmania* parasites, radioactive labelling using α^{32}P-isotope was employed. There are disadvantages in the use of radioactive compounds in general and α^{32}P in particular. Apart from the short half life (14 days), which requires an immediate use, there are health hazards in its use.

Other labelling methods of DNA probes have been described in the literature of nucleic acid hybridizations, such as those reviewed by Matthews and Kricka,(1988); Keller and Manak,(1989); Kozma and Adinolofy,(1987); and Van Eys, *et al.*(1987) as alternatives for radioactive labelling. However,although different non-radiolabelling systems are under investigation and their advantage is immense, none is yet ready for standard use.

CONCLUSION

In this study five clones, generated from *L. donovani* (IPB/399) minicircle kDNA and selected based on their non-reactivity with cutanotropic *Leishmania* by hybridization analysis, are reported. The clones showed differences in their sensitivity and specificity to Ethiopian *L. donovani* isolates and others.

(1) Restricted specificity by Clone 2.

Clone 2 detected only the parent *L.donovani* (IPB/399) and one Sudanise isolate. but no other Ethiopian *L. donovani* isolates. Therefore, the use of this clone is restricted, to *L. donovani* (IPB/399), an isolate from S.W. Ethiopia.

(2) Broad specificity:

(a) Less sensitive: Clones 6 and 13 are small clones in size and they are identical in 2/3 of their DNA base sequences. They have similar hybridization specificity and sensitivity on dot blots. Their sensitivity is lower(>100 parasites) as compared to the other Ethiopian *L. donovani* specific clones as observed from titration dot blot experements. Therefore, their use in the direct detection of Ethiopian *L. donovani* may not be recommended unless additional procedures which increase sensitivity of detection are used.

(b) More sensitive: Clones 14 and 18 are relatively large clones in size and they have no relation in their DNA base sequences (appendix-2). They have similar specificity and strong sensitivity (100 parasites) for Ethiopian *L. donovani* parasites.In order to know the differences among clone 14 and 18 as to their specificity and sensitivity, further analysis using viscearlizing *Leishmania* from different geographical regions of the world was conducted. This showed the broader application of these clones beyond the detection of

Ethiopian *L. donovani* parasites. These clones can be used for the direct detection of Ethiopian *L. donovani* parasites. It is also possible to maximize their sensitivities using different strategies such as PCR (appendix:3). The *L. donovani* kDNA clones generated in this study may have the following applications in Ethiopia.

1. For the direct detection of *L. donovani* in humans, insect vectors and reservoir hosts in large scale epidemiological studies.
2. For the non-invasive diagnosis of cutaneous and muco-cutaneous diseases due to *L. donovani*.
3. For the early diagnosis of asymptomatic, cryptic infections and co-infections of *L. donovani* with other parasites and AIDS (TDR,1991).
4. For monitoring chemotherapy and drug trials
5. In well equipped laboratories, it may be possible to use PCR as target amplification for more sensitive detection by hybrization probes (appendix:3) or to develop specific PCR-assays.

In general, these clones might be instrumental for the identification and diagnosis of Ethiopian *L. donovani* parasites when the proper diagnostic/identification strategy, among the several alternatives (appendix 3) is selected and optimized because sensitivity and specificity of a DNA probe are function of multiple parameters.

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APPENDIX

1. Appendix 1. List of *Leishmania* parasites, strains,and isolates used in this study and hybridization results.
pp 73-75.
N.B.
Ld = clone numbers
14 R and 14 F = Reverse and Forward PCR primers, respectively.
\+ = hybridizing
\- = not hybridizing
* = WHO referance strains.

2.Nucleotide Sequence of the *L.donovani* specific cloned minicircle kDNA probes: pp 76-78.

No	WHO or Donor's Code	Species	Geog. Origin	Cloned Miniciricle kDNA Probes					PCR14R/P
				Ld-2	Ld-3/6	Ld-13	Ld-14	Ld-18	
1	MHOM/IN/80/DD8'	L.d.d.	India	-	+/-	+/-	-	+	+
2	MHOM/IN/00/DEV 1	"	"	-	-	-	-	-	-
3	MHOM/IN/71/LRC-L51	"	"	NT	NT	NT	-	NT	+
4	MHOM/IN/WR 552	"	"	NT	NT	NT	-	NT	NT
5	MCAN/IT/76/Dora	L.d.Sl	Italy	NT	NT	NT	-	NT	+
6	MHOM/IQ/77/Basra 3	"	Iraq	NT	NT	NT	-	NT	+
7	MHOM/LB/84/Sakl 3	"	Lebanon	NT	NT	NT	-	+	+
8	MHOM/LB/84/Sakl 4	"	"	NT	NT	NT	-	+	+
9	MHOM/CN/00/Wanjie 1	"	China	-	+	+	+	+	+
10	IALE/CN/84/Turfan 10	"	"	NT	NT	NT	-	NT	+
11	MHOM/SA/81/Jeddah-KA	"	S.Arabia	-	-	-	-	-	+
12	MHOM/YE/86/LEM 953	"	Yemen	NT	NT	NT	+	NT	+
13	MHOM/YE/87/Lem 1085	"	"	NT	NT	NT	+	NT	+
14	MHOM/SD/68/1S	"	Sudan	+	+	+	+	+	+
15	MHOM/SD/62/3S	"	"	-	+	+	+	+	+
16	MHOM/SD/00/Khartoum	"	"	NT	NT	NT	+	NT	+
17	MHOM/SD/82/Orfan	"	"	NT	NT	NT	+	NT	+
18	MHOM/SD/90/D83	"	"	NT	NT	NT	+	NT	+
19	MHOM/SD/90/D73	"	"	NT	NT	NT	+	NT	+
20	MHOM/SD/91/D1809	"	"	NT	NT	NT	+	NT	+
21	MHOM/SD/00/Sudan IV	"	"	NT	+/-	+	+	+	+
22	MHOM/SD/75/LV 159	"	"	NT	NT	NT	+	NT	+
23	LRC-264	"	"	NT	NT	NT	+	NT	+
24	LRC-529	"	"	NT	NT	NT	+	NT	+
25	MHOM/KE/83/NLB 065	"	Kenya	-	+/-	+	+	+	+
26	MHOM/KE/00/NLB 218A	"	"	NT	-	±	+	+	+
27	MHOM/SD/75/L 61	"	Sudan	NT	NT	NT	+	NT	NT
28	MHOM/ET/89/PB399	"	Ethiopia	+	+	+	+	+	+
29	MHOM/ET/67/HU3	"	"	-	+	+	+	+	+
30	MHOM/ET/75/LV 175	"	"	-	+	+	+	+	NT
31	MHOM/ET/82/Leasa	"	"	-	+	+	+	+	+
32	MHOM/ET/83/Kotesa	"	"	-	+	+	+	+	+

№	WHO Donor's Code	Species	Geog. Origin	Cloned kDNA Probes					PCR 14R/P
				Ld 2	Ld5/6	Ld 13	Ld 14	Ld 18	
33	MHOM/ET/84/Addis 164	L.don.Sl.	Ethiopia	-	+	+	+	+	+
34	MHOM/ET/89/1PB 445	L.don.Sl.	Ethiopia	-	+	+	+	+	+
35	MHOM/ET/89/1PB 446	L.d.Sl.	Ethiopia	-	+	+	+	+	+
36	MHOM/ET/89/1PB 447	"	"	-	+	+	+	+	+
37	MHOM/ET/89/1PB 448	"	"	-	+	+	+	+	+
38	MHOM/ET/89/1PB 449	"	"	-	+	+	+	+	+
39	MHOM/ET/89/1PB 450	"	"	-	+	+	+	+	+
40	MHOM/ET/89/1PB 451	"	"	-	-	-	+	-	+/-
41	MHOM/ET/91/1PB 561(306)	"	"	-	+	+	+	+	+
42	MHOM/ET/91/1PB 565(301)	"	"	-	+	+	+	+	+
43	MHOM/ET/91/1PB 566(705)	"	"	-	+	+	+	+	+
44	MHOM/ET/91/1PB 568(602)	"	"	-	+	+	+	+	+
45	MHOM/ET/91/1PB 579(304)	"	"	-	+	+	+	+	+
46	MHOM/ET/91/1PB 580(106)	"	"	-	+	+	+	+	+
47	MHOM/ET/91/1PB 583(H-1)	"	"	-	+	+	+	+	+
48	MHOM/ET/91/1PB 601	"	"	NT	NT	NT	+	+	+
49	MHOM/ET/91/1PB 613	"	"	NT	NT	NT	+	+	+
50	MHOM/FR/88/LPMA 34	L.d.inf.	France	-	-	-	-	+/-	+
51	MHOM/FR/89/LPMA 73	"	"	-	-	-	-	-	+
52	MHOM/FR/89/LPMA 78	"	"	-	-	-	-	-	+
53	MHOM/FR/73/LPMA 56	"	"	-	-	-	-	+/-	+
54	MHOM/FR/83/LPMA 67	"	"	-	-	-	-	-	+
55	MHOM/FR/86/LPMA 71	"	"	-	-	-	-	-	+
56	MHOM/FR/87/LPMA 72	"	"	-	-	-	-	-	+
57	MHOM/FR/74/LPMA 57	"	"	-	-	-	-	-	+
58	MHOM/FR/83/LPMA66	"	France	-	-	-	-	-	+
59	MHOM/TN/80/1PT1*	"	Tunisia	-	-	-	-	+/-	+
60	MHOM/ES/81/BCN 1	"	Spain	-	-	-	-	-	NT
61	MHOM/CN/54/Peking	"	China	NT	NT	NT	-	NT	+
62	MHOM/BR/72/PP75*	L.d.cha.	Brazil	-	-	-	-	-	+

No	WHO Donor's Code	Species	Geog. Origin	Cloned Miniciricle kDNA Probes					PCR 14 RP
				Ld-2	Ld-5/6	Ld-13	Ld-14	Ld-18	
63	HR7	L.d.Cha.	Brazil	-	-	-	-	-	+
64	CR3	"	"	-	-	-	-	-	+
65	MHOM/ET/83/1467-83	L.inf.	Ethiopia	-	-	-	-	-	NT
66	MHOM/ET/72/L100*	"	"	-	-	-	-	-	+
67	IPHL/ET/89/SP169	"	"	NT	-	-	-	-	-
68	IPHL/ET/89/SP329	"	"	NT	NT	NT	-	-	-
69	Ouro Ouaa	"	"	NT	NT	NT	-	-	-
70	Wiadun	"	"	NT	NT	NT	-	-	+
71	Kasuya	"	"	NT	NT	NT	-	-	-
72	Chouadi	"	"	NT	NT	NT	-	-	-
73	Disco	"	"	NT	NT	NT	-	-	-
74	MHOM/SU/79/SASKH*	L.maj.	USSR	-	-	-	-	-	-
75	MHOM/SU/59/P	"	"	-	-	-	-	-	-
76	MHOM/IR/72/Nadia5	"	Iran	NT	NT	NT	-	NT	-
77	MHOM/SA/89/Hu	"	S.Arabia	NT	NT	NT	-	NT	-
78	MHOM/SN/00/DK1	"	Senegal	NT	NT	NT	-	NT	-
79	MHOM/IL/00/WR309	"	Israel	-	-	-	-	-	NT
80	MHOM/KE/00/NLB765	"	Kenya	NT	NT	NT	-	NT	-
81	MHOM/SU/74/K27	"	USSR	-	-	-	-	-	-
82	MHOM/SU/60/OD	L.trop.	"	-	-	-	-	-	-
83	MHOM/KE/00/NLB162	"	Kenya	NT	NT	NT	-	-	-
84	MHOM/SA/00/912-33	"	S.Arabia	NT	NT	NT	-	NT	-
85	MHOM/IQ/65/L75	"	Iraq	NT	NT	NT	-	-	-
86	MHOM/IN/79/DD7	"	India	NT	NT	NT	-	-	-
87	MHOM/IL/75/LV140	"	Israel	NT	NT	NT	-	-	-

leishman.2

```
        10          20          30          40          50          60
CCAGCCCCAC TCATCCCATA CAACCCAAAT AAACCCACTA ATAATTAAAT CAACCTCAAT
           F                                       M           M
           O                                       S           N
           K                                       E           L
           1                                       1           1

        70          80          90         100         110         120
ACCAAAGCCA CCAAAATCAG AAAACAAACA AACCTCCAGA GCATAAACCA GCGAAACACG
                                  M                               F
                                  N                               N
                                  L                               U
                                  1                               2

       130         140         150         160
CGAGTCCCAA ACCCAAAAGC AAAAAATCAA AGGCAAACTC TGG
 P
 L
 E
 1
```

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leishman.6

```
        10          20          30          40          50          60
CCGAAACAAA AACAAGCCTC CAGACCCAGG GGTCCAGGCT AAATATATAT ATATTAAAGC
               M       D   B   N A   B                       M
               N       R   S   L V   S                       S
               L       D   T   A A   T                       E
               1       1   1   4 2   1                       1

        70          80          90         100         110
AAAGGAAAGG TGTAAACCCC CAACACCATG CTAATAACCA GGCAACCCTA TCCAAGG
                  T       N           B         E     S
                  T       L           S         C     T
                  H       A           T         O     Y
                  2       3           1         1     1
```

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leishman.13

```
        10          20          30          40          50          60
CCAATCCAGC CCAGGGAAGC CCATTTCCAG CCACCACCCG GCTAAATATA TATATATTAA
           B                                   NH                  M
           S                                   CP                  S
           T                                   IA                  E
           1                                   12                  1

        70          80          90         100         110         120
AGCAAAGGAA AGGTGTAAAC CCCCAACACC ATGCTAATAA CCAGGCAACC CTATCCAAGG
                     T       N           B         E     S
                     T       L           S         C     T
                     H       A           T         O     Y
                     2       3           1         1     1
```

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leishman.14

        10         20         30         40         50         60
CCAGAGTTTG CCTTTGATTT TTTGCTTTTG GGTTTGGGAC TCGCGTGTTT CGCTGGTTTA
                                        P    F
                                        L    N
                                        E    U
                                        1    2

        70         80         90        100        110        120
TGCTCTGGAG GTTTGTTTGT TTTCTGATTT TGGTGGCTTT GGTATTGAGG TTGATTTAAT
       M                                          M         M
       N                                          N         S
       L                                          L         E
       1                                          1         1

       130        140        150        160        170        180
TATTAGTGGG TTTATTTGGG TTGTATGGGA TGAGTGGGGC TGGACATATA CGAGAGTATT
                             F
                             O
                             K
                             1

       190        200        210        220        230        240
ACATTCTACA CAGCACAGTC AAATTATAGT CAAATATAGA ACACGAATCA TAGACAAATA
                                                H
                                                I
                                                N
                                                1

       250        260        270        280        290        300
AGCGTATATA GACTAGTATT TATGTATTGT AGAATATTTA TAAACTATTA CCTATAAACT
            S                      S
            P                      S
            E                      P
            1                      1

       310        320        330        340        350        360
ATAAATATAA ACTAATTATA GCGGAGGACC CGAAACCCTA GGGTTACCAA CACCTTACCC
                         MPN           A     B    T            M
                         NPL           V     S    T            N
                         LUA           R     T    H            L
                         114           2     2    2            1

       370        380        390        400        410        420
TCCCCTATAA CTAATAATCA ATATTAAAGA GGCAGGTGTC CAGACCCCTT ATTACCCCTC
                    S    M    M   B                          M
                    S    S    N   S                          N
                    P    E    L   P                          L
                    1    1    1   1                          1

       430        440        450        460        470        480
CATCACCTCT TAACACCCTA AATCCATAAA CTCTAAAACC TAGACAGCCC AGTATAAGCC
  H  M   M                                          B          M
  P  N   S                                          S          N
  H  L   E                                          R          L
  1  1   1                                          1          1

       490        500        510        520        530        540
TCCGAACCCA TCTATTCACG CCCACCGCCT CCAAGCACAT CCACTTAACA CCCAGAACGC
                             M          F       M               M
                             N          O       S               N
                             L          K       E               L
                             1          1       1               1

leishman.14

       550        560        570
CTCCAATCCA GCCAACCCAG CCCCAAAAAG
```

leishman.18

```
        10         20         30         40         50         60
CCGGGAAATT CGGGGAAATT GCCCTTCCTG AGCCTCCTAG CCCGTTGTTT TGGTGGTTTC
N                             D     M
C                             D     N
I                             E     L
1                             1     1

        70         80         90        100        110        120
TAAATTTAGA TATTGAATTC TGACTTAAAT TTGCTGAATA TTGGTGGTTT TGGGATGTGT
                E          M          S                    F
                C          S          S                    O
                O          E          P                    K
                1          1          1                    1

       130        140        150        160        170        180
TTGGTCCTCG CGGCTGGTCT TGAGGCGGGC GGTGGCTGGT TTTAGATGTG GGCTGCGTTT
  A  M  FF             M                                   B
  V  N  NN             N                                   B
  A  L  UU             L                                   V
  2  1  21             1                                   1

       190        200        210        220        230        240
GGTGGTATGG GTATTCTTTA TGGATTGGTT TCCGACACTG GCTTGGCTTC ATTGGATTTG
                                           B
                                           S
                                           R
                                           1

       250        260        270        280        290        300
ATGGTGGTGG TTGGGATTGG ATGATAGTGA GCATAGGTTT GATATACAGA ATAAGTTATA
                    F
                    O
                    K
                    1

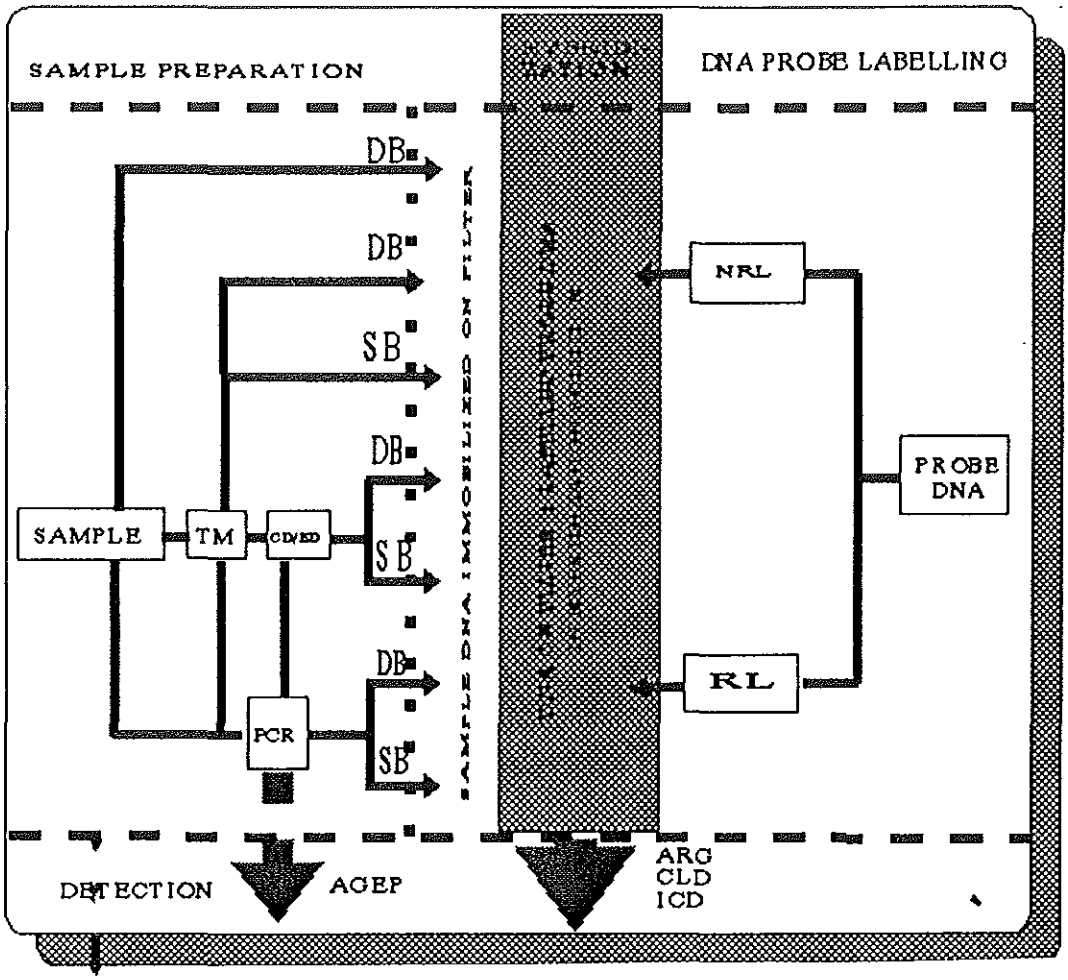
       310        320        330        340        350        360
TATTAAATAT GGCATTAGGG GTATTCAATA GGGGATACGA TAGCGTAGGT AGACTAGTTA
  M                                                 A    S
  S                                                 C    P
  E                                                 C    E
  1                                                 1    1

       370        380        390        400        410        420
TTTGTAGTTA TTATATTATT TATGATAACT CAACCTATAG ACATAACGTG CATAATATTT
                                                          S
                                                          S
                                                          P
                                                          1

       430        440        450        460        470        480
CTACTCTCTA ATTTACGATC TTTTTTATTG TATTGTATGG TAGCCTATTT GTGAGTATGT
                   S
                   A
                   U
                   A

       490
AGATGGTGTA G
```

Appendix 3. flow diagram showing possible strategies of detecting Leishmania using DNA probes.



N.B.

TM = Transport media; CD = Chemical digestion; ED = Enzyme digestion; DB = Dot blot; SB = Southern blot; NRAL = Non radioactive labelling; RAL = Radio labelling; AGEP = Agarose gel electrophoresis; ICD = Chromogenic substrate detection; CLD = chemiluminescent detection; ARG = Autoradiography.

Appendix 4.List of Enzymes used in this Study.

Enzyme	**Manufacturer**
Alu I	New England Biolabs (U.S.A.)
Hae III	" " " "
Rsa I	" " " "
Hinc II	Pharmacia (Sweden)
Proteinase K	Boehringer Mannheim (Germany)
Alkaline Phosphatase	Pharmacia (Sweden)
T4 DNA ligase	New England Biolabs (U.S.A.)
DNase	Amersham (U.K.)
E.coli DNA Polymerase	" " "
Pst I	New England Biolabs
Taq DNA polymerase	Cetus (U.S.A.)
Lysozyme	Sigma (U.S.A.)