CHARACTERIZATION OF *LEISHMANIA* PARASITES: MOLECULAR KARYOTYPE ANALYSIS.

BY

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DECLARATIONS

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATED TO:

MY HUSBAND GEORGE MESHACK NYAMBATI

AND CHILDREN

LEVI MORIASI, CLAIRE KEMUNTO AND

ALLAN SHITAKHA-MANOTI

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LIST OF ABBREVIATIONS

ARPPIS	African Regional Postgraduate Programme in Insect Science
CL	Cutaneous leishmaniasis
cm	centimetre
cpm	counts per minute
DDT	Dichloro Diethyl Trichloroethane
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
g	gramme
hr(s)	hour(s)
ICIPE	International Centre of Insect Physiology and Ecology
ILRAD	International Laboratory for Research in Animal Diseases
IPR	Institute of Primate Research
kb	kilobase
KEMRI	Kenya Medical Research Institute
l	litre
M	molar
mA	milliamp
MCL	Mucocutaneous leishmaniasis
mg	milligramme

min----------minute

ml----------millilitre

mm----------millimetre

mM----------millimolar

NNN----------Novy McNeal and Nicolls Medium

^{o}C----------degrees Centigrade

OFAGE----------Orthogonal Field Alternation Gel Electrophoresis

%----------percent

PFG----------Pulsed Field Gradient gel electrophoresis

pH----------hydrogen ion concentration

RPMI----------Rosewell Park Memorial Institute

sec----------second

ul----------microlitre

UV----------ultra violet light

VL----------Visceral leishmaniasis

w/v----------weight by volume

WHO----------World Health Organization

xg----------centrifugal force

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SUMMARY

Leishmaniasis is a zoonotic protozoal disease caused by intracellular parasites, known as ***Leishmania***, transmitted through the bite of female phlebotomine sandflies. Apart from human infections the same parasite species infect other mammals and reptiles. Research on leishmaniasis has been hampered by the difficulties in distinguishing between different species of ***Leishmania*** associated with various clinical manifestations.

The traditional classification of the species and subspecies, based mainly on clinical and epidemiological findings, has led to a lot of confusion. The taxonomy and identification of ***Leishmania*** species, which has been the subject of intensive studies in the last two decades, still needs clarification regarding the biology, epidemiology, immunology and chemosensitivity of all parasites in this genus. The ability to link leishmania parasites found in wild animals or in the sandfly vectors to those found in humans (or other hosts) is of paramount importance in the epidemiology and clinical investigations of the disease.

The objectives of this study were, therefore, to distinguish ***Leishmania*** species using a modern approach. This involved the examination of the molecular karyotypes and determination of the taxonomic relationships among

Leishmania species. Pulsed field gel electrophoresis techniques were applied to allow the separation of entire chromosome-sized or large DNA molecules. With these methods it is possible to localize and identify particular genes useful for species differentiation.

Investigations carried out here resulted in the determination and standardization of different parameters affecting the running of pulsed field gel electrophoresis. A good separation was obtained by using $1.5X10^7$ parasites at a pulse frequency of 40 sec, temperature 8^0C and electrophoresis was performed for 20 hrs at 300 volts.

Standard chromosome profiles have been established using six WHO reference strains for species identification and characterization of leishmania parasites. The profiles of the six strains could be divided into three different groups (I, II and III). Group I had four strains namely, ***L. infantum***, ***L. aethiopica***, ***L. major*** (Kenya) and ***L. donovani***. Groups II and III had one strain each and these were ***L. adleri*** and ***L. major*** (Israel) respectively. Chromosomal DNAs have also been analyzed from 25 cloned new leishmania isolates and their chromosome profiles compared with those of the WHO reference strains. The banding patterns of 13 isolates tested were similar to reference strains in Group I while 3 isolates fitted in Group II. None of the new isolates fitted in Group III. Nine isolates were different.

from the reference strains and therefore did not fit into any of the three groups.

The four reference strains which fitted in Group I could be distinguished from each other by digestion with restriction endonucleases.

Cloning of a fragment from reference strain ***L. major*** (ICIPE 235) showed both the insert and plasmid bands after separation by agarose gel electrophoresis.

The work carried out has demonstrated that orthogonal field alternation gel electrophoresis could be used for typing new ***Leishmania*** isolates. Restriction endonucleases could be used for intra-species differentiation.

CHAPTER 1:

LEISHMANIA AND LEISHMANIASES

LEISHMANIA AND LEISHMANIASES:

1. General Introduction

Leishmaniases are closely related diseases caused by several distinct species, subspecies and strains of flagellate protozoan parasites, known as ***Leishmania***, which are transmitted through the bite of female sandflies (Figure 1). Among protozoal diseases, leishmaniases are second in importance to malaria (Lainson and Shaw, 1978; Lee ***et al.***, 1985). An estimate by a WHO panel of 400,000 new cases per year has been reported (UNDP/WORLD BANK/WHO, 1981; Anon, 1984). Recently, UNDP/WORLD BANK/WHO (1990) reported that 350 million people are at risk of acquiring leishmaniases and that approximately 12 million are currently infected (WHO, 1990).

The geographical distribution of leishmaniases is determined by the composition of the whole parasite-vector-host system. The diseases have been reported to occur in over 100 countries (WHO, 1984a). Endemic foci of leishmaniases are scattered over wide areas of the globe in tropical and subtropical countries of Africa, Asia, Western Europe, Central and South America. These include China, India, Iran, Afghanistan, the Mediterranean region, the Middle East, Portugal, Mexico, the nothern part of Argentina, Sudan, Ethiopia, East and West Africa (Mutinga,

Fig. 1. The sandfly (Diptera, Phlebotominae. Picture courtesy of Dr. J.B. Kaddu).

1986a; Peters and Killick-Kendrick, 1987). The diseases strongly associated with occupation are found in isolated, impoverished rural areas. Not only are the leishmaniases a problem of the most economically under-developed and disadvantaged regions of the world, they also tend to be concentrated in the lowest socio-economic classes. People engaged in various farming practices, mining and fishing have a greater risk of being bitten by the sandfly vectors which transmit the causative agents of leishmaniases. High incidences of human leishmaniases which occur during movement of migrants, labourers and displaced people do not capture the attention of the public health authorities as do more dramatic outbreaks of "killer" diseases. Treatment for the diseases is expensive for many resource-poor people. They therefore forego treatment since the disease is a silent killer.

1.1 Historical background

Leishmanial amastigotes were first observed by Cunningham (1885) inside macrophages taken from a "Delhi boil" in India but he did not name them. Firth, in 1891, confirmed Cunningham's work and suggested the name ***Sporozoa furunculosa*** (cited by Wenyon, 1926). In 1903 Leishman described amastigotes that he had found three years earlier in a patient infected in Calcutta with "dum-dum fever".

Independently, Donovan (1903) also reported these parasites in spleen punctures from cases in Madras. Laveran and Mesnil (1903) saw Donovan's preparations and named the parasites ***Piroplasma donovani***. At the same time Ross (1903) proposed the name ***Leishmania***. A cutaneous lesion was noticed by Wright (1903) on a child who had come from Armenia; he proposed the name ***Helicosoma tropicum*** and suggested that the parasite was a protozoan. This name was modified to ***Leishmania tropica*** by Luhe (1906). Subsequently Nicolle (1908) observed a similar parasite in infants suffering from "black-disease" in the Mediterranean and named it ***Leishmania infantum***. Three years later, another parasite observed in cutaneous lesions in Brazil by Vianna (1911) was named ***Leishmania braziliensis***. Between 1903 and 1950 the naming of the species was based on geographical distribution and clinical manifestations in humans. Gardener (1977) gave a review of the numerous synonyms attached to parasites of this genus.

The discovery of the insect vector, the sandfly, was by workers in India (Swaminathan ***et al.***, 1942), who succeeded in transmitting ***Leishmania donovani*** to humans by the bite of experimentally infected ***Phlebotomus argentipes***. Adler and Ber (1941) transmitted ***Leishmania tropica*** to humans by the bite of ***Phlebotomus papatasi***.

1.2 Parasite

1.2.1. Taxonomy: (Honigberg *et al.*, 1964; Kudo, 1966).

Phylum :	Protozoa
Subphylum:	Sarcomastigophora
Superclass:	Mastigophora
Class:	Zoomastigophorea
Order:	Kinetoplastida
Suborder:	Trypanosomatina
Family:	Trypanosomatidae
Genus:	***Leishmania***

The classification of the genus ***Leishmania*** is shown in Figure 2. There are seven species and 11 subspecies of ***Leishmania*** infecting humans in the Old and New Worlds (Molyneux and Ashford, 1983; Barker, 1989). According to Lainson and Shaw (1979) these are ***L. aethiopica*** Bray, Ashford and Bray; ***L. donovani*** Leveran and Mensil; ***L. major*** Yakimov and Schokhov; ***L. tropica*** Wright; ***L. braziliensis*** Vianna; ***L. mexicana*** Biagi; and ***L. peruviana*** Velez.

Leishmania can also be grouped according to their development in the sandfly vectors. The life cycles in the sections Hypopylaria, Peripylaria and Suprapylaria vary in the initial sites of establishment in the sandfly. The

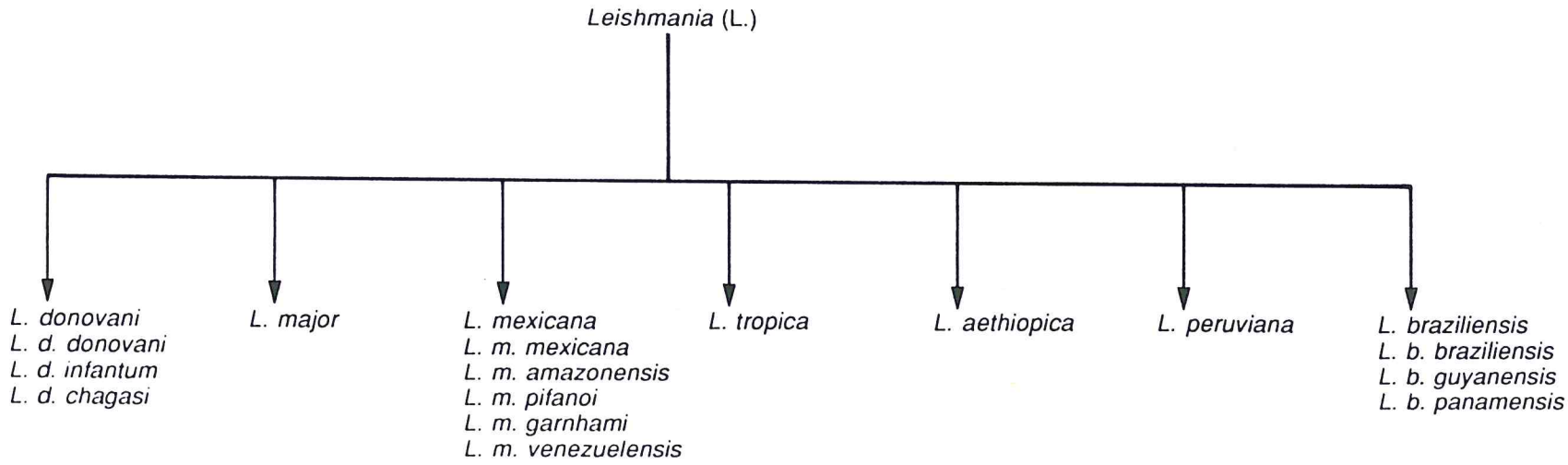


Fig. 2. Classification of the Genus *Leishmania*.

species in Hypopylaria are restricted to the hindgut. Peripylaria have retained the hindgut development but also migrate to an anterior station in the midgut and foregut. Suprapylaria occupy only the midgut and foregut of the sandfly vector. Hypopylaria are parasites of reptiles and include ***L. agamae*** David and ***L. ceramodactyli*** Adler and Theodor. Peripylaria include the following parasites of reptiles and mammals ***L. adleri, L. braziliensis, L. peruviana*** and ***L. tarentolae*** (Wenyon, 1926). Suprapylaria, parasites of mammals are ***L. aethiopica, L. donovani, L. major, L. mexicana, L. tropica, L. hertigi*** Herrer and ***L. enrietti*** Muniz and Medina (all names cited by Lainson and Shaw, 1979).

Lizard parasites of undefined section include ***L. gymnodactyli*** Khodukin and Sofiev; ***L. hemidactyli*** Mackie, Gupta and Swaminathan and ***L. hoogstraali*** McMillan and ***L. zmeevi*** Andrushko (all names cited by Lainson and Shaw, 1979).

1.2.2. Life cycle

There are two phases in the life cycle of the parasite, namely, the invertebrate (sandfly vector) and the vertebrate (animals) phases (Figure 3). ***Leishmania*** species multiply as extracellular flagellated, promastigotes (Figure 4) in the midgut of their sandfly vector and as non-

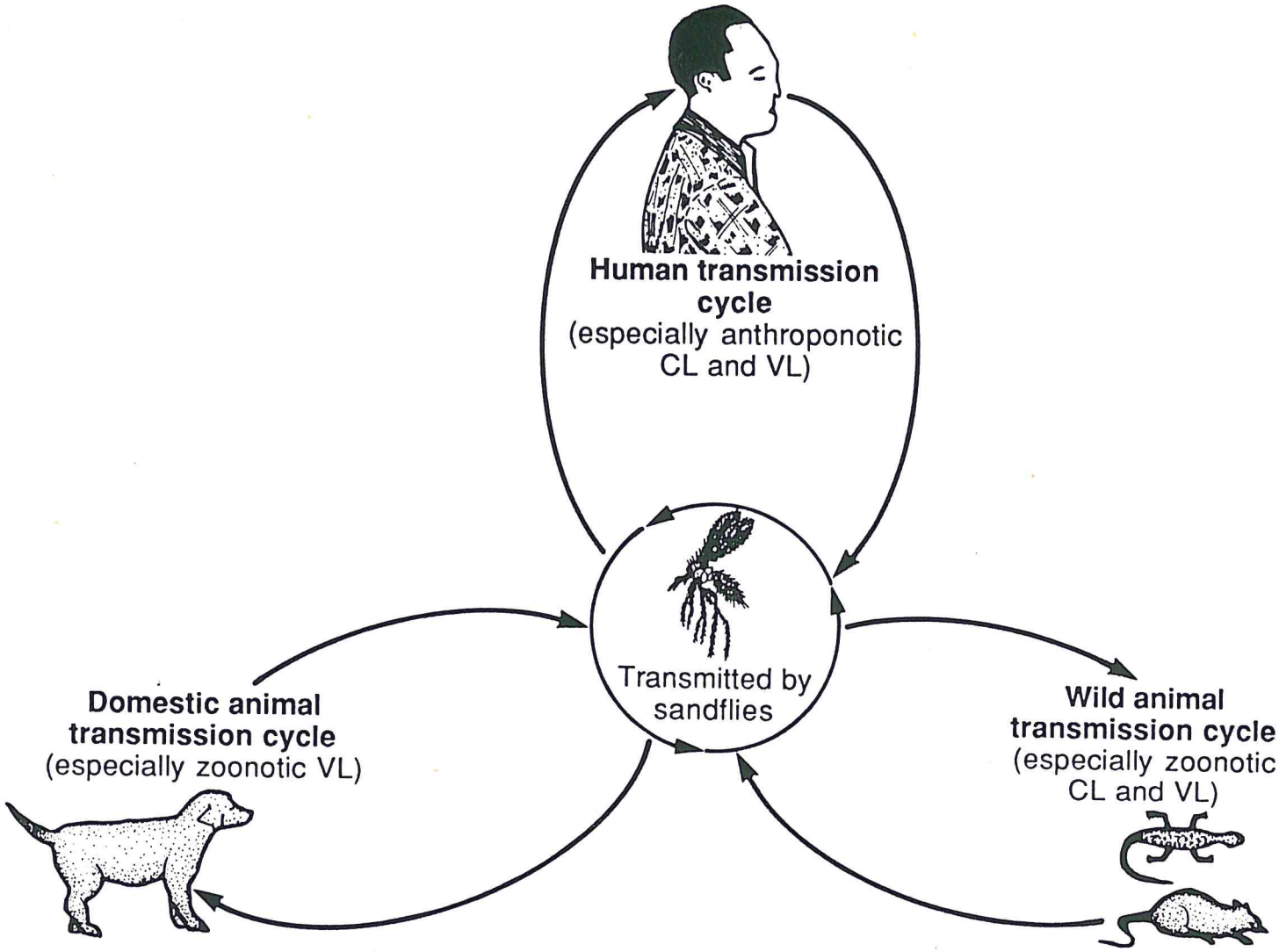
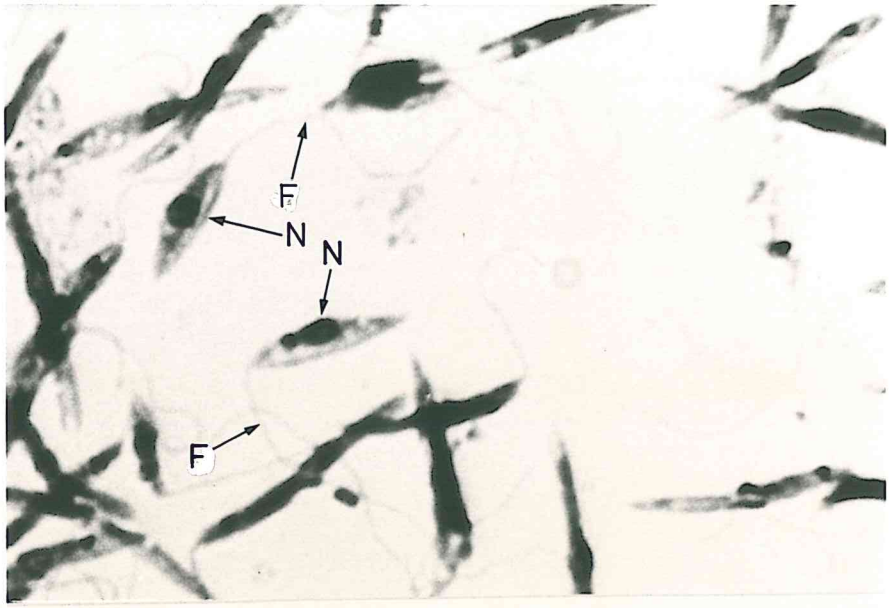


Fig.3. Transmission cycle of *Leishmania*

Fig. 4



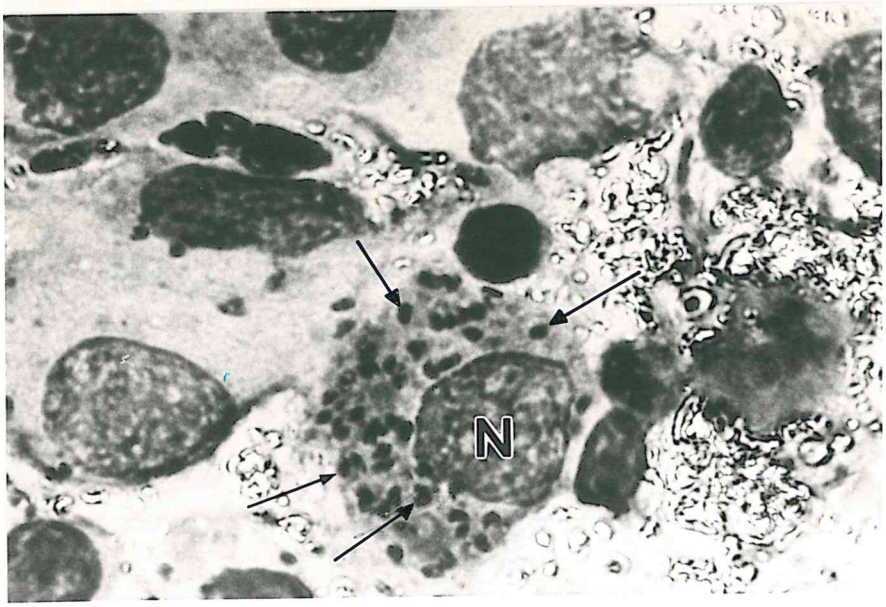


Fig. 5

flagellated amastigotes (Figure 5) in macrophages of their vertebrate hosts. The parasites thus exist in two morphologically distinct forms, namely, promastigotes and amastigotes.

1.2.2.1. Invertebrate phase

When a susceptible female sandfly feeds on an infected host, the amastigotes are taken up from the skin or peripheral blood. These enter the alimentary canal of the fly through the cibarium pharynx and the oesophagus into the midgut and become enclosed in a peritrophic membrane. The membrane gradually ruptures and the parasites lie free in the midgut. During the first 72 hrs, oval-shaped amastigotes (width 1.5 to 3.0μm and length 3.0 to 6.5μm) elongate within the gut contents, and the rudimentary flagellum grows out into a long whip-like structure to form the flagella on promastigotes. The promastigotes undergo extensive longitudinal binary fission which vary from 16.0 to 40.0 μm long by 1.5 to 3.0 μm wide. In the life cycle of most leishmania the promastigotes ultimately migrate to the anterior parts of the sandfly gut, where they become attached to the cardial wall, in vast numbers, by the flagellum. The parasites migrate further to the oesophagus, the pharynx and by the 5th day they are in the buccal cavity. It has been demonstrated that promastigotes undergo sequential development from a non-infective to an infective

stage during growth within the midgut (Sacks and Perkins, 1984; Sacks and Perkins, 1985). Sandfly saliva and sugar meals enhance the development of ***Leishmania*** within the gut (WHO, 1990). The time taken for the parasite to undergo its complete cycle in the sandfly vector depends on both the species of ***Leishmania*** involved and local conditions (for example, temperature and humidity affect the ability of sandflies to feed).

1.2.2.2. Vertebrate phase

When an infected sandfly is feeding it pumps saliva in the wound to prevent the blood from clotting and in doing so it injects the promastigotes into the skin of a new vertebrate host. The promastigotes are phagocytosed by macrophages where they are transformed into amastigotes in the lysosomal compartment and multiplication occurs by binary fission. Amastigotes are found mainly in the reticuloendothelial system of the visceral organs namely, the liver, spleen, intestinal mucosa, bone marrow and lymph nodes. The amastigotes may also be localized in the macrophages in cutaneous tissues. Occasionally, parasites occur in macrophages in the circulating blood and it is from here that the sandfly picks them when it is sucking blood.

1.3 Vectors and Reservoirs

The distribution and incidence of leishmaniases in various endemic areas is closely related to the distribution of specific sandflies (Kirk and Lewis, 1955). Most forms of leishmaniases are zoonotic; human beings are infected only secondarily. Wild and domestic animals have been incriminated as reservoirs of the disease. Although the same parasite species may be found in different geographical regions the dependence of the parasites on an insect vector and animal reservoir hosts indicates that each species is usually associated with specific environmental conditions. Sandflies are commonly found in the dry semi-arid savannah, close to termite hills or water holes. Temperature and humidity have a profound effect on larval development, breeding cycle, flight range and ability of sandflies to feed (Adler and Theodore, 1957; Lewis, 1971). Pifano ***et al.*** (1960) noted that ***Phlebotomus*** leaves its habitat when the ambient temperature and humidity approach those of its microhabitat. The breeding and resting sites of sandflies are diverse and widespread, and include rodent burrows, tree trunks, rock crevices, human dwellings, open fields and organic debris. Sandflies of the ***Phlebotomus***, the ***Sergentomyia*** and ***Lutzomyia*** genera are responsible for transmitting ***Leishmania*** to humans and/or to animals in the Old and New Worlds. ***Phlebotomus dubosqi***, a vector of

Leishmania major (Mutinga and Ngoka, 1983; Beach ***et al.***, 1984) rests inside rodent burrows.

The ecological systems maintaining the different ***Leishmania*** species in different areas vary from place to place. In Central Kenya and India man is thought to be the reservoir host for ***Leishmania donovani*** while in North-Western Africa, South America and southern Europe the dog is the reservoir (Adler and Theodor, 1932; Chance ***et al.***, 1978; Mutinga ***et al.***, 1980). Other reservoirs in various countries include rats, ***Rattus rattus*** (Yugoslavia, Italy); foxes, ***Vulpes vulpes*** (France, Italy, Spain, USSR and the Islamic Republic of Iran); jackals, ***Canis aureus*** (USSR, Islamic Republic of Iran); wolves, ***Canis lupus*** (USSR) and the racoon dog, ***Nyctereutes procynoides*** (China). ***Leishmania tropica*** has been isolated from dogs and rats, ***Rattus rattus***. All the leishmanias isolated from rodents in Kenya to date have been identified as ***Leishmania major*** (Peters ***et al.***, 1977; Chance ***et al.***, 1978; Mutinga and Ngoka, 1983; Githure ***et al.***, 1984; Mutinga ***et al.***, 1985; Le Blancq ***et al.***, 1986; Githure ***et al.***, 1986; Mutinga, 1986). The normal natural vertebrate host of ***Leishmania major*** in Central Asia is the great gerbil, ***Rhombomys optimus***. An isolate from a carnivore (genet cat) was typed to be ***Leishmania major*** (Mutinga ***et al.***, 1983; Okot-KotBer ***et al.***, 1989). In Senegal, ***L. major*** is maintained by ***Mastomys erthroleucus***, ***Tatera gambiana*** and ***Arvicanthis niloticus***. ***Leishmania***

aethiopica has been isolated from rock hyraxes, ***Procavia capensis***, in Ethiopia and Kenya. In Kenya, the tree hyrax (***Dendrohyrax arboreus***), the rock hyrax (***Procavia johnitoni***), and the giant rat (***Cricetomys gambianus***), were incriminated as reservoirs (Mutinga, 1975b). The sloth, ***Choloepus hoffmani***, is the reservoir host of ***L.b. panamensis*** in Panama, Costa Rica and Brazil. Dogs and rats, ***Rattus rattus***, have also been found infected in Panama and Brazil. The reservoir host of ***L. peruviana*** is not yet known while a number of mammals have been found to be naturally infected with ***L. mexicana***. These are mainly rodents, marsupials, primates and carnivores.

Experimental studies on ***Leishmania*** transmission by non-sandfly arthropod hosts have demonstrated that ***Leishmania donovani*** can be maintained in ***Aedes aegypti*** mosquito (Mutero ***et al.***, 1988). Natural occurence of ***Leishmania*** infestation has been reported in ***Anopheles gambiae*** indicating a possible means of disease transmission (Mutero ***et al.***, 1989).

1.3.1 Non-vectorial methods of parasite transmission

Nosocomial infections may occur through blood transfusion from infected donors and accidental innoculation especially in laboratories (by infected flies being squashed on the skin or being accidentally inhaled; WHO, 1984b).

Shortt (1945-1946) claimed that person to person transmission is theoretically possible. The organism may be present in a viable state in nasal secretions and in urine and they can survive for a day or more in moist soil and for several days in milk. They also appear to withstand desiccation for a few hours.

Transmission of kala-azar from person to person by way of the respiratory and alimentary tract could be possible (Forkener and Zia, 1934). Hendricks ***et al.*** (1985) showed that 52% of Kenyan patients with visceral leishmaniasis have amastigotes in their nasal and pharyngeal secretions and in 89% of cases the parasites were viable. There is experimental evidence of direct non-insect vector transmission of ***L. donovani*** in mice (Nuwayri and Khansa, 1985). These workers demonstrated vertical transmission of the parasite from a mother to her offspring. Maternal blood containing ***L. donovani*** parasites can escape into the foetal circulation through the cut umbilical cord and cause infection as has been shown by Chulay ***et al.*** (1985). Other possible methods of parasite transmission include sexual intercourse (Chatterjee, 1975), aerosols (Hendricks ***et al.***, 1985) and transplacental infections (Nyakundi ***et al.***, 1988).

1.4 Clinical Forms of Leishmaniases

The clinical outcome of infection with ***Leishmania*** depends on the nature and the potential of the infecting organism. The diseases are widespread in three main forms: visceral, cutaneous and mucocutaneous (Kirk and Lewis, 1955; Lainson and Shaw, 1978; Mutinga, 1985).

1.4.1 Visceral leishmaniasis

Visceral leishmaniasis (VL) sometimes called kala-azar ("black disease", "dum-dum fever" or "ponos") is endemic in large areas of North, Central and Eastern Africa, where many cases are reported annually. This form of disease is caused by ***Leishmania donovani*** and its subspecies and may be endemic, sporadic or epidemic. The parasite is transmitted by phlebotomine sandflies which may also infect animals (dogs and wild canidae) as secondary hosts. In some parts of Kenya, ***Sergentomyia*** sandflies, ***S. garnhami*** and ***S. ingrami*** have been reported as possible vectors of visceral leishmaniasis (Mutinga and Odhiambo, 1982; Mutinga and Kyai, 1985). The parasite invades internal organs which include the spleen, liver, bone marrow, lymph nodes and other lymphoid tissues. The disease is characterized by intermittent fever, hepatosplenomegaly, pancytopenia, anaemia, malnutrition, immune suppression and hypergammaglobinaemia (Kager, 1983).

The onset of kala-azar in the victim is slow and progresses through an incubation period of several months. Because of this slow development, the disease is not often diagnosed in time to effect proper cure. Non-indigenous people of any age, entering an endemic area may contract visceral leishmaniasis. Children and the productive age group of the population are susceptible to epidemic visceral leishmaniasis (Figure 6). Intercurrent infections are common and mortality rates of at least 70% have been reported in untreated cases (Gachihi ***et al.***, 1987).

1.4.2 Cutaneous leishmaniasis

Cutaneous leishmaniasis (CL) also called dermal leishmaniasis or oriental sore ("Baghdad boil", "Delhi sore" or "Aleppo button"), by far the most prevalent form of leishmaniasis, is caused by, among other species, ***Leishmania aethiopica, Leishmania major, Leishmania tropica*** and ***Leishmania braziliensis***. The parasites are transmitted by sandflies within the ***Phlebotomus*** and ***Lutzomyia*** genera and are found in Africa, Latin America, the Indian sub-continent, South-west Asia and parts of the Soviet Union (Adler and Theodor, 1926). Cutaneous leishmaniasis produces painful sores and lesions on the skin (Figure 7a) reducing the victim's ability to work and this can have important economic consequences. Uncomplicated cutaneous lesions heal

Fig. 6.

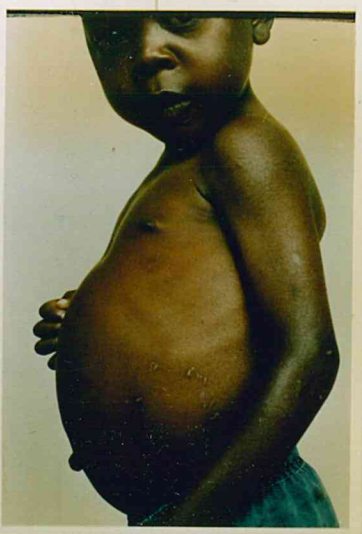


Fig. 7a.

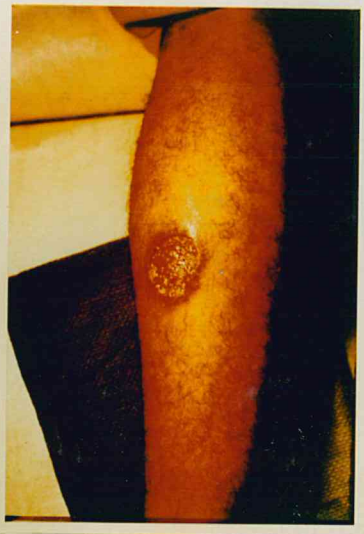


Fig. 7b.

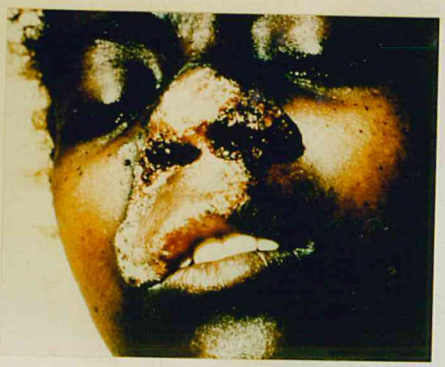


Fig. 8.

within nine months in South-west Asia and two years in Central and South America. However, non-healing lesions occur as in diffuse cutaneous leishmaniasis (Figure 7b) found in Ethiopia, and post kala-azar dermal leishmaniasis (PKDL) in East Africa and India. Post kala-azar dermal leishmaniasis may cause disfigurement and life-long scarring on the affected parts. Clinical features of cutaneous leishmaniasis tend to differ between and within regions, reflecting the different species of parasite or the type of zoonotic cycle concerned.

1.4.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (MCL) or espundia found in Central and South America is caused by ***Leishmania braziliensis braziliensis***. This parasite is transmitted by sandflies of the ***Lutzomyia*** genus. The disease destroys the soft tissue and cartilage of the oronasal/pharyngeal cavity and lips. In contrast to cutaneous leishmaniasis, the lesions do not heal spontaneously; the disease can mutilate the face so badly that victims become social outcasts resulting in a life-long psychological impact (Figure 8).

1.5 Approaches to control of leishmaniases

1.5.1 Diagnosis

The definite diagnosis of leishmaniases is established by demonstrating the presence of amastigotes in splenic and bone marrow aspirates, lymph nodes, liver biopsy, nasal secretions or peripheral blood in direct smears, by their growth in cultures or in experimental animals (Hess, 1930).

The scarcity or absence of the parasites in the peripheral blood make it difficult to demonstrate the parasites. In the case of kala-azar, the necessary splenic or bone marrow punctures and aspirations require expertise both in the performance and in the interpretation of the results of the smears which require laboratory facilities. Culture techniques are of limited value for diagnosis because of the low success rate in the culturing of certain species of ***Leishmania*** as well as the frequent microbial contamination of the cultures. Innoculation of susceptible animals with field material will give a result only after at least one month and that, only if the innoculum contains a high number of amastigotes infective to the particular animal used. Due to the inconsistency and possible unreliability of these techniques for the demonstration of

the parasites, many workers are using other, more definitive biochemical and molecular biological, methods.

1.5.2. Chemotherapy

The treatment objectives are to cure the patient (who is often malnourished and immunosuppressed) of intracellular parasitic infection, to prevent relapse and the development of unresponsiveness of parasites to drugs as well as keeping hospitalization and treatment costs to a minimum. To achieve these goals an appropriate drug must be given for a suitable period of time, at an adequate dose level and frequency.

Primary drug treatment is based on two pentavalent antimonials, namely, sodium stibogluconate and meglumine antimoniate. Before 1940, sodium antimonyl tartrate, organic trivalent antimonial, the urea stilbamine and an organic antimony pentavalent were used in leishmaniasis treatment (Cole, 1944; WHO, 1984a). Successful treatment was obtained with high doses of urea stilbamine as compared to the others. Cole (1942) described the use of stilbamidine in the treatment of leishmaniasis in East Africa. The use of the antimony gluconate has diminished due to its drawbacks which include high cost, toxicity, lower efficacy and adverse side effects (WHO, 1984a). Patients treated with this drug tend to relapse.

The drugs have to be given in daily injections for several weeks. The duration of treatment varies from one endemic area to another, but should be continued, in any case, for two weeks after anticipated parasitological cure, the exact length being determined for each country depending on the parasite responsible for the infection.

Other drugs include the antimicrobials amphotericin B, pentamidine (for kala-azar), metronidazole and nifurtimox (WHO, 1984b; Were ***et al.***, 1986). Primary unresponsiveness, defined as no clinical or parasitological improvement during or after the first course of treatment with pentavalent antimonials, is found in some of the patients. For drug resistant cases, allopurinol, in combination with sodium stibogluconate, has been used (Kager ***et al.***, 1983; Mebrahtu ***et al.***, 1990). According to Lainson and Shaw (1978), the control of leishmaniasis should depend on eliminating either the reservoirs or the insect vectors, or both, rather than on treatment with antimonial drugs.

The state of nutrition of the host has a profound effect on the immune capacity of the host (Bray, 1974). Bradley ***et al.*** (1977 and 1979) and Perez ***et al.*** (1979a and 1979b) confirmed that host factors (genetic, physiological or nutritional) influence the course of ***Leishmania*** infection. Non-specific treatment with high protein, vitamin-rich diets has been used. In some countries

patients with chronic kala-azar have undergone splenectomy, but with little success.

1.5.3. Control of Vectors and Animal Reservoirs

The main step to be taken in the prevention and control of leishmaniasis is the breaking of the transmission cycle: vertebrate reservoir-sandfly-vertebrate host. This is best effected by the elimination of the insect vector and the reduction of reservoirs of infection.

Efforts to control leishmaniases are hampered by the elusive nature of the sandfly vector and the diversity of animal reservoirs. Control of sandfly vectors is difficult because of the variability and general inaccessibility of the insects' breeding and resting sites. Individual protective measures, such as the use of insect repellents, bednets and window screens as well as the avoidance of outdoor activities, at times of greatest sandfly activity, may be effective. Insecticides have been used in the control of sandflies but according to Ward (1977) and Killick-Kendrick (1978), the prolonged exposure of sandflies to insecticides might lead to development of resistance. In some parts of the USSR, sandflies showed resistance to chlorinated insecticides (Killick-Kendrick, 1978). In the Peruvian Andes, Dichloro-Diethyl Trichloroethane was successfully used in the control of

Lutzomyia verrucarum, a vector of ***Leishmania peruviana*** (Lainson and Shaw, 1978). The use of insecticides on a large scale in tropical rainforest is highly uneconomical and may be harmful to man and the general environment. In Kitui, Kenya, limited applications of insecticides were carried out in houses, thorn bushes and termite hills in 1968 (Zahar, 1981; Mutinga, 1985) as a temporary control measure. Permethrin-impregnated fabrics (known as MBU cloth) have been successfully tested to control sandflies and mosquitos in Marigat, Baringo District by ICIPE (Mutinga - personal communication). A sticky trap, developed at ICIPE and consisting of plastic sheets covered with a thin film of locally produced castor oil (Mutinga, 1981), has been very effective in reducing the number of sandflies (Mutinga, 1986). Personal protection can be achieved by the use of efficient insect repellants such as diethyltoluamide, dimethylphthalate and trimethylpentanediol or the use of very fine-mesh nets for protection against sandfly bites.

Other control measures, that can be effective, include mechanical destruction of the animal reservoir habitats and poisoning of the animals themselves, especially rodents, as has been practised with success in the Soviet Union. In China, chemotherapy coupled with the elimination of infected dogs has proved highly effective.

The composition of plant extracts eaten by the sandfly has been found to affect the insect's vectorial capacity for leishmania, a finding which may offer a potential target for control activities (Schlein, 1986; Kaddu, 1986). Bacterial, fungal and viral infections prevent leishmania from developing normally within the sandfly vector (Schlein ***et al.***, 1985; Kaddu and Nyamori, 1991 - in press) and this could be explored as a method of control.

1.5.4: Vaccination

Many vaccination trials have been carried out all over the world (Manson-Bahr ***et al.***, 1963). Manson-Bahr and Southgate (1964) reported that repeated vaccination of volunteers with a non-virulent ***L. donovani*** strain (from a Kenyan ground squirrel) gave no protection against a natural infection. Various vaccines against visceral leishmaniasis are being tried in dogs in Europe as a means of reducing infection in the reservoir host (WHO, 1990).

Cutaneous leishmaniasis is generally a self-limiting disease followed by life-long immunity. Maybrink ***et al.*** (1979) used a vaccine containing killed promastigotes of five strains of ***Leishmania*** (polyvalent) on soldiers who were undergoing their training in an endemic area. They found a high protection in the soldiers who had shown a

positive leishmanin skin test three months after vaccination. The protected individuals had no circulating antibodies. The vaccine stimulated cell-mediated immunity rather than antibody production. In Israel, a successful trial of a killed promastigote vaccine against cutaneous leishmaniasis led to the adoption of the vaccine (Zuckerman, 1975). The deliberate infection (Leishmanization) of an individual has been used as a last resort for the control of cutaneous leishmaniasis in Israel, the Soviet Union (Greenblatt, 1988) and most recently, the Islamic Republic of Iran (WHO, 1990). Some individuals undergoing leishmanization develop large or persistent lesions which may require treatment. A person who has recovered from leishmanization is usually immune to natural infection.

Monoclonal antibodies against specific purified antigens from ***Leishmania*** are useful in the diagnosis of infections (and parasites isolated from vectors and reservoirs) and in the development of protective immunity. Cross-protection has been demonstrated between some species and subspecies in both man and experimental animals (Mauel and Behin, 1982). Research is being carried out for the development of killed leishmania vaccines and on parasite antigens that might be used as future vaccines.

1.6 Leishmania in Reptiles (Sauroleishmania)

Other than in the humans, leishmania parasites are also found in reptiles. These include ***Leishmania adleri*** and other species (See section 1.2.1). The parasites are transmitted through the bite of sandflies belonging to the genus ***Sergentomyia***. Although many leishmania species are capable of infecting a wide range of mammals, some are much more limited in their host range. The forms which are non-infective to humans are of little direct economic significance, but are important as experimental tools, models and to complete the overall picture of the genus. They cannot be entirely ignored for they are likely to be encountered in the course of surveys and it is essential that they should be differentiated from the pathogenic species.

1.7 Leishmaniases in Kenya

The main interest in leishmaniases, for this research, is in the problems presented by the disease in Kenya and approaches to their solution. The disease has been reported to occur in some of the countries bordering Kenya, namely Ethiopia, Sudan, Uganda and Tanzania. Research being conducted at ICIPE, KEMRI, IPR and the Division of Vector-borne Disease of the Ministry of Health,

Government of Kenya, reflects the magnitude of the problems presented by leishmaniases in Kenya.

1.7.1 History

The first recorded severe outbreaks of human leishmaniasis in Kenya occurred in the early 1940s (Heisch, 1954). During the Second World War, troops posted to Northern Kenya and to the frontier area with Sudan and Ethiopia contracted the disease and some sporadic cases were reported in the areas. The endemic and epidemic foci of the disease were established in mid 1950's and 1970's respectively (Heisch, 1954; Heisch, 1957; Ngoka and Mutinga, 1978). In 1976 increasing numbers of patients were seen from new foci (Rees ***et al.***, 1977) and in the north of Machakos district (Wasunna, 1977). At the Kenyatta National Hospital, Nairobi, only occasional, sporadic cases from the northern part of the country and from Kitui area had been seen for several years (Kager and Rees, 1983). The disease was later reported in other areas which include Embu (Clarke, 1949), Meru (Southgate and Oriedo, 1962; Wijers, 1963; Wijers, 1971; Mbugua and Arap Siongok, 1981), West Pokot, Marigat, in Baringo district (McKinnon, 1962a and 1962b; Mutinga and Ngoka, 1983) and Mt. Elgon (Mutinga, 1975a and 1975b). Sporadic cases of visceral and cutaneous leishmaniasis have been reported in many areas of the

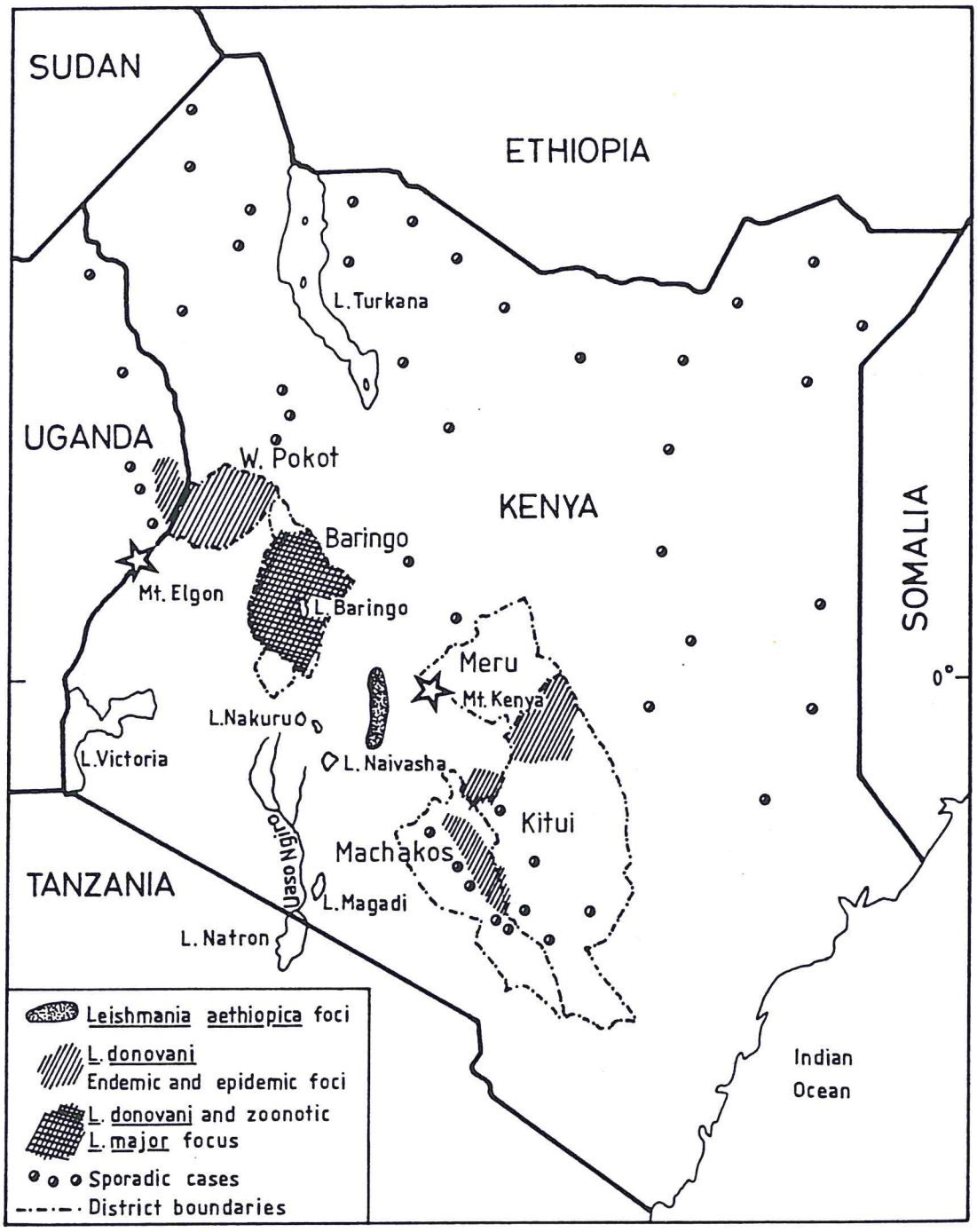


Fig. 9. Map of Kenya showing leishmaniases endemic areas.

country below 5000 ft. above sea level where sandflies responsible for disease transmission reside.

1.7.2 Geographical distribution

The distribution of the disease in Kenya is shown in Figure 9. The areas affected include the following districts: Baringo, Bungoma, Kitui, Machakos, Meru and West Pokot.

1.7.2.1 Baringo District

The presence of the endemic foci of visceral leishmaniasis in Baringo was observed in 1955 and 1956. Forty cases were reported during this period. The number increased to 104 in 1957 in the eastern foothills of Kamasia range, Kerio valley, Lake Bogoria (formerly Hannington) area and North of Lake Baringo (Kager and Rees, 1983). The disease still occurs at Marigat today and the incidence is around 100 cases per year (WHO, 1990). Cutaneous leishmaniasis caused by ***L. major*** was first confirmed by isoenzyme characterization of isolates made by Heisch and Mutinga (Chance ***et al.***, 1978). Later the disease was established to be zoonotic (Mutinga ***et al.***, 1986).

1.7.2.2 Kitui District

In 1952/53 there was a major epidemic outbreak affecting about 3,000 people in Tseikuru area. Control measures, which included treatment and spraying of termite hills, reduced the number of cases. A further upsurge of the disease occured between 1957 and 1960 and was later brought under control. The disease still occurs at this focus to date (Mutinga ***et al.***, 1986).

1.7.2.3 Machakos District

In Machakos District cases had been reported between 1942-1950 and it was assumed that the disease had been introduced by the Wakamba soldiers returning from World War II. An epidemic broke out in 1972 and 130 cases were reported mainly in hospitals and clinical centres located in the Athi River Valley. Between the epidemic foci of Machakos (Athi River Valley) and the old Kitui focus, cases were reported after the construction of dams. Fifty cases in young children were reported between 1978 and 1981 and this showed the disease was being transmitted in the focus (Mutinga ***et al.***, 1986).

1.7.2.4 Meru District

An epidemic outbreak occurred in Meru District between 1960 and 1962 in which 365 cases were reported. The number increased in 1963/64 by 597 cases and by 140 cases in 1965. By 1968 there were 57 cases in the sublocations of Tharaka location. The highest incidence of the disease was found in Kathangacini and Kamaguna which border Tana River. Few cases are reported sporadically every year (Mutinga ***et al.***, 1986).

1.7.2.5 West Pokot

The first case in West Pokot was reported in 1956. There was an outbreak in mid 1970's and since then more cases have been reported in Kapenguria and Ortum hospitals (Mutinga ***et al.***, 1986).

1.7.2.6 Bungoma District

In 1969 the first autochthonous case of ***L. aethiopica*** was reported (Mutinga and Ngoka, 1970; Kungu ***et al.***, 1972). Later an endemic focus of the disease was established in the eastern slopes of Mt. Elgon in Bungoma District and a sandfly, ***Phlebotomus longipes***, was incriminated as the vector of this parasite (Mutinga, 1971 and 1975a). The disease in this area is transmitted as a

zoonosis with the hyrax being the main animal reservoir and the giant rat (***Cricetomys***) serving as secondary animal reservoir.

1.7.3 Species of ***Leishmania*** in Kenya

Four species of ***Leishmania*** have been confirmed in Kenya, namely, ***Leishmania adleri, Leishmania donovani, Leishmania aethiopica*** and ***Leishmania major***. Recently, the presence of ***Leishmania tropica*** was also reported (Mebrahtu ***et al.*** 1988; Mebrahtu ***et al.***, 1989).

Heisch (1958) isolated ***L. adleri*** from lacertid lizards which live in the deserted burrows of gerbils. Human volunteers from Kitui district of Kenya were inoculated intradermally and subcutaneously with ***L. adleri*** promastigotes (Mansor-Bahr and Heisch, 1961). The parasites were isolated in culture from the nodule a week later. Scanty ***Leishmania*** were seen in smears up to the fifth day post-inoculation. No amastigotes of ***L. adleri*** were observed in organ smears from infected lizard blood smears. Since then, many isolates of lizard leishmania have been found.

Leishmania major was first reported in Kenya in a ground squirrel and gerbils (Heisch, 1963). This parasite remained unknown until 1977, when Mutinga and Ngoka (1983) isolated parasites from a gerbil in the same area and sent

it to Liverpool School of Tropical Medicine where they were identified together with those of Heisch as ***L. major*** (Chance ***et al.***, 1978). Later studies have shown other animals to be involved as reservoirs of the disease (Mutinga ***et al.***, 1983; Githure ***et al.***, 1984; Githure ***et al.***, 1986). The parasites were recently isolated from man (Muigai ***et al.***, 1987) as well as a naturally infected vervet monkey, ***Cercopithecus aethiops*** (Binhazim ***et al.***, 1987). The vectors of the disease were subsequently studied in detail (Mutinga ***et al.***, 1983; Beach ***et al.***, 1984; Mutinga ***et al.***, 1986).

1.7.4 Clinical Forms of leishmaniases found in Kenya

Visceral leishmaniasis, the most important form in Kenya, was first diagnozed in 1933 in a patient from Elgeyo, although apparently clinical cases had been reported before. Wright (1943) and Fendall (1953) reported the first cases from Machakos and Kitui districts in 1942 and 1946 respectively. The disease had also been found in Baringo district, Kerio valley and Perkerra Irrigation scheme in Marigat (McKinnol and Fendall, 1956; Heisch, 1963; Chance ***et al.***, 1978; Mutinga ***et al.***, 1980; Mutinga and Ngoka, 1983) and West Pokot (Mutinga ***et al.***, 1984; Mutinga, 1985). The incriminated vector of visceral leishmaniasis is ***Phlebotomus martini*** (Minter ***et al.***, 1962; Mutinga and Ngoka, 1978b; Perkins ***et al.***, 1988). The wild maintainance hosts of ***L. donovani*** remain to be fully established although Mutinga ***et***

al. (1980) found two out of 288 dogs naturally infected with ***L. donovani,*** one of which had the same enzymatic and serological characteristics as those found in the human ***L. donovani.***

Acquisition of kala-azar has been connected with herdsmen and their association with termite hills where ***P. martini*** are found (Manson-Bahr and Southgate, 1964). Solid immunity against re-infection with Kenya ***L. donovani*** is acquired after effective chemotherapy (Hoogstraal and Heyneman, 1969). ***Sergentomyia garnhami*** has been shown to be a potential vector in the Machakos focus of visceral leishmaniasis (Mutinga and Odhiambo, 1982).

Cutaneous leishmaniasis caused by ***Leishmania major*** has been reported in wild animals and, although the disease had not been found in humans (Kaddu, 1986; Mutinga, 1986), some cases have been reported recently (Muigai ***et al.***, 1987). Several cases of cutaneous leishmaniasis caused, by ***Leishmania aethiopica,*** have been reported from around Mount Elgon, the Aberdare mountain range near Solai, Elmentaita and Nakuru areas of Kenya (Mutinga and Ngoka, 1970; Mutinga, 1975a; Mutinga ***et al.***, 1980; Molyneux and Ashford, 1983; Mutinga, 1986). This species is taxonomically unique among Old World ***Leishmania*** in causing both localized and diffuse cutaneous leishmaniasis (Le Blancq ***et al.***, 1986a). Solid

immunity against a homologous strain has been reported after healing.

1.8. The Importance of *Leishmania* Identification and Characterization

Control of leishmaniases depends mainly on the demonstration of parasites in the host, appropriate treatment and vector elimination. In order to do this effectively the parasite, ***Leishmania***, has to be identified.

Research on leishmaniases dates from the discovery of the leishmanial parasite in 1903 (Garnham, 1987). Basic work has been conducted on vectors and reservoirs as important elements of the epidemiology. To establish that an animal is either a vector or a reservoir host in the epidemiology of human infections, the identity of the parasites it harbours and transmits has to be established. Identification of ***Leishmania*** species is a world health problem of increasing importance, in view of the resurgence of the disease, following the widespread ban on the use of the insecticide, Dichloro-Diethyl Trichloroethane (DDT) in the early 1960's (Killick-Kendrick, 1978; Seal, 1977; Kusaoimi, 1979; Bhattacharya, 1981). This has led to the reappearance of human leishmaniasis in countries where it had been controlled. Thus the disease re-appeared in Zaire (Gigase ***et al.***, 1978), Zambia (Naik ***et al.***, 1976), Namibia

(Grove, 1970; Grove, 1978; Rutherford and Uys, 1978), Honduras (Nuernberger ***et al.***, 1975), Japan (Okano ***et al.***, 1977) and the United States (Stewart and Pilcher, 1945; Anderson ***et al.***, 1980).

International business and recreational travel can result in the introduction of human and animal leishmaniases into non-endemic regions and different forms of clinical disease into endemic regions (Price and Silvers, 1977; Garett, 1978; Jones, 1979; Schalm, 1979; Al-Taqi and Behbehani, 1980; Geracci ***et al.***, 1980). These situations pose serious diagnostic and chemotherapeutic problems to the physician. In a non-endemic region the clinician, unfamiliar with disease symptoms, may not diagnose leishmaniasis at an early stage. In endemic areas, inadequate or over-treatment may result when the physician makes a disease prognosis and initiates chemotherapy based upon experience with the local leishmanial species. Identification is thus essential in deciding on the course of treatment (Molyneux and Ashford, 1983). The drug of choice will depend on the type of leishmania responsible for the infection and it is necessary to identify the parasite so that international control programmes can be planned.

Accurate taxonomic knowledge is of great practical importance since the expansion of information concerning the parasite and the disease is possible only when new facts can

be related to organisms that have been adequately characterized. Correct characterization of the parasites in animals is vital in determining the role of the animals as potential reservoir hosts. Considerable progress could be made in planning control campaigns, if highly sensitive and specific tests could be developed for identifying the leishmania parasites infective to humans, from batches of sandflies found in different ecological situations. Additionally, different vaccines may be required against leishmanial infections caused by different parasites, which must therefore be identified as precisely as possible.

In the New World, each ***Leishmania*** species represents a complex of strains with different biological properties (Lainson, 1983; WHO, 1984a; Lainson and Shaw, 1987). In Africa, very little is known about the ***Leishmania*** parasite characteristics such as infectivity and capacity to survive in humans. However, it is clear that the various species of ***Leishmania*** express these characteristics to different degrees. The species and subspecies of the parasite and immunogenetic background of the host affect disease development. Only certain species of ***Leishmania*** may cause a given clinical syndrome. For this reason, it is vital to elucidate the genetics of ***Leishmania*** and to classify the causative organism for epidemiological studies and clinical investigations of the disease (Godfrey, 1978).

The early history, nomenclature, characterization and classification of strains by morphology, culture characteristics, clinical and epidemiological aspects of infections in human and other natural hosts, infections and behaviour in animals, cross-immunity and serological tests were described by Kirk in 1949. The identification and taxonomy of ***Leishmania*** parasites has been described by different workers (Adler, 1964; Bray, 1974; Gardener, 1977; Zuckerman and Lainson, 1977; Hommel, 1978; Lainson and Shaw, 1979). Identification of ***Leishmania*** has been hampered by deficiencies in the classical criteria used, such as morphology (Marsden, 1979; Lainson, 1982) and clinical manifestations (Chance ***et al.***, 1974). Therefore, different biological, biochemical and molecular biological methods have been introduced for identifying and studying leishmania parasites (Schnur and Chance, 1976; Gardener, 1977; Chance, 1979; Peters, 1981; Chance and Walton, 1982; Godfrey, 1984).

1.8.1. Methods for the characterization of ***Leishmania***

Several methods for identification and characterization of leishmania have been developed and utilized. Currently, the most common method of parasite identification is by isoenzyme electrophoresis using cellulose acetate or polyacrylamide gels (Gardener ***et al.***, 1974; Kreutzer ***et al.***, 1983). Other methods for parasite identification are restriction enzyme digestion of kinetoplast DNA (Decker-

Jackson ***et al.***, 1977; Arnot and Barker, 1981; Wirth and McMahon Pratt, 1982; Barker and Butcher, 1983; Jackson ***et al.***, 1984; Kennedy, 1984; Spithill and Grumont, 1984; Lawrie ***et al.***, 1985; Barker ***et al.***, 1986; Lopez and Wirth, 1986; Barker, 1987), DNA buoyant density analyses (Chance ***et al.***, 1974), the excreted factor assay (Schnur ***et al.***, 1972; Schnur and Zuckerman, 1977; El-On ***et al.***, 1979), radiospirometry (Decker-Jackson ***et al.***, 1977) and on reactivity with monoclonal antibodies (McMahon Pratt ***et al.***, 1981; Mcmahon-Pratt and David, 1982; Greenblatt ***et al.***, 1983). DNA analysis, using restriction enzymes followed by hybridization with DNA probes is being used to identify parasites and study the relationships between parasites and the clinical forms of diseases (Barker and Butcher, 1983; Kennedy, 1984; Massamba and Williams, 1984; Lawrie ***et al.***, 1985; Barker ***et al.***, 1986; Jackson ***et al.***, 1986; Barker, 1987; Van Eys ***et al.***, 1987; Van Eys ***et al.***, 1989; Majiwa, 1989).

Identification of ***Leishmania***, or any other parasite by isoenzyme analysis should be based on data from many different enzymes rather than just a few (Kreutzer ***et al.***, 1983). The greater the number of enzymes included in the identification the greater the likelihood of classifying the isolates into the correct groups.

Restriction endonucleases are used for specific cleavage and fragmentation of DNA. The set of DNA fragments produced by a particular restriction enzyme is characteristic of a given DNA, and the electrophoretic pattern of these can be used for taxonomic purposes (Rivkin, 1982). Analysis of kDNA seems to stress dissimilarities between strains rather than relationships since patterns of isolates belonging to the same species show differences (Van Eys ***et al.***, 1989).

Although monoclonal antibodies are useful in the taxonomic identification of different species of ***Leishmania***, they are specific and bind to one antigen, resulting in a battery of several antibodies being required to identify a particular organism (McMahon-Pratt and David, 1981). Different stages in the life cycle of the organisms produce different surface antigens (Fong and Chang, 1982) and cross-reactions have been reported between 'specific' monoclonal antibodies and other unrelated cells (De Ibarra ***et al.***, 1982).

Other identification methods, which include geographical distribution, clinical outcome of the disease in humans, response of the parasite to drugs, differing characteristic growth patterns of the parasite in culture and the behaviour of the parasite in sandfly and laboratory animals, are not accurate. There is thus an urgent need to

establish a rapid and accurate means of identification to aid both clinicians and epidemiologists, particularly with ***Leishmania*** isolates, which are difficult or impossible to passage in laboratory animals or to grow in culture.

Methods based on proteins (such as isoenzymes) and lipids are subject to phenotypic variation and can be affected qualitatively and quantitatively by gene expression or rearrangements. Genetic heterogeneity of leishmania that has not been detected by these methods may be responsible for some of the diversity seen in clinical infections (Scholler ***et al.***, 1986). Methods which utilize DNA represent the most direct analysis of the genetic material that is possible and, as such, are highly unlikely to show any life-stage or environmentally mediated variation. The same DNA is present in the parasite whether it is in a free-living stage, in an invertebrate vector or in a vertebrate host.

1.9 Aims of the present Work

The present study was undertaken with the following objectives:

- to distinguish ***Leishmania*** species by examination of their molecular karyotypes.

- to study the taxonomic (phylogenetic) relationships amongst leishmania species.

1.10 Pulsed Field Gradient Gel Electrophoresis

An electrophoretic technique has been used to analyse chromosome organization in the protozoa. The technique, pulsed field gradient gel electrophoresis (PFG) is capable of separating intact chromosomal DNA molecules in the size range 50 kilobases up to several million base pairs which is well above the resolving power of conventional gel electrophoresis (Schwartz ***et al.***, 1983). Conventional gel electrophoretic techniques for DNA analysis are effectively limited to molecules less than 20,000 base pairs in size. Above this size all DNA molecules have such similar mobilities in ordinary agarose gels that no separations can be achieved (Fangman, 1978; Serwer, 1980).

The PFG technique was based on the consideration that the native structure of long-chained molecules undergoes conformational changes in an electrical field, showing a length dependent relaxation behaviour. As a result, corresponding differences in mobility in the gel were observed. Hence DNA separation is primarily dependent on the change of direction of the electrical field and the interval that the molecule needs to alter its direction of motion (Holzwarth ***et al.***, 1987). In traditional agarose gel techniques such as submarine gels, molecules larger than 50 kilobases undergo a conformational change and move through the gel as a broad, unresolved band. In PFG gel electrophoresis large molecules of DNA obtained from the lysed organisms are subjected to alternate electrical pulses at an angle of 90^0 or more. Because these molecules are larger than the pore size of the agarose gel, they are forced to stretch out and align with the field in order to migrate through the gel. The net movement of the molecule will be in the direction resulting between the two applied fields. The time required for this molecular reorientation is dependent on the DNA size, so that large molecules spend proportionally more of each pulse cycle reorientating and less time in foward migration. This results in very large DNA molecules remaining near the loading slot while smaller molecules migrate fowards because they can turn corners in the gel matrix more rapidly. The DNA thus separated can be visualized by staining the gel with ethidium bromide and

blotting on nitrocellulose filter membranes and the genes located by hybridization techniques.

1.10.1 Applications of Pulsed Field Gradient Gel electrophoresis

The PFG technique has enabled several groups not only to enumerate chromosomes but also to separate and identify them as well as elucidate some of their functions. DNA insertions, deletions and reciprocal translocations can all be visualized directly by changes in the sizes of chromosomal DNAs and confirmed by observing a shift in the size of known chromosome-specific DNA probes. Chromosome length polymorphisms and rearrangements have been found among closely related strains of parasitic protozoa. In some parasitic organisms these DNA rearrangements are exploited to generate antigenic diversity that is valuable to the survival of the parasite against the hostile environment of the immune defence system of the host (Van der Ploeg ***et al.***, 1984c). Since useful genes for species differentiation can be localized and identified with these methods, it should be possible to distinguish between genes encoding antigens important in protection and those used by parasites to evade the host immune response. It might also be possible to distinguish between virulent and avirulent strains of parasites and to pinpoint the basis of drug

resistance, if the genes responsible for such phenotypes can be identified.

The technique was first applied to the analysis of yeast chromosomes (Schwartz and Cantor, 1984; Carle and Olson, 1985). Since the yeast genetic map was already known, it was possible to show that the high molecular weight DNA bands were really intact chromosomal DNA molecules. Genes that code for various proteins could be localized to specific bands in the gels. The technique has also been applied to the study of antigenic variation in trypanosomes (Van der Ploeg ***et al.***, 1984a;b;c) and has shown that there is more than one expression site for variant surface glycoprotein (VSG) genes in ***Trypanosoma brucei*** (Van der Ploeg ***et al.***, 1984c) and that transcription of VSG genes is discontinuos (Van der Ploeg ***et al.***, 1984b; Guyaux ***et al.***, 1985). The VSG genes are seen most readily in the minichromosomes (Cox, 1985).

The technique has demonstrated that, at the molecular level, ***Trypanosoma (Nannomonas) congolense*** and ***Trypanosoma (Nannomonas) simiae*** differ significantly from each other, and can be distinguished by molecular hybridization using appropriate DNA hybridization probes (Majiwa ***et al.***, 1985; Majiwa and Webster, 1987). Characterization and identification of ***Trypanosoma***

congolense by molecular karyotyping has been described by Majiwa ***et al.*** (1986) and Masake ***et al.*** (1988).

Direct comparisons of the molecular karyotypes (the patterns of chromosomal DNA bands seen with ethidium bromide fluorescence) of different species provide useful information for the evolutionary relationships. For example, ***Trypanosoma vivax*** and ***Trypanosoma cruzi*** differ from ***Trypanosoma brucei*** in having no minichromosomes visible by ethidium bromide staining of their chromosomal DNA (Van der Ploeg ***et al.***, 1984a; Gibson and Miles, 1986), whereas the chromosomal organization of ***Trypanosoma congolense*** and ***Trypanosoma brucei*** is similar (Gibson and Borst, 1986). In contrast to ***Trypanosoma brucei***, ***Trypanosoma equiperdum*** has fewer minichromosomes (Van der Ploeg ***et al.***, 1984a). Since all the trypanosomes undergo antigenic variation it seems that minichromosomes are not essential for this process.

The technique has also been used in distinguishing different isolates of ***Plasmodium falciparum*** (Kemp ***et al.***, 1985; Van der Ploeg ***et al.***, 1985) and to compare the karyotypes of ***Plasmodium falciparum*** and ***Plasmodium chabaudi*** (Langsley ***et al.***, 1987). The sequence analyses of chromosomes of ***Plasmodium falciparum*** have revealed short repeats of amino acids that constitute important antigens. The genes for these repetitive antigens are distributed among several chromosomes (Cox, 1985). The molecular

karyotypes of the rodent malaria parasites have been characterized by similar methods (Sheppard ***et al.***, 1989).

Modifications of this technique have been used in the typing of leishmania isolates (Van der Ploeg ***et al.***, 1984a; Spithill and Samaras, 1985; Comeau ***et al.***, 1986; Giannini ***et al.***, 1986; Scholler ***et al.***, 1986; Bishop and Miles, 1987; Galindo and Ochoa, 1989) and has shown that the DNA karyotype is useful for stock identification, taxonomy and localization of specific genes of leishmania (Scholler ***et al.***, 1986).

Apart from separating whole chromosomes, PFG is useful in the large scale restriction mapping of chromosome regions as well as DNA fragment purification for cloning (Stewart ***et al.***, 1988). Chromosomal DNAs of some organisms are too large to be handled as intact molecules. For this reason, intact chromosomal DNAs are cut by restriction nucleases with relatively rare cutting sites, into well defined fragments that fall in the size range separable only by PFG. There are two restriction nucleases commercially available that have eight base pair recognition sites. These enzymes, Not I and Sfi I, have been used to cleave ***Escherichia coli*** DNA , mouse and human DNA into large fragments. There are a number of restriction enzymes that have six base pair recognition sites but these sites are relatively rare in particular genomes and include Mlu I and

Sac II among others. Individual fragments can be identified by Southern blotting and hybridization with cloned single copy DNA probes. It is possible to tell whether two genes believed to be closely linked appear in the same size DNA fragment. Physical maps of complete genomes of organisms can be developed by the generation of the large fragments using restriction nucleases.

1.10.2 Pulsed Field Gradient Gel Electrophoresis techniques

Four different PFG techniques have been described up to date. The first method for pulse field separations was described by Carle and Olson, (1984) as well as Schwartz and Cantor, (1984). Orthogonal Field Alternation Gel Electrophoresis (OFAGE) formed the basis for the first commercially produced instruments. This consists of four electrodes placed at the corners of the gel where two non-uniform electric fields are switched on and off (Figure 10). After electrophoresis a series of bands are formed in curvillinear lanes. The advantage of this method lay in the size of the agarose gel which could conveniently be used for further processing or treatment of separated material. In the OFAGE apparatus, different parts of the gel are subjected to different reorientation angles, mostly between 120^0 and 150^0 (Chu ***et al.***, 1986). The technique is suited

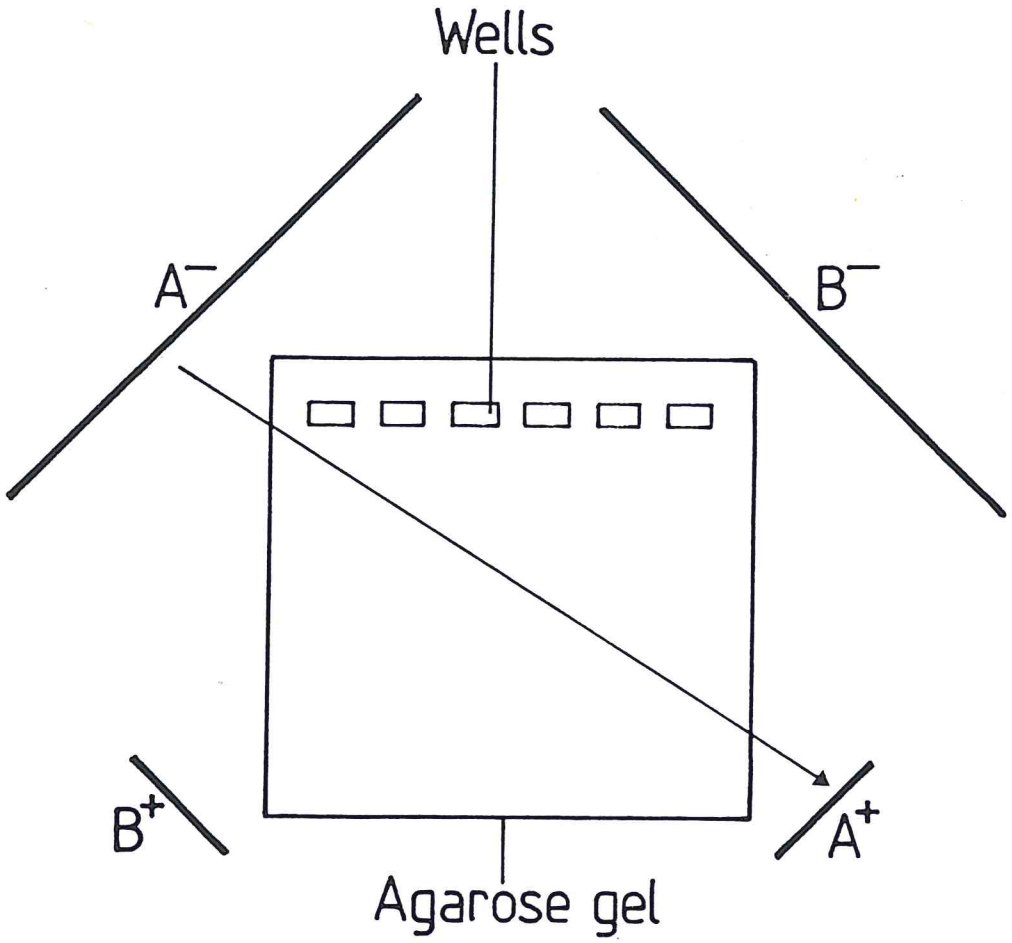


Fig. 10. The Principle of Orthogonal Field Alternation Gel Electrophoresis. (The arrow indicates one of the electrical field directions. A and B are electrodes).

for resolving chromosomes greater than one million base pairs.

Towards the end of 1985, Carle ***et al.*** (1986) developed Field Inversion Gel Electrophoresis (FIGE), featuring linear and logarithmic changes in current direction by reversal of electrode polarity. The electric field is periodically inverted so that a single pair of electrodes can be used to generate homogenous electric fields. The system resolves large DNA with a pattern of separation independent of its position across the gel. The fact that the migration of the DNA is not a monotonic function of size leads to the unexpected comigration of molecules that differ greatly in size. The problem can be avoided only by choosing an appropriate range of pulse times during electrophoresis. The apparatus which consists of a single field reverses direction through a 180^0 angle every one to thirty seconds (Carle ***et al.***, 1986; Larson ***et al.***, 1987). The range of molecular weights that can be resolved by FIGE is limited to about one million base pairs with satisfactory resolution only up to 800,000 base pairs. Although straight lanes of bands are formed after electrophoresis, the mobilities of molecules separated by the technique are not a simple function of molecular weight (Ellis ***et al.***, 1987). Size determination is thus difficult since larger DNAs can migrate faster than smaller ones.

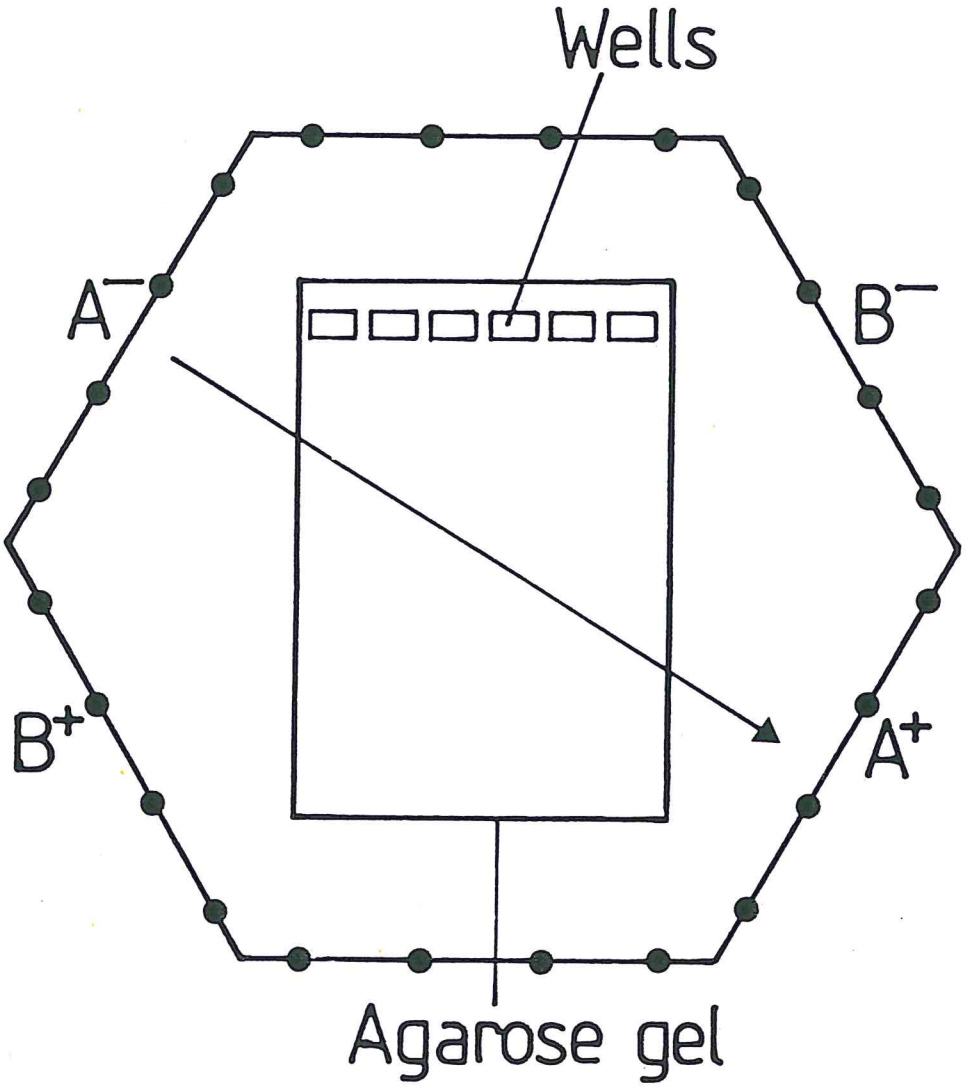


Fig. 11. The Principle of Contour–clamped Homogenous Electric Field Electrophoresis. (The arrow indicates one of the electrical field directions. A and B are electrodes).

DNA molecules as large as 2 megabases can be well separated with Contour-clamped Homogeneous Electric Field (CHEF; Chu ***et al.***, 1986) between two orientations 120^0 apart (Figure 11). Small gels of 12x14cm are used and the pattern of separation is independent of position in the gel thus giving bands in straight lanes. The electric field is generated by a method in which multiple electrodes are arranged along a polygonal contour and clamped to predetermined electric potentials. A homogenous electric field is generated by two parallel, infinitely long electrodes. Compared to FIGE, the CHEF system alternating with a single pulse time can produce excellent resolution of large DNA. In addition, the simple relation between migration and molecular weight seems to be generally preserved (Carle and Olson, 1984; Schwartz and Cantor, 1984). Contour-clamping can also be used for electrophoresis in non-uniform fields (Chu ***et al.***, 1986). Contour-clamped fields can be used in identification of secondary structures in DNA as well as other macromolecules.

Transverse Alternating Field Electrophoresis (TAFE), a simple, high resolution technique providing a straight lane geometry was first described by Gardiner ***et al.*** (1986). The gel (10x10cm) is placed vertically in a buffer chamber so that both faces are exposed to electrolyte while electrodes are positioned so that the alternating fields pass through the thickness of the gel, rather than

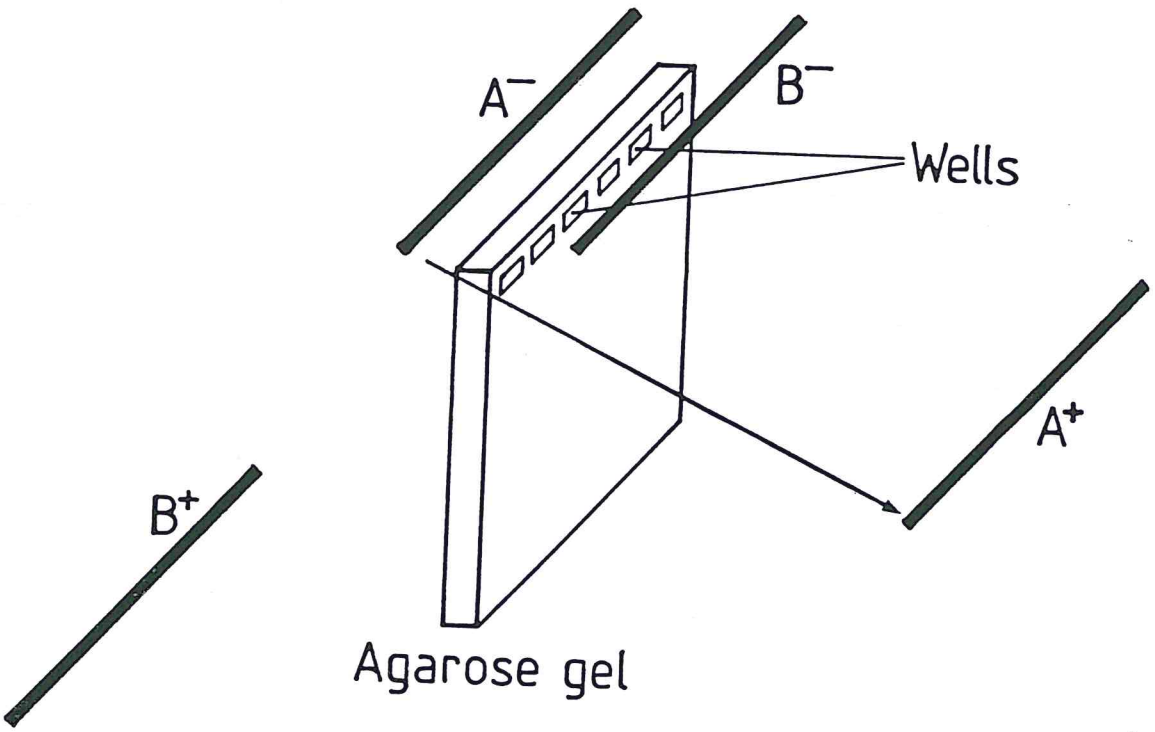


Fig.12. The Principle of Transverse Alternating Field Electrophoresis. (The arrow indicates one of the electrical field directions. A and B are electrodes).

down its length. This ensures that all gel lanes are exposed to equivalent fields so that there is no distortion of the DNA banding pattern. There is a fixed separation angle of 115^0 between the alternating fields (Figure 12). The techniques can separate very large DNA molecules up to 9 million base pairs (Stewart ***et al.***, 1988). An additional advantage of this system is that it can be used to perform direct electrophoretic transfers by placing the nitrocellulose blotting membrane next to the gel. The electrodes are designed to provide a graded field: stronger near the top of the gel where the larger DNA fragments are found, to ensure efficient transfer; weaker near the gel bottom, to prevent the smaller fragments from being driven through the nitrocellulose membrane. The traditional blotting procedures require fragmentation of large DNA molecules by depurination / hydrolysis, a procedure which is difficult to control and can result in significant loss of signal.

In this study, the orthogonal field alternation gel electrophoresis technique was used to analyse chromosome size DNA molecules from new leishmania isolates using WHO ***Leishmania*** reference strains for comparison.

CHAPTER 2:

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 MATERIALS I

2.1.1 Leishmania Promastigote Cultivation Medium

2.1.1.1 N,N,N- MEDIUM

(i) Liquid phase (Lock's solution)

Sodium chloride	8g
Potassium chloride	0.2g
Calcium chloride	0.2g
Potassium monophosphate	0.3g
Glucose/Dextrose	2.5g
Distilled water	1 litre
Penicillin	200 units/ml
Streptomycin	200 mg/ml

(ii) Solid phase

Nutrient agar	20g
Peptone	20g
Beef extracts	25g
Sodium chloride	5g
Distilled water	1 litre
Fresh rabbit blood	1 to 5%

2.1.1.2 RPMI MEDIUM

RPMI 1640 (Gibco)	10.43g/l
Sodium bicarbonate	25mM
Sodium chloride	30mM
Hepes	20mM
Penicillin	200 units/ml
Streptomycin	200mg/ml.
Foetal calf serum	20%

2.1.2 10xTBE buffer (stock solution).

0.9M Tris

0.9M Boric Acid

25mM Ethylenediaminetetraacetate, disodium salt.

Working solution is 0.5xTBE buffer.

TABLE 1

WHO REFERENCE STRAINS

ICIPE REF. NO.	WHO REF. NO.	SPECIES	ORIGIN	HOST	CLINICAL MANIFEST-ATION
227	MHOM/FR/62 (LRC-L47)	***L. infantum***	France	Man	VL
228	MHOM/ET/72/ (L100. LRC-L147)	***L. aethiopica***	Ethiopia	Man	CL
235	MHOM/IL/67/ JERICHO- II (LRC-L137)	***L. major***	Israel	Man	CL
236	LRC-L119	***L. major***	Kenya	Rat	CL
244	LRC-L123	***L. adleri***	Kenya	Lizard	-
245	MHOM/IN/80/ DD8	***L. donovani***	India	Man	VL

CL = Cutaneous leishmaniasis

VL = Visceral leishmaniasis

TABLE 2

NEW LEISHMANIA ISOLATES

ICIPE REF. NO.	HOST	ORIGIN	DATE COLLECTED
47	Lizard	West Pokot	1975
51	"	" "	"
60	"	" "	"
70	"	" "	"
74	"	" "	"
79	"	" "	"
80	"	" "	"
81	"	" "	"
82	"	" "	"
140	"	" "	"
73	Man	" "	"
126	"	Machakos	1979
261	"	Baringo	1986
262	"	"	"
263	Goat	West Pokot	1987
264	"	" "	"

TABLE 2 continued:

265	"	Trans Mara	"
269	"	Baringo	1988
268	"	West Pokot	1987
271	"	" "	1988
274	"	" "	"
281	"	" "	"
283	"	" "	"
277	Sheep	" "	"
267	Rat	Baringo	1987

2.2 METHODS I

2.2.1 Cultivation of Leishmania parasites

Parasites from the cryobank were grown at 25°C in RPMI 1640 culture medium (2.1.1.2.). The culture-adapted ***Leishmania*** isolates were then mass-cultured, pelleted by centrifugation at 600xg for 10 min at 4°C. Some of these parasites were cryopreserved in 2ml plastic tubes in RPMI 1640 culture medium containing 10% glycerol and stored in liquid nitrogen for future use. The remaining parasites were used in cloning while others were stored in sealed tubes at -20°C after washing twice in normal saline. The latter were used for DNA extraction.

2.2.2 Cloning of Leishmania

Serial dilutions of the parasites from culture were made starting from 1:10 to about 1:100,000. These were examined under a light microscope using a x40 eyepiece and counted. The dilution showing 10 parasites per ml was used for cloning. Droplets of the diluted parasite suspension were spotted onto sterile microscope slides and examined. Drops which contained a single parasite were transferred to sterile eppendorf tubes containing 500µl RPMI 1640 culture medium and grown. One hundred tubes were required for each isolate. These tubes were transferred to an incubator

maintained at 25°C. A drop of media from each tube was examined after 6 to 8 days to determine if there were parasites growing therein. The tubes that showed growth were treated as follows: 250µl of the parasite suspension were transferred to clean tubes containing 750µl of fresh RPMI 1640 culture medium. An equal volume of medium (750µl) was added to the original tube of the clone so that the parasites would continue growing. The parasites were then subcultured into clean 50ml culture flasks containing 5ml RPMI 1640 culture medium and left to grow.

2.2.3 Mass cultivation of Leishmania parasites

The parasites from the 50ml flasks were mass-cultured in 200ml culture flasks. After growth these parasites were pelleted by centrifugation at 600xg for 10 min at 4°C and washed twice in normal saline (0.85% sodium chloride). Some of these parasites were then cryopreserved as described earlier while the rest were used in the preparation of agarose blocks for orthogonal field alternation gel electrophoresis (OFAGE).

2.2.4 Preparation of chromosome-sized DNA molecules

The parasite pellet obtained after washing in normal saline was resuspended in the same solution and warmed in a 37°C water bath. These were added to an equal

volume of pre-cooled molten 1.3% low melting point agarose in 0.5xTBE buffer (2.1.2) in a final concentration of $3x10^8$ parasites per ml. One hundred microlitres of this mixture was poured in each well of dimensions 10x6x2 mm and allowed to set on ice. The agarose blocks were then placed in lysis buffer (0.5M EDTA pH 9, 1% SDS and 1mg/ml pronase) and incubated in a water bath at 50°C for 36-48 hrs. On completion of incubation, the blocks were washed extensively with 0.5xTBE buffer. They were then stored in the same buffer at 4°C until use.

2.2.5 Orthogonal Field Alternation Gel Electrophoresis

The conditions suitable for separation of chromosome-sized DNA molecules from ***Leishmania*** by the OFAGE technique were first determined. These included the number of parasites per ml (concentration), pulse frequency, temperature of the buffer and the total electrophoresis time. The method which gave the best separation is described below.

Electrophoretic fractionation was carried out by slicing agarose blocks in halves and placing them in slots (7x3 mm) of agarose gel measuring 14x14 cm. The gel was placed in an electrophoresis tank containing 2.8 litres of 0.5xTBE buffer maintained at 13°C by recirculating through tubing immersed in a controlled temperature water bath at

8°C. Electrophoresis was carried out at 300v for 20 hrs with a pulse frequency of 40 sec. Upon completion of the electrophoresis the gel was stained with 0.5µg/ml ethidium bromide in distilled water and photographed using a C667 high speed polaroid film (ISO 3000/36°) under ultraviolet light.

2.3 MATERIALS II

2.3.1 Restriction endonucleases (from Serva Feinbiochemica, Heidelberg, Germany).

2.3.1.1 BssH II

2.3.1.2 Mlu I

2.3.1.3 Not I

2.3.1.4 Sfi I

TABLE 3

Restriction nucleases useful for generating large DNA fragments (Smith ***et al.***, 1987).

		Source of DNA				
Enzyme	Sequence	Phages	Bacteria	Viruses	Mammals	Others
Not I	GC/GGCCGC	0	1	1	1	1
Sfi I	$GGCCN_4/NGGCC$	1	2	3	1	1
Pvu I	CGAT/CG	3	13	4	1	2
Sal I	G/TCGAC	2	7	6	2	4
Mlu I	A/CGCGT	4	14	4	1	2
Sac I	GAGCT/C	4	5	1	4	6
Nru I	TCG/CGA	5	12	7	1	3

The numbers represent the source of DNA for enzymes.
The source of DNA for enzymes that especially cut rarely are indicated by underlined numbers.
The position where the enzymes cut the sequence specifically is shown by the line drawn in the brackets (/).

2.4 METHODS II

Table 3 shows a list of restriction enzymes useful for generating large DNA fragments. Chromosome samples from WHO reference strains 227, 228, 236 and 245 were digested with restriction enzymes BssH II, Mlu I, Not I and Sfi I according to the Manufacturer's instructions (Serva Feinbiochemica, Heidelberg, Germany). For each sample, 20 units of the enzyme were added in a final volume of 250µl in an eppendorf tube. The tubes were then incubated in a water bath at 37^0C (for Mlu I and Not I) or 50^0C (BssH II and Sfi I) overnight. Twenty five microlitres of 1M EDTA, pH 9 were added to each sample to stop the reaction and the tubes incubated again for 1hr at the same temperature. The samples were then washed extensively with TE buffer (10mM Tris; 0.1M EDTA) before performing OFAGE as described in 2.2.5. Electrophoresis of BssH II, Not I and Sfi I was carried out for 20 hrs while Mlu I was 10 hrs. A control of the intact undigested blocks was included in the gel.

2.5 MATERIALS III

2.5.1 SOLUTIONS AND BUFFERS

2.5.1.1 L-BROTH (1 litre).

Trypton or NZ-Amine	10g
Yeast extract	5g
Sodium chloride	10g
Agar	15g
4N Sodium hydroxide	750µl
Magnesium sulphate (hydrated)	2.5g

2.5.1.2 Pre-hybridization solution

	Volume	Final concentration
20xSSC	3.3 ml	6x
50xDenhardt	1.1 ml	5x
10% SDS	0.1 ml	0.1%
10% Na PPi	0.1 ml	0.1%
Sterile distilled water	6.4 ml	

2.5.1.3 Hybridization solution

Pre-hybridization solution plus one tenth volume of competitor sonicated calf thymus DNA (10µg/ml) and probe (0.5×10^6 cpm/ml).

2.5.1.4 20xSSC stock solution (per litre)

175.3g sodium chloride and 88.2g sodium citrate; pH 7.

2.5.1.5 Washing buffer

Stock solution	Working solution
20xSSC	0.1xSSC
10% SDS	1% SDS

2.5.1.6 50xDenhardt's solution (stock solution)

1% Bovine serum albumin

1% Ficoll 400

1% Polyvinylpyrrolidone

Stir for about six hours at room temperature and sterilize by filtration.

2.5.1.7 10xTAE buffer, pH 7.6 (stock solution).

0.4M Tris base

0.05M Sodium acetate

0.01M EDTA

Working solution is 1xTAE buffer.

2.5.1.8 TCM buffer

10mM Tris-HCl (pH 7.4)

10mM Calcium chloride

10mM Magnesium chloride

2.5.1.9 10xNick translation buffer

500mM Tris-HCl pH 7.8

100mM Magnesium chloride

70mM 2-mercaptoethanol

1mg/ml Bovine serum albumin

2.5.1.10 10xLigation buffer

500mM Tris-HCl (pH 7.5)

100mM Magnesium chloride

100mM 2-mercaptoethanol

10mM Adenosine triphosphate

2.6 METHODS III

2.6.1 Preparation of chromosomal DNA fragment from OFAGE gel

Isolation of the fragment of interest from WHO reference strain 235 (***L. major***, Israel) chromosome blocks was carried out by using three different techniques. In order to isolate the required fragment OFAGE was perfomed on many chromosome samples of strain 235 using low melting point agarose. After electrophoresis the fragments were cut from the agarose gel and kept at 4^0C in 0.5xTBE buffer until use. Several samples were collected and then pooled.

2.6.1.1 Technique 1

The pooled samples in agarose were sliced to long narrow portions and placed in a piece of dialysis tubing containing 0.5xTBE buffer. The dialysis tubing and contents were then placed in the OFAGE gel tank containing 0.5xTBE buffer and electroelution carried out overnight under the standardized conditions. The samples were centrifuged at 7,000xg for 30 min at 4^0C. The volume of the supernatant was reduced to 10ml by extractions with equal volumes of n-butyl alcohol. The lower layer was kept after each addition. The sample was then extracted twice with an equal volume of phenol and ether respectively. The upper and lower layers were kept after the two extractions

respectively. The sample volume was then reduced to 2ml using n-butyl alcohol before adding 200µl of 3M sodium acetate pH 5.2 and 5ml absolute ethanol. The sample was left to precipitate overnight at -20^{0}C. It was then centrifuged at 7,000xg for 30 min at 4^{0}C and the precipitate cleaned with 5ml of 75% ethanol before drying in a lyophilizer for 20 min. The dried sample (labelled I) was resuspended in 200µl of TE buffer (10mM Tris; 0.1M EDTA) and kept at -20^{0}C until used for cloning.

2.6.1.2. Technique 2

The pooled samples in agarose were melted in a water bath at 65^{0}C and transfered at 37^{0}C. The melted sample was diluted three times with 5xTE buffer (pre-warmed in a 37^{0}C water bath). The sample was then extracted twice with an equal volume of phenol (pre-warmed in a water bath at 37^{0}C) as described previously before mixing with an equal volume of chloroform. After ten minutes the upper layer was extracted three times with ether as already described and the sample volume reduced to 5ml by adding n-butyl alcohol. Precipitation was then carried out overnight after adding 500µl of 3M sodium acetate, pH 5.2 and 12ml absolute ethanol. The precipitate obtained after centrifugation at 7,000xg for 30 min at 4^{0}C was washed with 5ml of 75% ethanol, lyophilized for 20 min and then resuspended in 200µl TE buffer. A mini DEAE-52 column was used to clean

the sample further by eluting with 0.5M Tris, pH 8 containing 1.5M sodium acetate. The sample was precipitated by adding 750µl absolute ethanol and leaving it at -70^{0}C for one hour. Centrifugation was carried out at 7,000xg for 30 min at 4^{0}C and the precipitate lyophilized for 20 min. The dried precipitate (labelled II) was resuspended in 100µl TE buffer and kept at -20^{0}C until use.

2.6.1.3. Technique 3

The pooled samples in agarose were sliced lengthwise into narrow portions and placed in a piece of dialysis tubing containing 1xTAE buffer. As control the agarose gel left after cutting out the required fragments was placed in a different dialysis tubing. The dialysis bags were placed in a normal submarine electrophoresis tank containing 1xTAE buffer. Electroelution of the DNA from agarose was perfomed at 400 volts and 300mA in the cold room at 4^{0}C. After 1 hr the control tube was examined under UV illumination for the presence of DNA bands. Since the bands in the gel could still be seen electroelution was continued until there were no DNA bands observed with a UV transilluminator. Buffer containing the electroeluted fragment of DNA was then pipetted from the dialysis bag into a centrifuge tube. The volume of the sample was reduced to 5ml with butanol and extracted twice with an equal volume of phenol and ether respectively. 3M sodium acetate diluted to

a final concentration of 0.3M and 2.5 volumes of cold absolute ethanol were then added to the sample and left at -20^{0}C overnight. The sample was centrifuged at 7,000xg for 30 min at 4^{0}C before lyophilizing for 20 min. It was then resuspended in 250µl TE buffer. Since the sample was not clear it was left to dialyse overnight in one litre of TE buffer in a 'finger tip' dialysis tube at 4^{0}C. Electrophoresis was carried out before and after dialysis so as to determine the size of DNA present in order to select the vector to be used in cloning. The sample (labelled III) was then kept at -20^{0}C until use.

2.6.2 Cloning of Isolated Chromosomal DNA Fragment

Chromosomal DNA samples I, II and III were digested separately with Pst I. The total volume of the samples were made up to 100µl and 200µl respectively by adding sterile distilled water. These were left overnight in a 37^{0}C water bath. 10µl of each digested sample was then used for electrophoresis. The rest of the samples I, II and III were extracted once with phenol and three times with diethyl ether. Precipitation was then carried out for 4 hrs at -70^{0}C by adding 3M sodium acetate, pH 5.2 to a final concentration of 0.3M and 2.5 volumes of cold absolute ethanol. The precipitate was collected by centrifugation at 7,000xg for 30 min at 4^{0}C and then dried for 20 min in a lyophilizer before resuspending in 20µl TE buffer. 10µl of

each sample was used in the ligation step while the remaining was kept at -20^{0}C.

2.6.3 Ligation.

10µl of each sample was ligated to 100ng of pUC18 vector (which had been digested with Pst I restriction endonuclease) in the presence of T4 DNA ligase and 2µl of 10xligation buffer (2.5.1.10). The total volume was made up to 20µl with sterile distilled water before incubating overnight in a water bath at 14^{0}C.

2.6.4 Preculture of ***E. coli*** JM 83 bacterial cells

Ten microlitres of ***E. coli*** JM 83 cells were added to a tube containing 10ml L-broth (without antibiotics). The tube was placed in a cooled orbital incubator with a shaker at 37^{o}C and left overnight. The following morning 1ml was transferred to a flask containing 100ml L-broth (without antibiotics) and left in the incubator at 37^{o}C with a shaker for 3-4hrs. The cells were then centrifuged at 600xg for 10 min at 4^{o}C and the pellet resuspended in 5ml of 50mM calcium chloride. These were left on ice for 20 min before spinning at 600xg for 10 min at 4^{o}C. The pellet was then resuspended in 10ml of 50mM calcium chloride and left on ice to be used for transformation.

2.6.5 Transformation

Each sample (10µl) was mixed with 50µl TCM buffer (2.5.1.8) in sterile tubes. 100µl JM 83 competent bacteria cells were added to the DNA-TCM mixture and incubated on ice for 15 min. The tubes were transferred to a water bath at 42^0C for 2 min, left on ice for 5 min and then 800µl L-broth was added. The samples were then incubated in a water bath at 37^0C for 45 min before carrying out the plating operation.

2.6.6 Preparation of L-Broth plates

One litre of freshly prepared L-broth and agar (2.5.1.1) was cooled to 45^0C before adding 10ml Ampicillin (10mg/ml). The L-broth was then poured onto Petri dishes and left overnight upside down at 37^0C. The mixture containing the sample was poured onto the agar plates and spread evenly by using a glass L-shaped loop. After 15 min the plates were incubated overnight at 37^0C. The transformed bacteria colonies were transferred to new agar plates in duplicate, one covered with nitrocellulose filter and the other free (master plate). The colonies to be tested were stabbed using sterile tooth picks onto the two plates. The colonies were transferred in a grid pattern and on identical positions on both plates. The plates were then incubated overnight at 37^0C. The master plate was kept at

4^0C while the filters were removed from the plates and soaked in 0.5M sodium hydroxide containing 1M sodium chloride for 10 min with the colony side up. The filters were then transferrred to 1M Tris-HCl, pH 7.4 containing 3M sodium chloride and 10mM EDTA for 10 min before soaking them in 4xSSC solution (2.5.1.4). The filters were dried by blotting with 3MM filter paper and baked under vacuum at 80^0C for 2hrs. The filters were immersed in pre-hybridization solution (2.5.1.2) in a sealed plastic bag and left overnight at 65^0C. The filters were then transferred to fresh pre-hybridization solution, just enough to cover the filters and boiled probe (total DNA from strain 235) with carrier (2.5.1.3) added into it for hybridization at 65^0C overnight. This was carried out in a sealed plastic bag. The filters were washed with 0.1xSSC containing 1% SDS at 65^0C for 2 to 3hrs. They were then dried and set up for autoradiography. The autoradiogram was compared with the master plate in order to select the recombinants that had hybridized with the probe. The colonies whose DNA gave a positive autoradiographic signal were then recovered from the master plates. These colonies were transfered to L-broth media containing Ampicillin and left to grow overnight in an orbital shaker incubator.

2.6.7 Miniscreen of Recombinant Plasmids

Five hundred microlitres of the clones that had been grown overnight were preserved in an equal volume of glycerol and kept at -20^{0}C. The rest were centrifuged at 1,100xg for 5 min. The supernatant was discarded while the precipitate was resuspended by vortexing in 1ml of an ice-cold solution (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH 8, 4mg/ml lysozyme). The tubes were left at room temperature for 5 min before adding 2ml of an ice cold solution of 0.2N sodium hydroxide and 1% SDS. The contents of the tube were mixed before adding 1.5ml of an ice-cold solution of potassium acetate, pH 4.8 (6ml of 5M potassium acetate added to 1.15ml of glacial acetic acid and 1.85ml of water; the resulting solution was thus 3M potassium and 5M acetate). Tubes were vortexed for 10 sec and left on ice for 5 min before centrifuging at 1,100xg at 4^{0}C for 5 min. The supernatant was transferred to clean tubes and extracted with an equal volume of phenol/chloroform mixture. Tubes were then centrifuged at 1,100xg for 5 min at 4^{0}C and the supernatant transferred to a clean tube. Two and a half volumes of absolute ethanol were added to each sample and precipitation allowed to take place overnight at room temperature. The precipitate was obtained after centrifugation at 1,100xg for 5 min at 4^{0}C and lyophilized. Each precipitate was then resuspended in 200 μl TE buffer and kept in eppendorf tubes at -20^{0}C. Electrophoresis of the samples was carried out in 1xTAE buffer (2.5.1.7).

CHAPTER 3:

MOLECULAR KARYOTYPE ANALYSIS OF *LEISHMANIA*

3. MOLECULAR KARYOTYPE ANALYSIS OF LEISHMANIA

After the conditions required for performing OFAGE were standardized, chromosomal DNAs were analysed from new ***Leishmania*** isolates (Table 2); the WHO ***Leishmania*** reference strains (Table 1) were included in the analysis for comparison.

3.1 RESULTS

3.1.1 Determination and standardization of running conditions for OFAGE

The optimum conditions for OFAGE were determined as described in the Materials I and Methods I section (Chapter 2). The results obtained using the different parameters are shown below.

3.1.1.1 Concentration

The optimum concentration of the parasites was determined by preparing different concentrations of one isolate and performing electrophoresis at a constant voltage of 300v at 8^{o}C for 22 hrs and a pulse frequency of 40sec. OFAGE was carried out using $7.5x10^{7}$ to $1.25x10^{9}$ parasites per ml (Figure 13). The bands could be seen clearly in the lower region at concentrations of $1.25x10^{9}$ and $6.0x10^{8}$

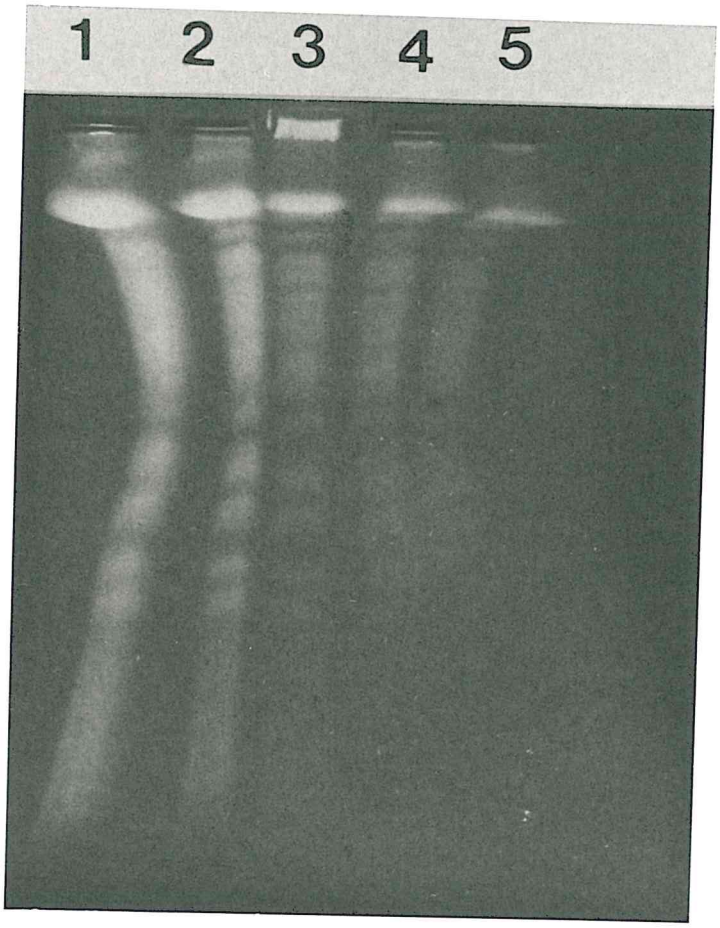


Fig. 13. The Chromosome Profiles of one isolate at different concentrations:

Lane 1:	1.25×10^9	parasites per ml.
" 2:	6.0×10^8	" " "
" 3:	3.0×10^8	" " "
" 4:	1.5×10^8	" " "
" 5:	7.5×10^7	" " "

parasites per ml (lanes 1 and 2 respectively). Bands in the upper region were not resolved (lanes 1 and 2). The best resolution of bands in all the regions was obtained using $3x10^8$ parasites per ml (lane 3). At the lower concentrations, the bands in the lower region could not be seen clearly. Therefore, the selected working concentration for performing electrophoresis was $3x10^8$ parasites per ml. Since half of the 100µl parasite-agarose block was used, the actual concentration was $1.5x10^7$ parasites per slot.

3.1.1.2 Pulse Frequency

The electrophoretic mobility of DNA subjected to OFAGE depends strongly on the pulse time (duration of applied field). Different isolates at a fixed concentration of $3x10^8$ parasites per ml were used. Electrophoresis was carried out at pulse frequencies of 20, 40, 60, 80 and 100 sec at 8^0C for 22 hrs. At 20 sec chromosome-sized DNA bands stayed between the loading slots and medium molecular weight regions (Figure 14a). A pulse frequency of 40 sec was suitable since bands could be clearly seen in all the regions (i.e. low, medium and high molecular weight regions) as compared to the others which had bands in specific areas (Figure 14b). The bands at 60 sec were scattered between the loading slots and the medium region while those at the lower region were squeezed together (Figure 14c). There was a smear from the high to the medium molecular weight region

Fig. 14a. The Chromosome Profiles of isolates at a pulse frequency of 20 sec.

Lanes 1 to 6 have different isolates.

Fig. 14b. The Chromosome Profiles of isolates at a pulse frequency of 40 sec.

Lanes 1 to 6 have different isolates

Fig. 14c. The Chromosome Profiles of isolates at a pulse frequency of 60 sec.

Lanes 1 to 6 have different isolates.

Fig. 14d. The Chromosome Profiles of isolates at a pulse frequency of 80 sec.

Lanes 1 to 6 have different isolates.

Fig. 14a

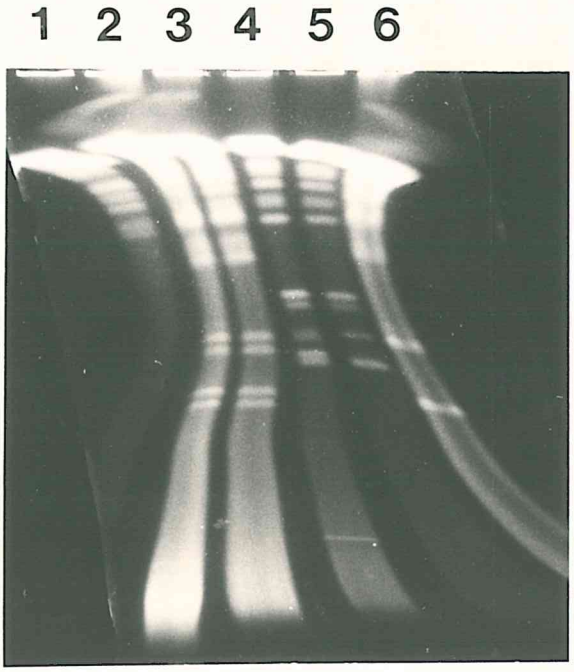
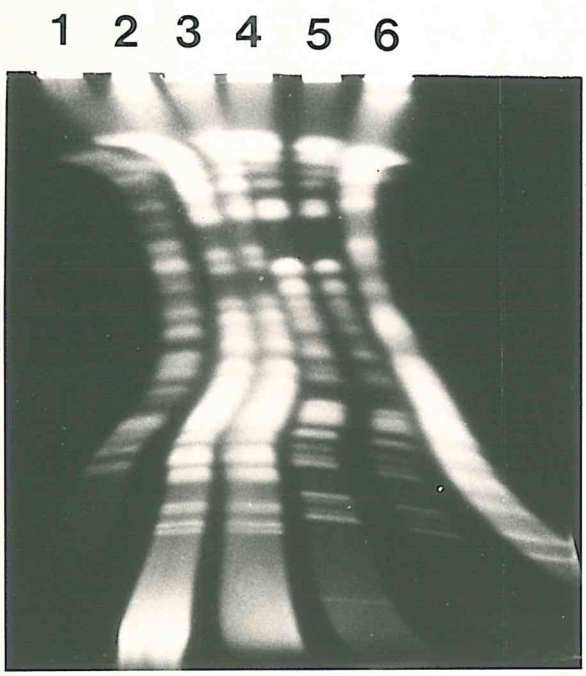


Fig. 14b



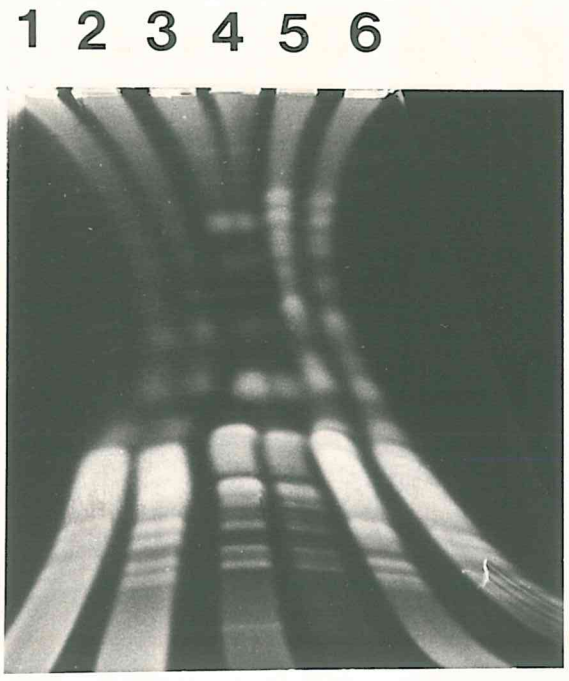


Fig. 14c

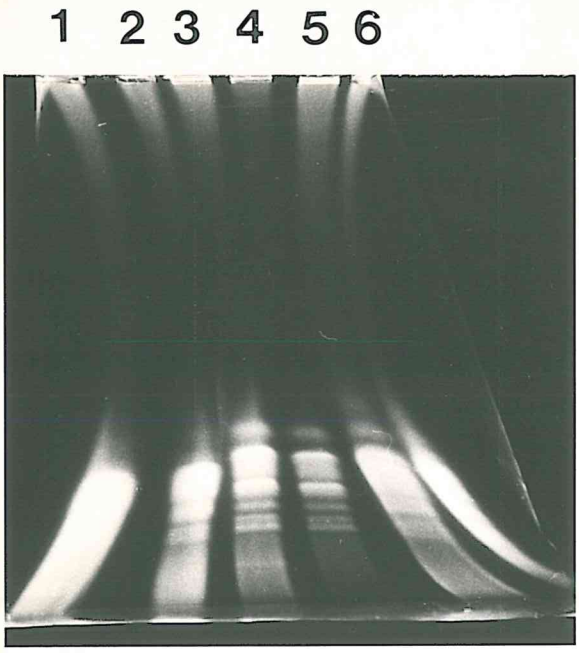


Fig. 14d

at 80 and 100 sec and bands were seen only at the lower region (Figure 14d).

3.1.1.3. Temperature

A cooling system was set at the following temperatures: 4^{o} , 6^{o} , 8^{o} , 10^{o} and $12^{o}C$ respectively. One isolate at a fixed concentration of $3x10^{8}$ parasites per ml was used. Electrophoresis was carried out at a pulse frequency of 40 sec for 22 hrs. The pattern of bands seen at $4^{o}C$ and $6^{o}C$ appeared blurred while those at 8^{o}, 10^{o} , and $12^{o}C$ were clear (Figures 15a, b, c and d). The temperature appropriate for the electrophoresis was the lowest of the last three, which is $8^{o}C$, since the high voltage of 300v used in the technique requires rapid and efficient cooling. There is also less disintegration of the DNA at the lower temperature. The temperature difference between the water bath and buffer in the gel tank was $5^{o}C$. Thus the temperature in the gel tank was $13^{o}C$ at the selected water bath temperature of $8^{o}C$.

3.1.1.4. Duration of running the electrophoresis

Electrophoresis of one isolate containing $3x10^{8}$ parasites per ml was carried out for different durations (from 16 to 24 hrs) at a pulse frequency of 40 sec and $8^{0}C$. The separation of bands at 16 and 18 hrs was not very

Fig.15a

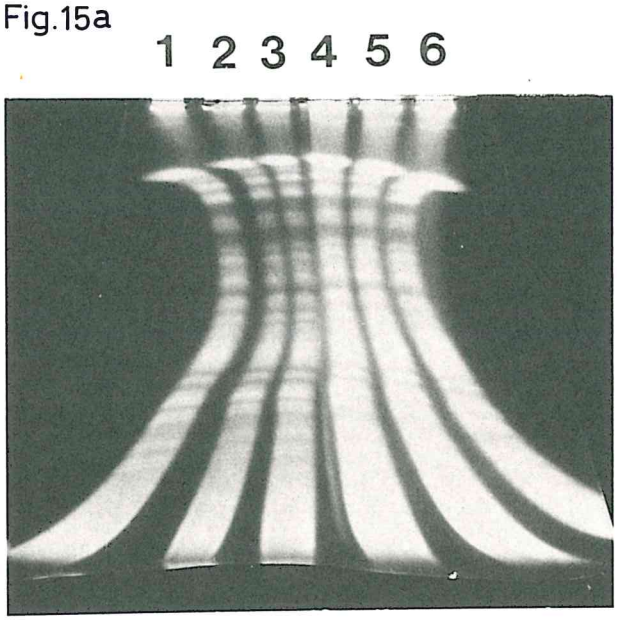
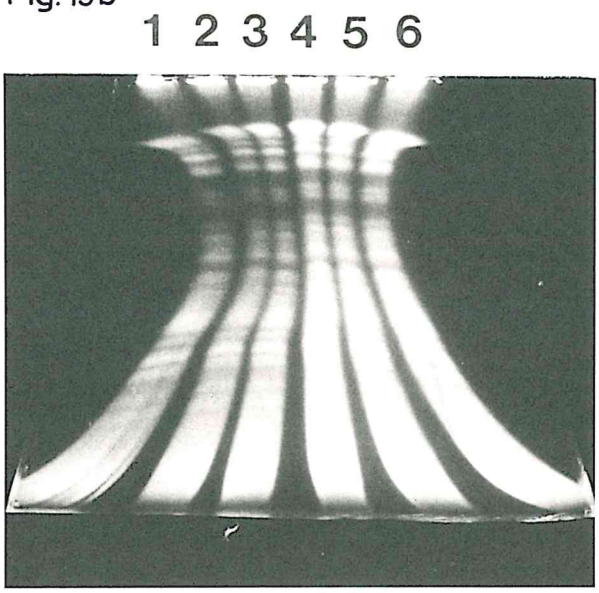


Fig.15b



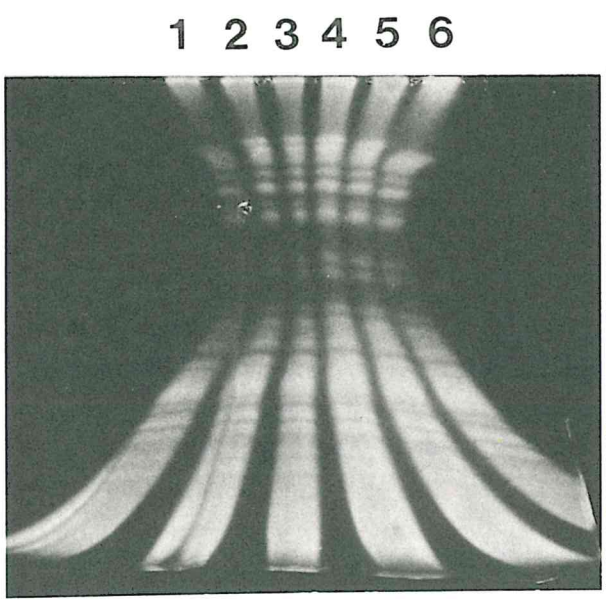


Fig.15c

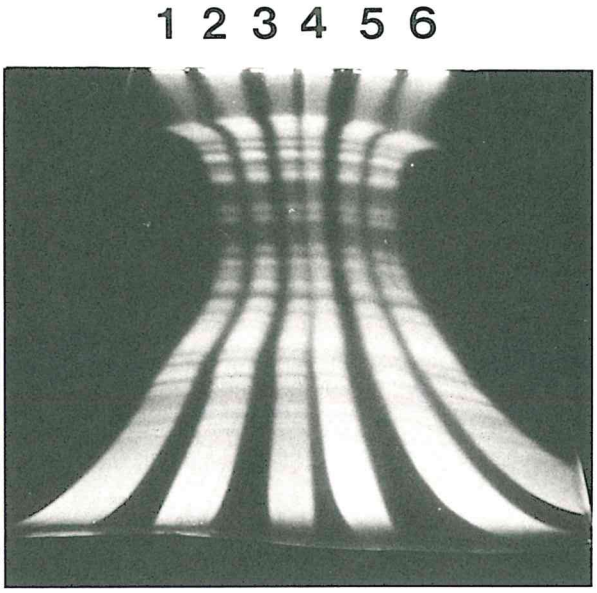


Fig.15d

Fig.16a

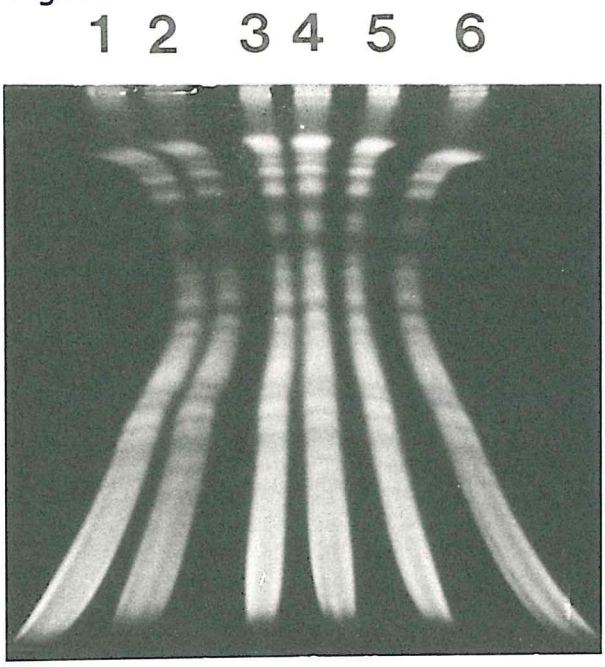
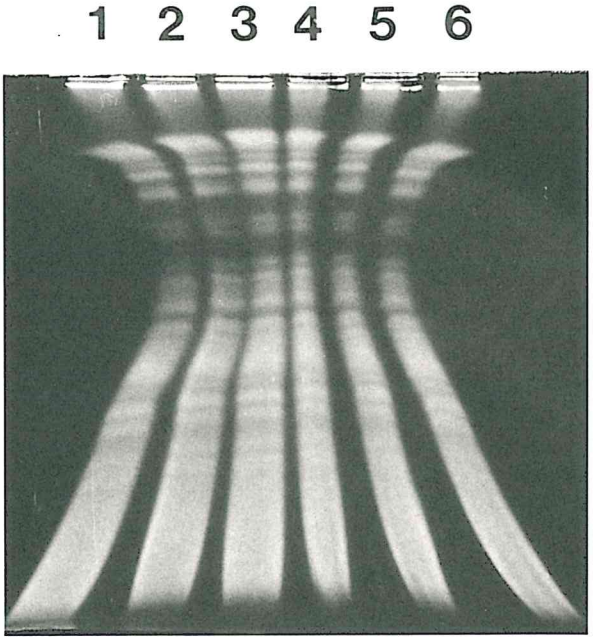


Fig.16b



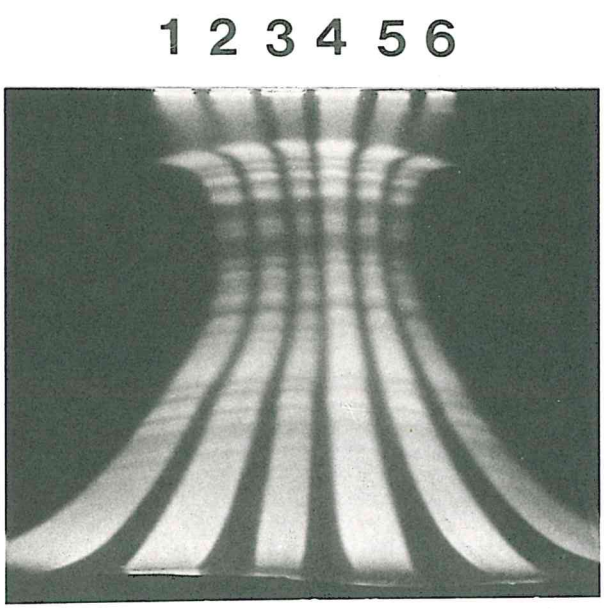


Fig.16c

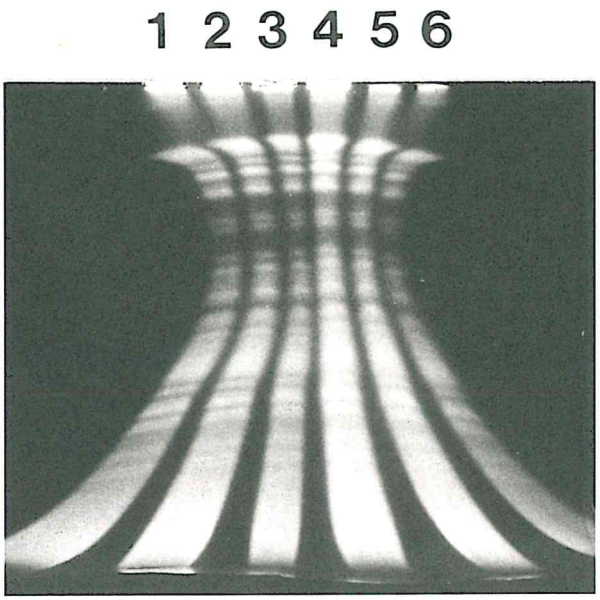


Fig.16d

distinct and the bands were closer to each other (Figures 16a and 16b). The pattern of bands at 20, 22 and 24 hrs were the same (Figures 16c and 16d). Electrophoresis of all the isolates was thus carried out for 20 hrs.

3.1.2 Analysis of Molecular Karyotypes of the WHO *Leishmania* Reference Strains

Studies were carried out to analyse by OFAGE the chromosomal DNAs from WHO reference strains and to determine the relationships amongst themselves.

The Materials I and Methods I used have been described in Chapter 2. The WHO ***Leishmania*** reference strains for this work were obtained from the ICIPE cryobank (Table 1). These reference strains were used to study the taxonomic relationships among new leishmania isolates.

The chromosome profiles of the WHO reference strains is shown in Figure 17. The chromosome-sized DNA banding patterns in the medium and low molecular weight regions of these marker strains could be divided into three groups as shown below:

Group I	227 (***L. infantum***)
	228 (***L. aethiopica***)
	236 (***L. major***, Kenya)
	245 (***L. donovani***)
Group II	244 (***L. adleri***)
Group III	235 (***L. major***, Israel)

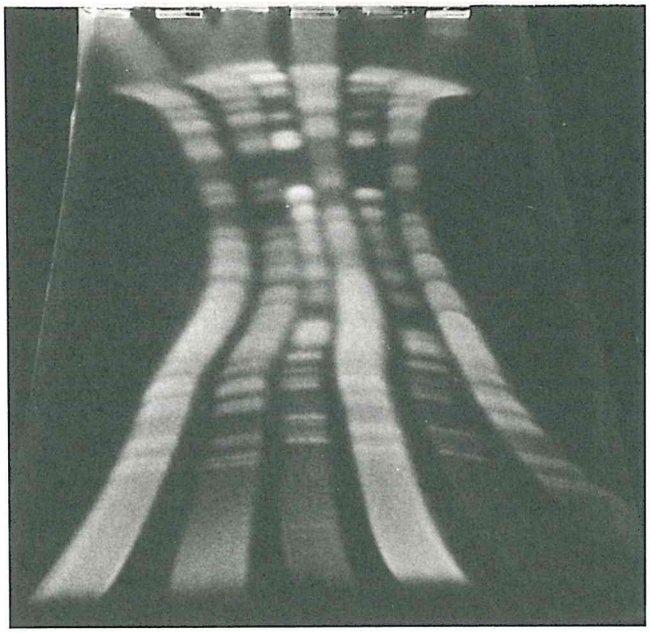
All the chromosome profiles had the megabase band which represents large DNA molecules that only just enter the gel about 1cm from the loading slot. Most of the bands were spread 1.5-5cm from the origin which is the medium-sized range between 200-500 kilo base pairs. No mini-chromosomes were present except in strain 235 (***L. major***, Israel). Apart from these, very large DNA molecules remained in or near the loading slot.

3.1.3 Analysis of Molecular Karyotypes of New *Leishmania* Isolates collected from the disease endemic areas in Kenya

The OFAGE technique was used to analyse chromosomal DNAs from twenty five cloned new leishmania isolates as described in Materials I and Methods I in Chapter 2.

Fig.17

1 2 3 4 5 6



1 2 3 4

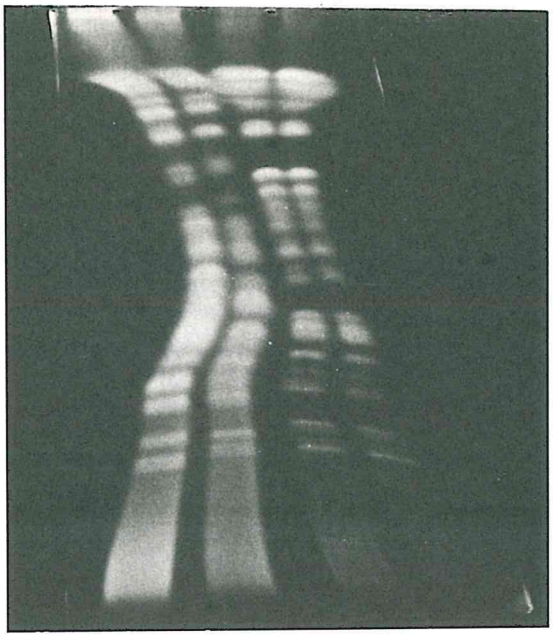


Fig.18

The new leishmania isolates were from humans and other animals (Table 2). These were obtained from the liver or spleen of the host and grown in ***in vitro*** culture in N,N,N media (2.1.1.1) before preserving in liquid nitrogen.

Electrophoresis of the twenty five cloned new leishmania isolates (Table 2) was performed and their chromosome profiles were compared to those of the reference strains (Figures 18 to 26). The banding patterns of thirteen isolates tested were similar to reference strains in Group I while three fitted in Group II (Table 4a). None of the isolates were similar to the reference strain in Group III (Table 4a). The remaining nine isolates were different from the reference strains and therefore did not fit in any of the three Groups (Table 4b).

3.1.3.1 New Leishmania Isolates that were similar to reference strains

The grouping of the chromosome profiles of new leishmania isolates that were similar to reference strains is shown in Table 4a.

3.1.3.1.1 *Leishmania* isolates that fitted in Group I

One of the isolates which fell under Group I (isolate 140) was run on the same gel with reference strains

from Groups I, II and III. The results of the chromosomal DNA bands in Figure 18 show that 140 (lane 2) is similar to reference strains in Group I in the low and medium molecular weight regions (lane 1 - 228 fitted in Group I).

The chromosomal DNA molecules of isolates 73, 74, 80, 81 and 82 were similar to each other (Figure 19). The chromosome profiles of isolates 60, 70, 140, 79, and 265 were similar to each other (Figure 20). All the isolates in the two figures (19 and 20) fitted in Group I.

Isolates 283, 274, 281, 47 and 51 were run against reference strain 245 (***L. donovani***) in Group I. The banding patterns of isolates 281, 47 and 51 were similar to that of reference strain 245 (Figure 21).

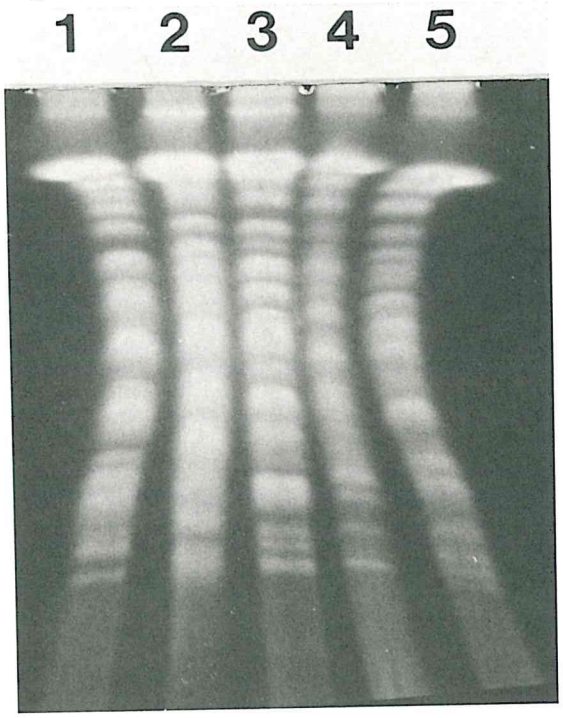
3.1.3.1.2 ***Leishmania*** isolates that fitted in Group II

In Figure 22, reference strains 244 and 493 (both ***L. adleri***) in Group II have the same banding patterns as isolates 268, 269 and 271 (Table 4a).

3.1.3.2 New Leishmania Isolates that were different from reference strains

The banding patterns of nine isolates that did not fit in any of the three groups could be divided into

Fig.19



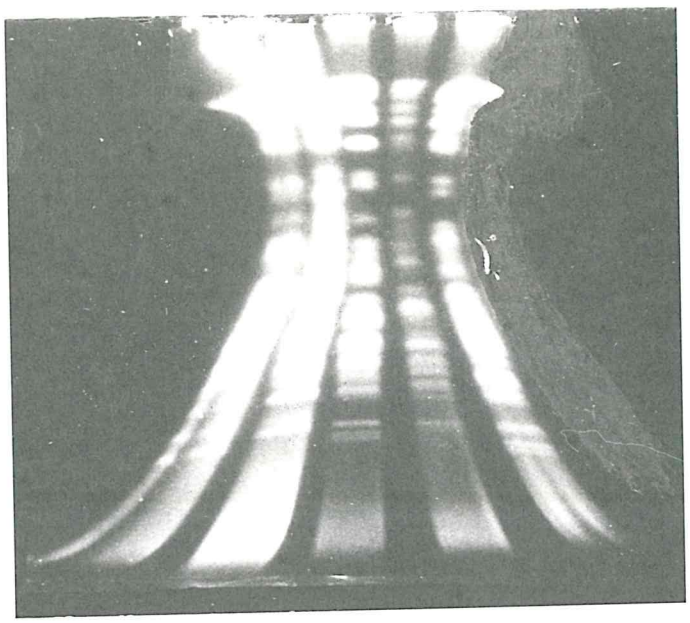
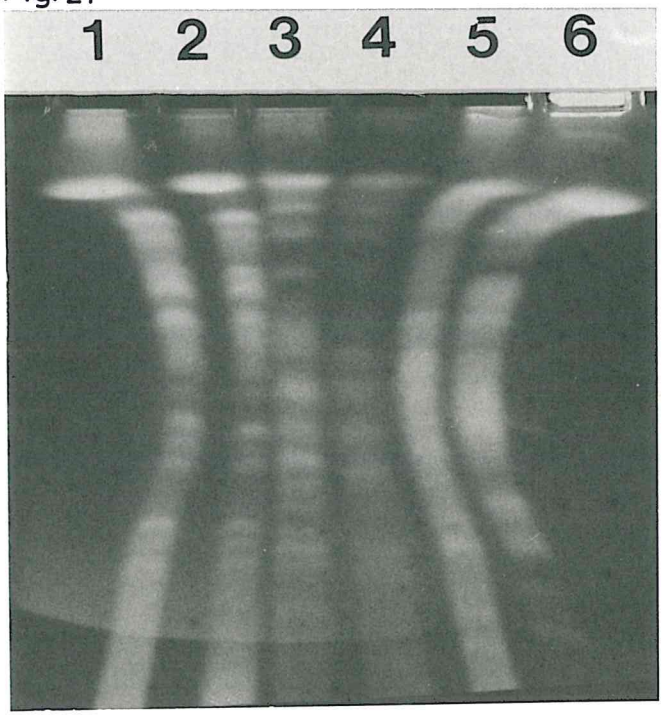


Fig. 20

Fig. 21



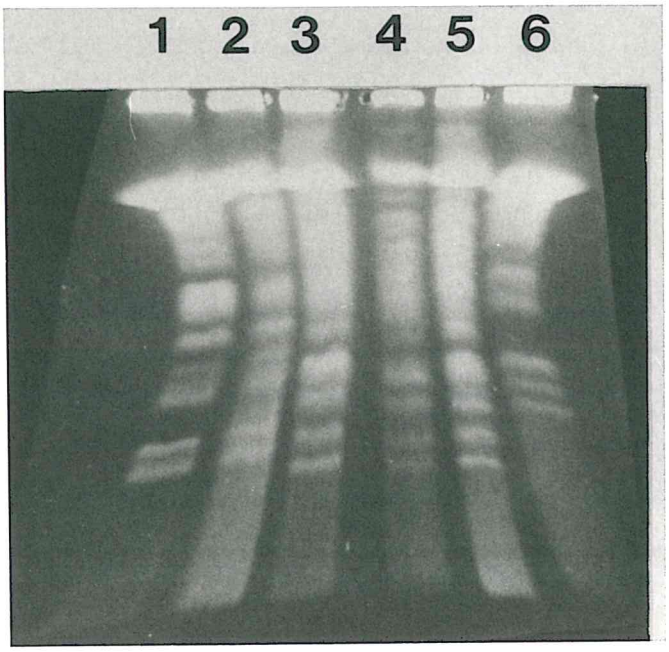


Fig. 22

five separate groups shown in Table 4b. The five groups had three, two, two, one and one isolates respectively. Isolate 126 was run on the same gel with reference strains in Groups I, II and III. The results in Figure 23 show that the chromosome profile of this isolate is different from that of all the three Groups.

In Figures 24 and 25 the chromosomal banding patterns of isolates 262, 263 and 264 are shown to be similar. Isolates 261 and 267 have the same chromosome profiles as shown in Figures 24 and 26 while 126 is different. Isolates 283 and 274 had similar banding patterns (Table 4b) as shown in Figure 21. Isolate 277 had fewer chromosomes compared to all the isolates tested (Table 4b). No small chromosomes were observed in this isolate (Figure 22).

Fig. 23

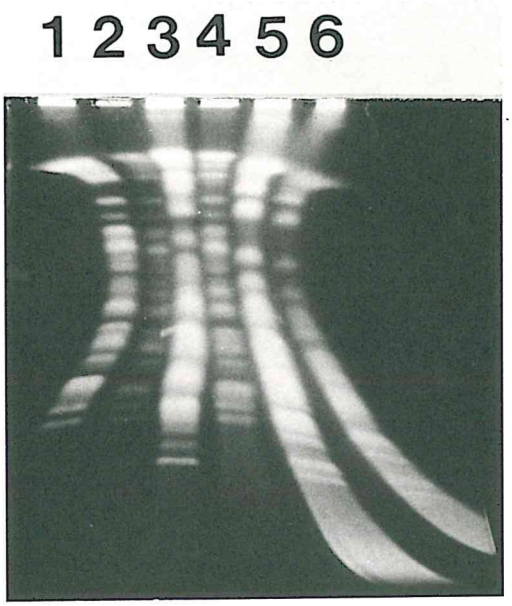


Fig. 24

Fig. 25

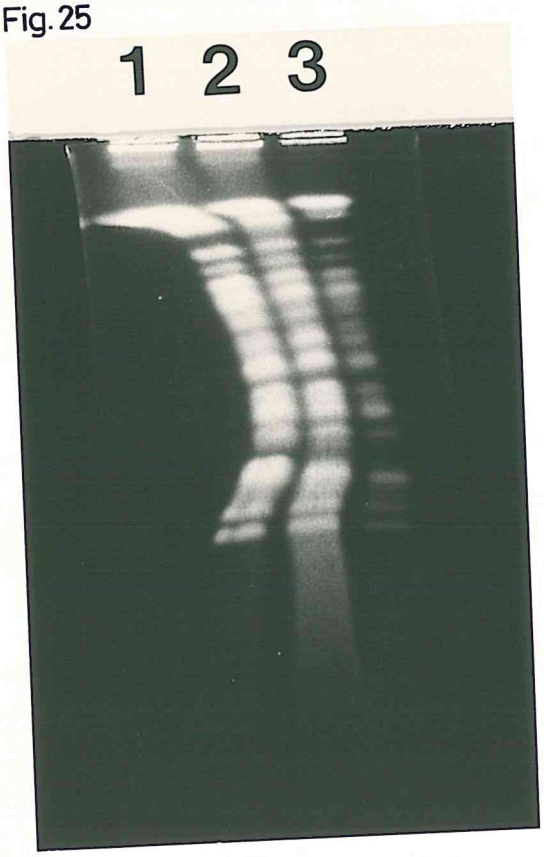


Fig. 26

TABLE 4

(a) GROUPING OF THE CHROMOSOME PROFILES OF NEW ***LEISHMANIA*** ISOLATES THAT WERE SIMILAR TO REFERENCE STRAINS

GROUP I	GROUP II	GROUP III
47, 51, 60, 70, 73,	268	-
74, 79, 80, 81, 82,	269	-
140, 265, 281.	271	-

(b) GROUPING OF THE CHROMOSOME PROFILES OF NEW ***LEISHMANIA*** ISOLATES THAT WERE DIFFERENT FROM REFERENCE STRAINS

A	B	C	D	E
262	261	274	277	126
263	267	283		
264				

3.2 Discussion and Conclusions

The chromosomal banding patterns of the six WHO reference strains could be divided into three groups. Four strains namely, ***L. infantum***, ***L. aethiopica***, ***L. major*** (Kenya) and ***L. donovani*** fitted in Group I. ***L. adleri*** and ***L. major*** (Israel) were in Groups II and III respectively. The chromosome profile of ***L. major*** (Israel) had a unique small fragment (Figure 17).

The results have demonstrated that, as can be revealed by comparison of chromosomes separated by OFAGE, ***L. major*** (Kenya) is different from ***L. major*** (Israel) although both cause cutaneous leishmaniasis (Figure 17). A study in Israel showed a variation in isoenzyme patterns between ***L. major*** isolates obtained from different areas (Le Blancq ***et al.***, 1985). Massamba ***et al.*** (in preparation) using isoenzyme analysis and molecular hybridization techniques found that ***L. major*** (Kenya) and ***L. major*** (Israel) were different ***Leishmania*** species. Surface carbohydrates of ***L. major*** isolates have also been found to be different as demonstrated by the variation in excreted factor serotypes (Schnur, 1982; Schnur and Jacobson, 1989). Thus the results have shown that species which originate from different geographical regions and show a similar clinical picture can have different molecular karyotypes. The four strains in Group I could not be distinguished by OFAGE.

The chromosome profiles of the new leishmania isolates were analysed using WHO reference strains for comparison. Thirteen isolates fell under Group I while three fitted in Group II. None of the isolates fitted in Group III. Nine isolates were different from all the reference strains tested and therefore did not fit into any of the three groups.

All the isolates collected from West Pokot did not fit in the same genetic group. This indicates that different ***Leishmania*** species are present in the same focus, whose vectors are yet to be established. Parasites isolated from ten lizards, a goat and one human in West Pokot as well as a goat from Trans Mara fitted in the same group (Group I). The goats and human isolates which fitted in Group I could belong to one of the three species, that is, ***L. aethiopica***, ***L. major*** or ***L. donovani***. An isolate from a goat (265) was studied by Mutinga ***et al.*** (1988) and Mutinga ***et al.*** (1989). According to their data the parasites isolated from it could be ***L. aethiopica***. The parasites from the human and goats in this group need further analysis to classify them into specific species. The two goats (265 and 281) were from different geographical regions, Trans Mara and West Pokot respectively. The human isolate (73) was from the same region as the ten lizards isolates, that is, West Pokot. The humans and lizards could have acquired the

parasite from sandflies feeding on lizards and humans. Further analysis of these isolates would help in classifying them into specific species.

Isolates that fitted in Group II were from three goats; two of these were found in West Pokot while the other one was from Baringo. This may indicate that ***L. adleri*** is also found in other animals apart from reptiles. It is possible that sandflies infected with ***L. adleri*** had fed on these goats since the insects can feed on a variety of hosts. It is also possible that either of the host, that is, reptile and goat may have been "accidental" hosts and this needs to be studied to resolve the riddle.

None of the isolates from the lizards fitted into Group II (***L. adleri***). This may indicate that apart from ***L. adleri***, other species of ***Leishmania*** are present in reptiles. It is possible that lizards (and may be other reptiles) are reservoir hosts of the species that infect man. It would be interesting to study isolates from other reptiles and determine whether they also act as reservoir hosts for the human ***Leishmania*** or if ***L. adleri*** circulates among them. It is also possible that sandflies belonging to the genus ***Sergentomyia*** which are vectors of ***L. adleri*** may not be present in West Pokot and this would explain why the lizards harbour other ***Leishmania*** species. If this is the case then the lizards may be incidental hosts. Furthermore,

it may be possible that other routes of infection apart from the sandfly bite might exist, that is, infection through the gut by lizards feeding on infected flies.

None of the isolates were similar to Group III (***L. major*** - Israel). This may be explained by the fact that it is from a different geographical region. It is further supported by the results obtained for the reference strains ***L. major*** from Kenya and Israel which fitted in different genetic groups.

The nine isolates that did not fit in any of the three groups of the reference strains could be subdivided into five separate groups (Table 4b). These included isolates from humans, goats, sheep and a rat. The isolates fitted in the following groups- A: two isolates from goats (in West Pokot) and one from a human (in Baringo); B: one from a human and another from a rat (both from Baringo); C: two from goats in West Pokot; D: one from a sheep in West Pokot and E: one from a human in Machakos.

Some of the isolates which fitted in the same chromosome profile group were initially isolated from different regions of Kenya while others were from the same region. It is possible that some of the isolates that did not fit into the three groups may be similar to other

strains, for example, ***L. tropica*** which was recently reported to be present in Kenya (Mebrahtu ***et al.***, 1988; Mebrahtu ***et al.***, 1989). The isolates could also have been similar to other strains which have shown genetic diversity, for example, ***L. donovani***. The ***L. donovani*** included among the reference strains was from India. Bishop and Akinsehiwa (1989) demonstrated that African stocks of ***L. donovani*** were genetically distinct from other ***L. donovani*** isolated from South America, the Mediterranean and India. In view of these authors' findings and the observations from this study, it would be useful to review the current classification of these species of Protozoa. It would also be interesting to compare the chromosome profiles of the isolates to a Kenyan strain of ***L. donovani***. More reference strains are thus needed for comparison.

CHAPTER 4:

RESTRICTION ENDONUCLEASE DIGESTION OF DNA IN THE CHARACTERIZATION OF *LEISHMANIA* SPECIES

4. Restriction Endonuclease digestion of DNA in the characterization of *Leishmania* species

Restriction enzymes are a group of endonucleases which cleave phosphodiester bonds of a double-stranded DNA at a specific site. This property leads to the use of enzymes in a specific cleavage and subsequent construction of a recombinant DNA. Analysis of parasite species and strains can be conveniently done by examination of the patterns of their DNA digested with specific restriction endonucleases. A restriction enzyme cuts a pure DNA sample into a consistently reproducible set of fragments that can be easily separated by gel electrophoresis. The comparison of DNA fragments produced by restriction analysis is a powerful tool for taxonomic purposes. Restriction enzyme analysis can reveal even relatively minor differences between DNAs from various species, such as deletions, rearrangements or substitutions. The set of DNA fragments produced by a restriction enzyme is characteristic for a given DNA and the electrophoretic pattern of these fragments can be used for taxonomic and diagnostic purposes.

The biological function of these enzymes is to protect the bacterial cell from foreign DNA by digesting it. The proper cell DNA is protected from restriction by the site-specific enzymatic methylation of base residues within restriction site. The enzymes are usually designated after

microorganisms from which they are isolated, for example, Pst I from ***Providencia stuartii***. Different enzymes obtained from the same strain are indicated by Roman numerals (for example, Hind II and Hind III).

The Materials II and Methods II are described in Chapter 2.

4.1 Results

4.1.1 Not I chromosomal DNA digestion

Digestion with Not I showed a smear in all the four reference strains (Figure 27). Differences were observed in the mobilities of the smears. According to their mobilities the strains fitted in two groups: one was 227 (***L. infantum***) and 236 (***L. major***, Kenya); two was 228 (***L. aethiopica***) and 245 (***L. donovani***). A control of intact block had bands in all the regions.

4.1.2 BssH II chromosomal DNA digestion

In the digestion with BssH II differences were observed in the mobilities of the smears (Figure 28). The three reference strains 228, 236 and 245 were similar while 227 was different. The control of undigested blocks had all the bands in the high and low molecular weight regions.

Fig. 27

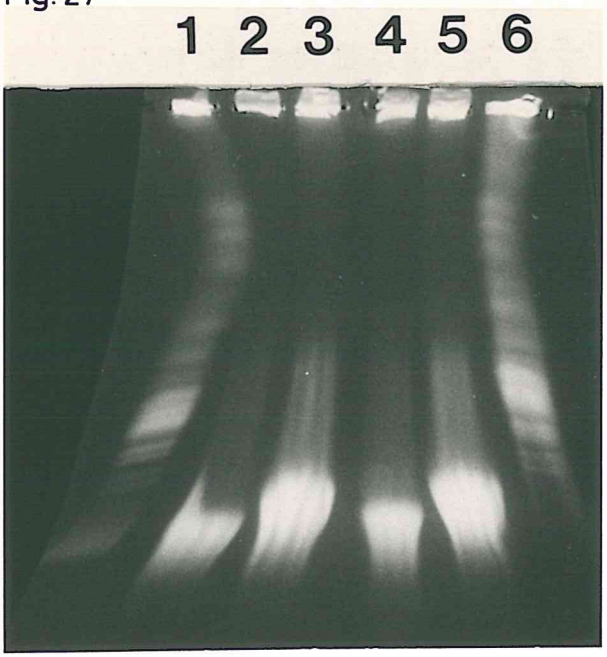
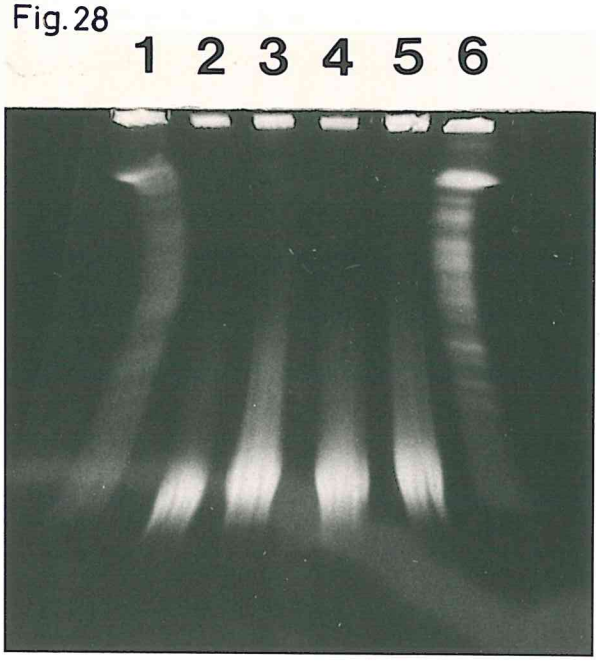


Fig. 28



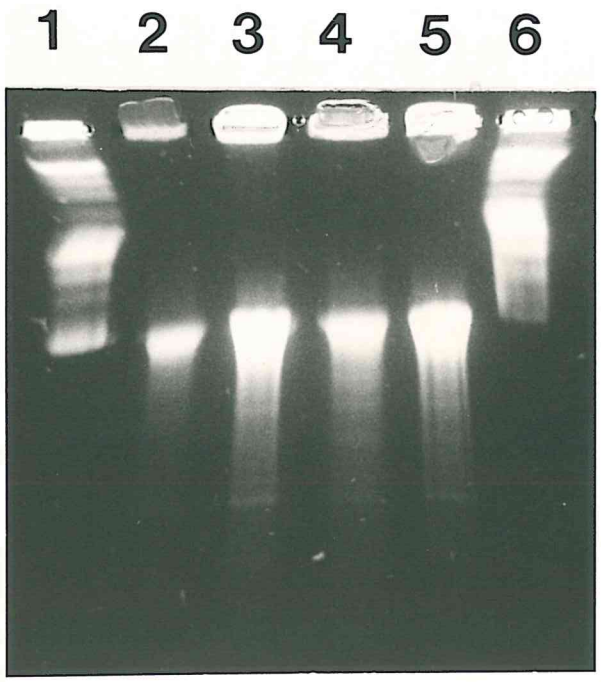


Fig. 29

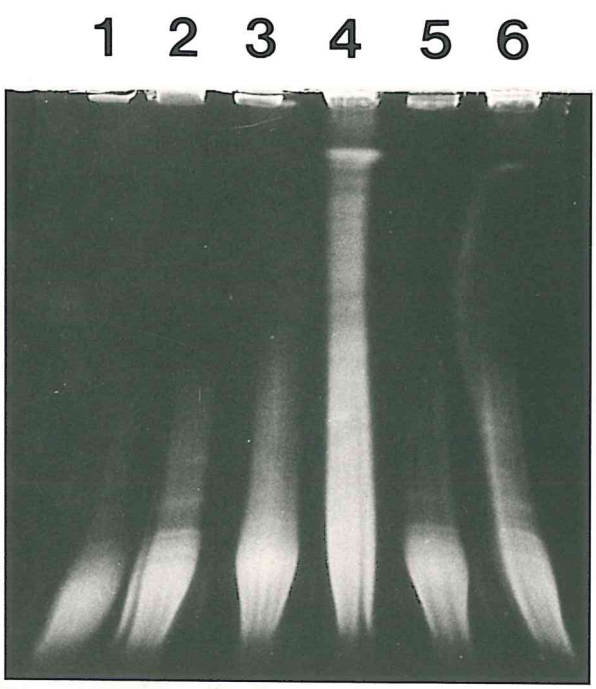


Fig. 30

4.1.3 Mlu I chromosomal DNA digestion

The control of undigested block had all the bands while the samples digested with Mlu I had no bands in the high and medium molecular weight regions. Differences were observed in the four reference strains (Figure 29). The mobility of the smear of strain 227 was different from the other three. Strain 236 had more bands than 228 and 245. This endonuclease showed the presence of a band in the low molecular weight region of strain 228. The band was not found in strains 245, 236 and 227.

4.1.4. Sfi I chromosomal DNA digestion

Digestion with Sfi I showed that 227 and 236 were different (Figure 30). There were more bands in strain 236 as compared to 227. This result is similar to the one observed in the digestion with Mlu I with respect to 236 and 227. A smear was formed in strain 245 digested with Sfi I. The control of intact block had all the bands.

4.2 Discussion and Conclusions

The smears obtained by chromosomal DNA digestion with Not I and BssH II means that these enzymes cut the DNA into several fragments. Digestion with Mlu I could differentiate 228 from the other three reference strains namely, 227, 236 and 245. Sfi I distinguished 227 from 236 and it also cut strain 245 into several fragments as shown by the generation of a smear.

Restriction endonucleases cut DNA into fragments which are then analysed by PFG. Strains of the parasite can be grouped in subpopulations having similar patterns of restriction endonuclease products. Experiments with these endonucleases have shown that Sfi I and Mlu I can be used to distinguish the four reference strains that fitted in Group I. Digestion with Not I and BssH II could not show differences among the strains. Sfi I and Mlu I can thus be used to digest blocks of isolates that fitted in this group for further analysis into specific marker strains.

The results obtained have shown that the four reference strains 227, 228, 236 and 245 differ in the restriction enzyme sites. This could imply that the four strains are genetically different. Jackson ***et al.*** (1984) distinguished parasites that cause different forms of the

disease (visceral and cutaneous leishmaniases) by using restriction enzyme digestion of kinetoplast DNA. The restriction enzymes used in this work could distinguish ***L. major*** from ***L. donovani***, the parasites which cause cutaneous and visceral leishmaniases respectively. Beverley ***et al.*** (1987) demonstrated genetic heterogeneity between and within ***Leishmania*** species by comparing nuclear DNA restriction fragment patterns. The four reference strains in Group I that had similar karyotypes could be distinguished by the enzymes. Further analysis of the new isolates that fitted in Group I can be done by using the same restriction enzymes.

CHAPTER 5:

CLONING OF CHROMOSOME-SIZED DNA FRAGMENT FROM *L. MAJOR* (ICIPE 235)

5. Cloning of chromosome-sized DNA fragments (*L. major*, ICIPE 235)

The previous results of OFAGE on WHO reference strains showed that strain 235 (***L. major***, Israel) had a unique small fragment (Figure 17). This fragment was isolated in order to find out if it can be used as a species-specific chromosome DNA probe in hybridization experiments for the identification of strains that are similar to 235. OFAGE distinguishes some of the strains, but not all. There is thus a need to get a probe since this would be specific and more sensitive.

Species-specific DNA probes have been used to detect and distinguish the parasites even when they occur as mixed infections in tsetse flies or mammalian hosts and in trypanosome populations grown in culture (Kukla ***et al.***, 1987; Gibson ***et al.***, 1988). The identification of parasites like ***Taenia*** species has been accomplished using probes from a genomic library of size-selected DNA fragments (Barker, 1989). The use of probes combined with PFG electrophoresis for the separation of the chromosomes of parasites has opened up new avenues of research. Double probes for the simultaneous identification of the insect and the parasites they transmit have recently been reported (Ready ***et al.***, 1988). DNA probes for use in the field are

now available to identify many of the most important vector-borne parasites (Post and Crampton, 1987).

5.1 Results

5.1.1 Fragments of *Leishmania* reference strain ICIPE 235

OFAGE of chromosome blocks from reference strain 235 was repeatedly carried out in all the six lanes and the small fragments cut from the agarose gel (Figure 31). This was done in order to collect enough material for extraction of DNA.

5.1.2 Electrophoretic analysis of Recombinant Plasmids

There were no detectable DNAs after running electrophoresis of sample I. Two samples (1 and 2) obtained after cloning sample II showed growth in liquid broth. These were digested with Pst I before running electrophoresis. In both samples 1 and 2 (lanes 3 and 5 respectively) there was a smear in the upper region indicating presence of bacterial DNA in the plasmid preparation and a plasmid band at 2.7kb (Figure 32). No insert was apparent unless it was 2.7, and thus identical to pUC 18 vector. The results obtained for sample III are described below:

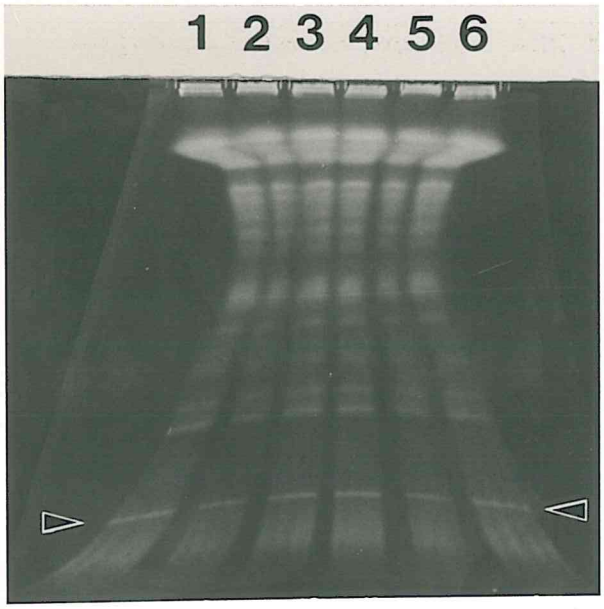


Fig. 31

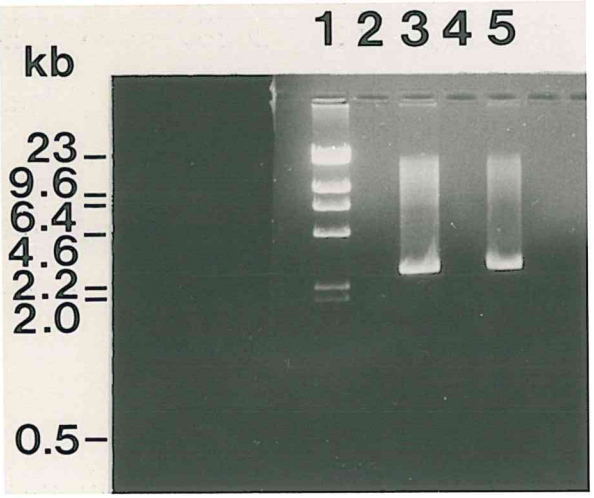


Fig. 32

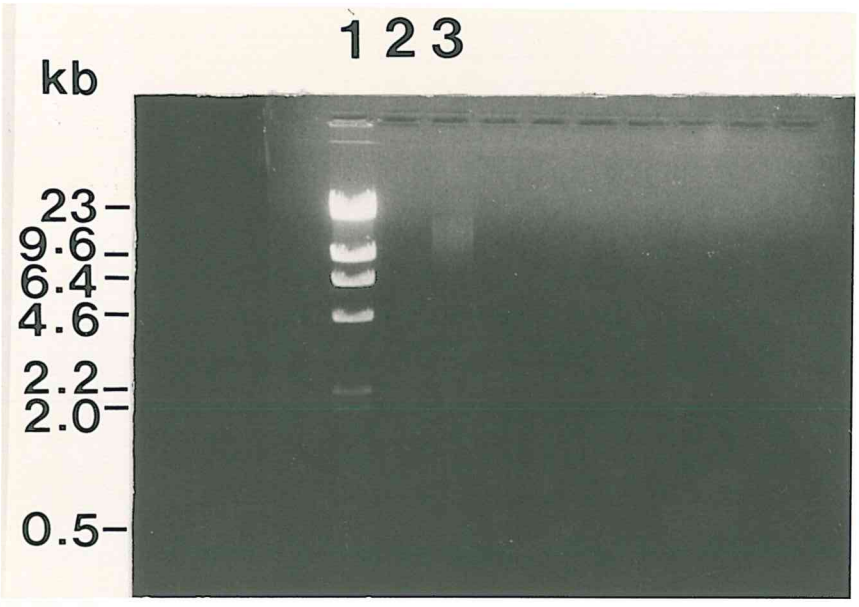


Fig. 33

After electroelution and extraction of DNA following the procedure already presented, there was a smear between 2.0 and 9.6kb (Figure 33). After ligation, transformation and plating on selective plates, 16 putative candidate recombinants showed growth of bacteria in the liquid broth. A miniscreen for recombinant plasmids was then carried out. Electrophoresis of the 16 samples digested with Pst I is shown in Figures 34a and 34b. DNA samples 4 (lane 6- Figure 34a) and 14 (lane 4- Figure 34b) had two bands. All the other samples had one band each (the vector) except samples 2, 8, 9, 11 and 16 which had none. Markers were included in order to determine the size of the bands. The bands in sample 4 were of equal intensity at around 2.6 kb region. In sample 14 there was a strong band at around 2.6 kb and a weak band detected at around 0.4 kb. Electrophoresis of samples 4 and 14 as well as the vector pUC 18 in which the DNA was cloned is shown in Figure 35. There were two bands in sample 4 (lanes 3 and 4) representing the vector and insert. Only one band (the vector) was found in sample 14 (lanes 6 and 7). On hybridization of sample 4 with a radiolabelled total DNA probe from strain 235 there was no positive autoradiographic signal.

Fig.34a

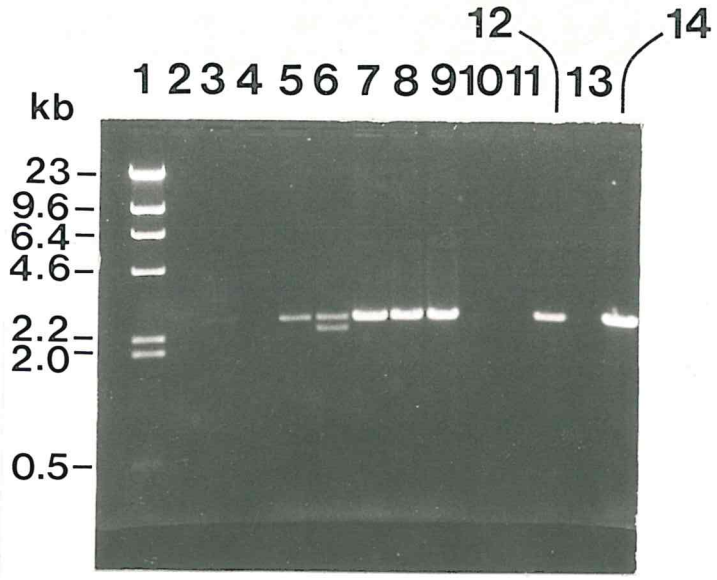


Fig.34b

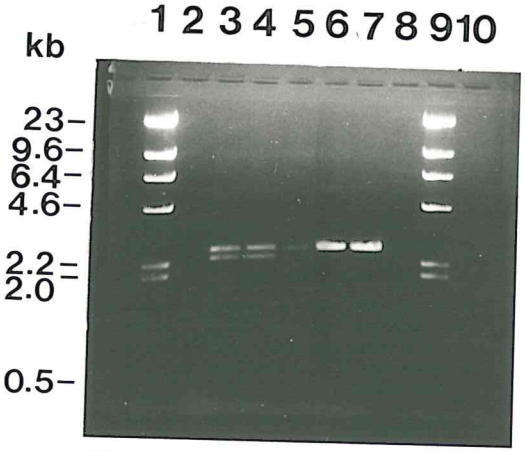


Fig.35

5.2 Discussion and Conclusions

The amount of DNA electroeluted from sample I could not be detected after electrophoresis. DNA electroeluted from sample II was detected after electrophoresis, but on cloning only the plasmid band was observed. There was no insert. In sample III, two bands were observed (Figure 34a - lane 6). One of the bands represents the plasmid while the second band is the insert (Figure 35 - lanes 3 and 4). On hybridization however no positive autoradiographic signal was obtained. This may be explained by the fact that the insert is probably not represented in many copies. If the insert was in many copies a strong hybridization would have been obtained with radiolabelled total DNA from strain 235. It is also possible that hybridization of the putative recombinant with parasite DNA separated by OFAGE would have produced a positive autoradiographic signal.

Alternatively, the second band may not be an insert. It may represent a DNA fragment from a plasmid which has undergone deletion.

Successful cloning with DNA vectors (plasmids or phages) requires formation, via ligation, of hybrid molecules which have both the correct fragment composition and the appropriate conformation. The majority of the

transformants had only vector DNA without any insert as shown by the presence of only one band. This is due to the ability of the linear plasmid DNA molecules generated after digestion with restriction endonucleases to recircularize when incubated in the presence of DNA ligase, leading to transformants containing the parental circular plasmid. It would be interesting to try cloning using different vectors, bacterial cells and restriction endonucleases sites.

CHAPTER 6:

GENERAL DISCUSSION AND CONCLUSIONS

6. General Discussion

OFAGE was used to examine the electrophoretic profiles of chromosome-sized DNA molecules of new leishmania isolates. The profiles were compared to WHO ***Leishmania*** reference strains. The patterns of chromosomal DNAs of morphologically indistinguishable ***Leishmania*** species and new isolates fitted into different genetic groups. The isolates within each group had a similar chromosome profile with respect to number and relative position of the bands. The significance of the differences in molecular karyotype is not clear at present. These observations demonstrate that OFAGE could be used in the identification of ***Leishmania***. The technique is of limited usefulness by itself for any absolute classification as suggested by Scholler ***et al.*** (1986) because of the large diversity of karyotypes. However, the initial grouping of the new isolates into genetically related clusters is cost-effective since restriction enzymes are not used. This method has been used in the characterization and identification of different Protozoan parasites including ***Trypanosoma congolense*** (Majiwa ***et al.***, 1986; Masake ***et al.***, 1988), ***Plasmodium falciparum*** (Kemp ***et al.***, 1985; Van der Ploeg ***et al.***, 1985) among others.

Although the number of chromosomes cannot be assessed accurately because some material always remains

trapped in the slot and not all bands consist of single chromosomes, each karyotypic profile represents a well defined genetic group. The pulse time sets the limit to the size of DNA molecules that can be fractionated (Smith ***et al.***, 1987). Scholler ***et al.*** (1986) resolved 14-24 chromosomal DNA bands depending on the isolate and pulse frequency used. At a pulse frequency of 40 sec which was used in this work, 15-17 bands were obtained in most of the isolates tested. The number of chromosomal DNA bands is close to that reported by other workers; Spithill and Samaras (1985) reported 17 bands while Galindo and Ochoa (1989) obtained a total of 18 bands.

Normally there is just one major vector and vertebrate host for a given leishmania species in a particular area. The results showed that two goats and a rat fitted in the same groups as humans (Table 3b). These animals may be "accidental" or "dead-end" hosts from which the parasite may never regain entrace into its primary vector, the sandfly. Alternatively, they could function as a source of infection for sandflies. In Kenya, rats have been reported to harbour ***Leishmania*** parasites and probably act as reservoirs (Mutinga, 1975b; Githure ***et al.***, 1986). The incidence of leishmaniasis in goats reported by Mutinga ***et al.*** (1988) needs further investigation to establish their role in disease transmission.

Isolates from all the goats were genetically different from that of the single sheep which was studied. The first clinical case of leishmaniasis in domestic animals (goat) was reported by Mutinga ***et al.*** (1989). Although it is possible that these animals harbour different species of leishmania parasites it is important to study more isolates from sheep.

Animal reservoirs are frequently infected with mixtures of pathogenic and non-pathogenic flagellates (Lainson ***et al.***, 1981; Arias and Naiff, 1981) as are the sandflies that feed on them. One consequence of this heterogeneity is that when isolates are cultured ***in vitro***, changes in various parasite characters may seem to occur, due to overgrowth of one or more parasite clones at the expense of other slower-growing clones. It is desirable to have available, for research purposes cultures of leishmania parasites which are stable with regard to their biological and biochemical properties. Thus cloning of parasites was done from the naturally occuring mixed isolates. However, cloning selects a single parasite population which may obscure important epidemiological and ecological information. Recently a concurrent infection with ***L. donovani*** and ***L. major*** was reported in a patient (Mebrahtu ***et al.***, 1990). Such a mixed infection would be detected by species-specific DNA probes.

New isolates that do not grow in culture cannot be analysed by OFAGE. For this reason the results obtained in this work may not accurately reflect the picture of leishmaniases in Kenya. This may be overcome by using species-specific DNA probes which are more sensitive and may even detect parasites in their vectors and hosts without having to grow them in culture.

The work carried out represents the first systematic study by OFAGE of ***Leishmania*** parasites from different parts of Kenya. The results have demonstrated that OFAGE could be used in the classification of ***Leishmania***. Isolates collected from the same region may not fit in the same group (as shown by isolates obtained from West Pokot). This could imply that there are different ***Leishmania*** species in a given region and hence a heterogenous population of sandflies. It is therefore not surprising to find a mixed infection in an animal as reported by Mebrahtu ***et al***. (1990).

Parasites found in different animals fitted in the same group. Probably these animals were bitten by sandflies of the same species. Alternatively, they could harbour different ***Leishmania*** species which are related (like the four WHO reference strains that fitted in the same group). It would be interesting to study isolates collected from sandflies in different regions of Kenya and compare the

findings to those of animals that were examined in this work.

Isolates that were collected from lizards fitted in the same group (***L. aethiopica, L. donovani, L. infantum*** and ***L. major,*** Kenya) . It is possible that lizards harbour the same or different species of ***Leishmania*** which could be genetically related. The fact that their chromosome profiles did not fit in Group II (***L. adleri***) probably indicates that lizards could be reservoir hosts of species that infect humans. By using molecular karyotyping, membrane lipids and kinetoplast DNAs sequences, Gomez-Eichelman ***et al.*** (1988) found that lizard isolates were related to the mammalian ***L. major.*** Okot-Kotber ***et al.*** (1989) demonstrated the presence of L. major in a lizard by using isoelectrofocusing technique. Recently, Massamba ***et al.*** 1991 (in press) showed that most of the lizard isolates collected in Kenya were ***L. major.*** From the observations described by the workers mentioned above it is possible that the lizards' isolates tested could be reservoir hosts of ***L. major*** or probably other species that infect humans. Further analysis needs to be carried out on these lizard isolates to fit them into specific species.

6.1 Conclusions

The conditions for setting up OFAGE were determined and standardized. This included temperature, pulse frequency, the parasite concentration and duration of performing the electrophoresis. Using the standardized conditions, molecular karyotypes of six WHO ***Leishmania*** reference strains were analysed. The chromosome profiles of these strains fitted in three different groups. Four strains fitted in group I while groups II and III had one strain each. Molecular karyotypes of 25 cloned new ***Leishmania*** isolates analysed were compared with those of the reference strains. Sixteen of these isolates had banding patterns similar to reference strains in Groups I and II. None of the isolates was similar to the reference strain in Group III. Nine of the isolates were different from all the reference strains in the three groups.

The four reference strains which fitted in Group I could be distinguished by using restriction endonuclease digestion.

Attempts to clone a fragment from ***L. major*** (Israel) showed both the insert and plasmid bands after performing electrophoresis but no positive autoradiographic signal was obtained on hybridization with the parasite DNA..

The work carried out has shown that OFAGE could be used for typing new ***Leishmania*** isolates. This technique could differentiate some morphologically indistinguishable ***Leishmania*** isolates by molecular karyotypes and classify them into groups. However for intra-species differentiation restriction endonuclease digestion was carried out. Individual species could be identified by using species specific DNA probes if these were available.

Research always gives rise to new questions as it attempts to answer old ones. The following topics are among the possibilities for further investigations:

1. Determine the sizes of the chromosomes from the isolates examined here.

2. Analyse molecular karyotypes from more WHO ***Leishmania*** reference strains to be used as standard chromosome profiles. Chromosome profiles of the nine isolates that did not fit in the three groups of reference strains tested could be similar to other reference strains.

3. Isolation of characteristic bands for investigation into their usefulness as DNA probes for characterization of new isolates.

4. Carry out infectivity tests of the new isolates in order to determine the correlation, if any, between the clinical outcome and the groups in which they fitted.

All of this work, that is already accomplished and that which could be accomplished starting from this basis, could make a major contribution to the control of leishmaniases in Kenya and elsewhere.

REFERENCES

REFERENCES

ADLER, S. (1964). Leishmania. ***Adv. Parasitol. 2***: 35-96.

ADLER, S. and BER, M. (1941). The transmission of ***Leishmania tropica*** by the bite of ***Phlebotomus papatasi***. ***Indian J. Med. Res. 29:*** 803-809.

ADLER, S. and THEODORE, O. (1926). Further observations on the transmission of cutaneous leishmaniasis to man from ***Phlebotomus papatasi***. ***Ann. Trop. Med. Parasit. 20***: 175-194.

ADLER, S. and THEODOR, O. (1932). Investigations on Mediterranean kala-azar. VI. Canine visceral leishmaniasis. ***Proc. Roy. Soc. London. B 110***: 402-412.

ADLER, S. and THEODOR, O. (1957). Transmission of disease agents by Phlebotomine sandflies. ***Ann. Rev. Entom. 2***: 203-226.

AL-TAQI, M. and BEHBEHANI, K. (1980). Cutaneous leishmaniasis in Kuwait. ***Ann. Trop. Med. Hyg. 74***: 495-501.

ANON (1984). The Leishmaniases. Report of a WHO Expert Committee. ***WHO Technical Report series 701:*** 1-140.

ANDERSON, D.C., BUCKNER, R.G., GLENN, B.L. and MACVEAN, D.W. (1980). Endemic canine leishmaniasis. ***Vet. Pathol. 17***: 94-96.

ARIAS, J.R. and NAIFF, R.D. (1981). The Principal reservoir host of the cutaneous leishmaniasis in the urban areas of Manaùs, Central Amazon of Brazil. ***Mem. Ins. Oswaldo Cruz. 76***: 279-286. UI:83088038.

ARNOT, D.E. and BARKER, D.C. (1981). Biochemical identification of cutaneous leishmanias by analysis of kinetoplast DNA. II. Sequence homologies in ***Leishmania*** kDNA. ***Mol. Biochem. Parasitol. 3***: 47-56.

BARKER, D.C. (1987). DNA diagnosis of human leishmaniasis. ***Parasit. Today 3 (6)***: 177-184.

BARKER, D.C. (1989). Molecular approaches to DNA diagnosis. ***Parasitol. 99***: S125-S146.

BARKER, D.C. and BUTCHER, J. (1983). The use of DNA probes in the identification of leishmanias: discrimination between isolates of the ***Leishmania mexicana*** and ***Leishmania braziliensis*** complexes. ***Trans. R. Soc. Trop. Med. Hyg.*** **77**: 285-297.

BARKER, D.C., GIBSON, L.J., KENNEDY, W.P.K., NASSER, A.A.A. and WILLIAMS, R.H. (1986). The potential of using recombinant DNA species-specific probes for the identification of tropical ***Leishmania***. ***Parasitol.*** ***91***: S139-S174.

BEACH, R., KIILU, G., HENDRICKS, L., OSTER, C. and LEEUWENBERG, J. (1984). Cutaneous leishmaniasis in Kenya: transmission of ***Leishmania major*** to man by the bite of ***Phlebotomus duboscqi***. ***Trans. Roy. Soc. Trop. Med. Hyg.*** ***78***: 747-751.

BEVERLEY, S.M., ISMACH, R.B. and McMAHON PRATT, D. (1987). Evolution of the genus ***Leishmania*** as revealed by comparisons of nuclear DNA restriction fragment patterns. ***Proc. Nat. Acad. Sci. 84***: 484-488.

BHATTACHARYYA, P.K. (1981). Distribution of kala-azar in India. ***Trans. Roy. Soc. Trop. Med. Hyg.*** ***75***: 333-334.

BINHAZIM, A.A., GITHURE, J.I., MUCHEMI, G.K. and REID, G.D.F. (1987). Isolation of ***Leishmania major*** from a naturally infected vervet monkey (***Cercopothecus aethiops***) caught in Kiambu District, Kenya. ***J. Parasitol. 73***(6): 1278-1279.

BISHOP, R.P. and AKINSEHIWA, F. (1989). Characterization of ***Leishmania donovani*** stocks by genomic DNA heterogeneity and molecular karyotype. ***Trans. Roy. Soc. Trop. Med. Hyg. 83***: 629-634.

BISHOP, R.P. and MILES, M.A. (1987). Chromosome size polymorphisms of ***Leishmania donovani***. ***Mol. Biochem. Parasitol. 24***: 263-272.

BRADLEY, D.J. and KIRKLEY, J. (1977). Regulation of ***Leishmania*** population within the host. II. Genetic control of acute susceptibility to ***Leishmania donovani*** infection. ***Clin. Exp. Immunol. 30***: 130-140.

BRADLEY, D.J., TAYLOR, B.A., BLACKWELL, J., EVANS, E.P. and FREEMAN, J. (1979). Regulation of ***Leishmania*** population within the host. III. Mapping of the locus controlling susceptibility to visceral leshmaniasis in the mouse. ***Clin. Exp. Immunol. 37***: 7-14

BRAY, R.S. (1974). ***Leishmania. Ann. Rev. Microbiol. 28***: 189-217.

CARLE, F.G., and OLSON, M.V., (1984). Separation of chromosomal DNA molecules from yeast by orthogonal field alternation gel electrophoresis. ***Nucleic Acids Research 12***: 5647-5665.

CARLE, G.F. and OLSON, M.V. (1985). An electrophoretic karyotype for yeast. ***Proc. Natl. Acad. Sci. 82***: 3756-3760.

CARLE, G.F., FRANK, F. and OLSON, M.O.(1986). Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. ***Science 232***: 65-68.

CHANCE, M.L. (1979). The identification of Leishmania. In: Problems in the Identification of Parasites and their Vectors (Edited by Taylor A.E.R. and Muller R.) pp. 55-74. Blackwell Scientific Publications, Oxford.

CHANCE, M.L., PETERS, W. and SHCHORY, L. (1974). Biochemical taxonomy of ***Leishmania.*** I. Observations on DNA. ***Ann. Trop. Med. Parasit. 68***: 307-316.

CHANCE, M.L., SCHNUR, L.F., THOMAS, S.C. and PETERS, W. (1978). The biochemical and serological taxonomy of ***Leishmania*** from Aethiopian zoogeographical region of Africa. ***Ann. Trop. Med. Parasit. 72***: 533-542.

CHANCE, M.D. and WALTON, B.C., eds. (1982). In: Proceedings of a Workshop held at the Pan American Health Organization, Washington, D.C. 9-11 December, 1980. UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland.

CHATTERJEE, K.D. (1975). Blood and tissue flagellates. In: Parasitology (Protozoology and Helminthology) in relation to clinical medicine. pp.58. S.N. Guha Ray, Sree Saraswaty Press Ltd. Calcutta, India.

CHU, G.D., VOLLRATH, D. and DAVIS, R.S. (1986). Separation of large DNA molecules by contour-clamped homogeneous electric fields. ***Science 234***: 1582-1585.

CHULAY, J.D., ADOYO, M.A., GITHURE,J.I. (1985). ***Leishmania donovani*** parasitaemia in Kenyan visceral leishmaniasis. ***Trans. Roy. Soc. Trop. Med. Hyg. 79***: 218-222.

CLARKE, M. (1949). A case of kala-azar. ***E. Afr. Med. J. 26:*** 219-221.

COLE, A.C.E. (1942). Cutaneous leishmaniasis in East Africa. ***E. Afr. Med. J. 19***: 199-200.

COLE, A.C.E. (1944). Kala-azar in East Africa. ***Trans. R. Soc. Trop. Med. Hyg. 37 (6):*** 409-435.

COMEAU, A.M., MILLER, S.I. and WIRTH, D.F. (1986). Chromosome location of four genes in ***Leishmania***. ***Mol. Biochem. Parasitol. 21***: 161-169.

COX, F.E.G. (1985). Chromosomes of malaria parasites and trypanosomes. ***Nature 315***: 280-281.

CUNNINGHAM, D.D. (1885). On the presence of peculiar parasitic organisms in the tissue of a specimen of "Delhi boil". ***Scientific Memoirs by Medical Officers of the Army of India 1***: 21-31.

DE IBARRA, A.A.L., HOWARD, J.G. AND SNARY, D. (1982). Monoclonal antibodies to ***Leishmania tropica major***: specificities and antigen location. ***Parasitol. 85***: 523-531.

DECKER-JACKSON, J.E., SCHROT, J.R. and LEVIN, G.V. (1977). Identification of ***Leishmania*** spp. by radiorespirometry. ***J. Protozool. 24***: 463-470.

DONOVAN, C. (1903). On the possibility of trypanosomiasis in India. ***Brit. Med. J. 2***: 79-82.

ELLIS, T.H.N., CLEARY, W.G., BURCHAM, K.W.G. and BOWEN, B.A. (1987). Ramped field inversion gel electrophoresis: a cautionary note. ***Nucleic Acid Res. 15***: 5489.

EL-ON, J., SCHNUR, L. and GREENBLATT, C.L. (1979). ***Leishmania donovani***: Physiochemical, immunological and biological characterization of excreted factor from promastigotes. ***Exp. Parasitol. 47***: 254-269.

FANGMAN, W.L. (1978). Separation of very large DNA molecules by gel electrophoresis. ***Nucleic Acids Research 5*** (3): 653-665.

FENDALL, N.R.E. (1953). The history and character of the kala-azar outbreak in the Kitui district. ***E. Afr. Med. J. 30***: 269-285.

FONG, D. and CHANG, K.P. (1982). Surface antigenic change during differentiation of a parasitic protozoan, ***Leishmania mexicana*** : Identification by monoclonal antibodies. ***Proc. Nat. Acad. Sci. 79:*** 7366-7370.

FORKNER, C.E. and ZIA, L.S. (1934). Viable ***Leishmania donovani*** in nasal and oral secretions of patients with kala-azar and the bearing of this finding on the transmission of the disease. ***J. Exp. Med. 59:*** 491-499.

GACHIHI, G.S., CHUNGE, C.N., BRYCESON, A.D.M., CHULAY, J.D., OSTER, C.N., WERE, J.B.O. and LEEUWENBURG, J. (1987). A comparative trial of four dosage regimens of sodium stibogluconate (Pentostam) in treatment of visceral leishmaniasis in several geographical areas of Kenya. In: Proceedings of the eighth Annual Medical Scientific Conference Nairobi, Kenya; p 280-283.

GALINDO, I. and OCHOA, J.L.R. (1989). Study of ***Leishmania mexicana*** electrokaryotype by clamped homogeneous electric field electophoresis. ***Mol. Bioch. Parasitol. 34*** (3): 245-252.

GARDENER, P.J. (1977). Taxonomy of the genus ***Leishmania***: A review of nomenclature and classification. ***Trop. Dis. Bull. 74***: 1069-1088.

GARDENER, P.J., CHANCE, M.L., and PETERS, W. (1974). Biochemical taxonomy of ***Leishmania***. ***II***. Electrophoretic variation of malate dehydrogenase. ***Ann. Trop. Med. Parasit. 68:*** 317-325.

GARDINER, K.W., LAAS, W. and PATTERSON, D. (1986). Fractionation of large mammalian DNA restriction fragments using vertical pulsed-field gradient gel electrophoresis. ***Somatic Cell Mol. Genet. 12***: 185-195.

GARNHAM, P.C.C. (1987). Introduction to the Leishmaniasis in Biology and Medicine. In: Peters and Killick-Kendrick (eds). The Leishmaniasis in Biology and Medicine. Vol. 1, pp xiii-xxv. Academic Press, New York.

GARETT, A. (1978). Visceral leishmaniasis. ***Southwestern Vet. 31***: 125-128.

GERACI, J.E., WILSON, W.R. and THOMPSON, J.H., Jr. (1980) Visceral leishmaniasis (kala-azar) as a cause of fever of unknown origin. ***Mayo Clinic Proc. 55***: 455-458.

GIANNINI, S.H., SCHITTINI, M., KEITHLY, J.S., WARBURTON, P.W., CANTON, C.R. and VAN DER PLOEG, L.H.T. (19-86). Karyotype analysis of ***Leishmania*** species and its use in classification and clinical diagnosis. ***Science 232***: 762-765.

GIBSON, W.C. and MILES, M.A. (1986). The karyotype and ploidy of ***Trypanosoma cruzi***. ***Embo J. 4***: 1299-1305.

GIBSON, W.C. and BORST, P. (1986). Size fractionation of the small chromosomes of ***Trypanozoon*** and ***Nannomans*** trypanosomes by PFG electrophoresis. ***Mol. Biochem. Parasitol. 18***: 127-140.

GIGASE, P., MOENS, F., VAN EMELEN, J., VAN MARCK, E. and VAN MULLEM, J. (1978). Autochthonous visceral leishmaniasis in Zaire. ***Ann. Soc. belge. Med. Trop. 58***: 235-240.

GIBSON, W.C., DUKES, P. and GASHUMBA, T.K. (1988). Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. ***Parasitol. 97***: 63-73.

GITHURE, J.I., BEACH, R.F. and LIGHTNER, L.K. (1984). The isolation of ***Leishmania major*** from rodents in Baringo District, Kenya. ***Trans. Roy. Trop. Med. Hyg. 78***: 283.

GITHURE, J.I., SCHNUR, L.F., LE BLANCQ, S.M. and HENDRICKS, L.D. (1986). Characterization of Kenyan Leishmania spp. and identification of ***Mastomys natalensis, Taterillus emini major. Ann. Trop. Med. Parasit. 80*** (5): 501-507.

GODFREY, D.G. (1978). Identification of economically important parasites. ***Nature 273***: 600-604.

GODFREY, D.G. (1984). Molecular biochemical characterization of human parasites. In: ***Recent Advances in Tropical Medicine*** (Edited by Giles H.M.) pp. 289-319. Churchill Livingstone, London.

GOMEZ-EICHELMAN, C.M., HOLZ, Jr. G., BEACH, D., SIMPSON, A.M. and SIMPSON, L. (1988). Comparison of several lizard ***Leishmania*** species and strains in terms of kinetoplast minicircle and maxicircle DNA sequences, nuclear chromosomes and membrane lipids. ***Mol. Biochem. Parasitol. 27***: 143-158.

GREENBLATT, C.L. (1988). Cutaneous leishmaniasis: The prospects for a killed vaccine. ***Parasitol. Today 4*** (2): 53-54.

GREENBLATT, C.L., SLUTZKY, G.M., DE IBARRA, A.A.L. and SNARY, D. (1983). Monoclonal antibodies for serotyping ***Leishmania*** strains. ***J. Clin. Microb. 18 (1)***: 191-193.

GROVE, S.S. (1970). Cutaneous leishmaniasis in South West Africa. ***S. African Med. J. 44***: 206-207.

GROVE, S.S. (1978). The clinical and histological features of South West African cutaneous leishmaniasis. ***S. Africane Med. J. 53***: 712-715.

GUYAUX, M., CORNELISSEN, A.W.C.A., PAYS, E., STEINERT, M. and BORST, P. (1985). ***Trypanosoma brucei***: a surface antigen mRNA is discontinuously transcribed from two distinct chromosomes. ***EMBO J. 4***: 995-998.

HEISCH, R.B. (1954). Studies in leishmaniasis in East Africa. 1. The epidemiology of an outbreak of kala-azar in Kenya. ***Trans. R. Soc. Trop. Med. Hyg. 48***: 449-469.

HEISCH, R.B. (1957). The isolation of ***Leishmania*** from a ground squirrel in Kenya. ***East Afr. Med. J. 34***: 183

HEISCH, R.B. (1958). On ***Leishmania adleri*** species from lacertid lizards (***Latastia*** sp) in Kenya. ***Ann. Trop. Med. Parasitol. 52***: 68-71.

HEISCH, R.B. (1963). Is there an animal reservoir of kala-azar in Kenya? ***E. Afr. Med. J. 40***: 359-362.

HENDRICKS, L.D., OSTER, C.N., MEBRAHTU, Y. CHUNGE, C.N., MUIGAI, R., RASHID, J.R., WASSUNA, K., ADOYO, M.,KIBET, F. and KOECH, D. (1985). Presence of leishmaniasis in Kenya. In: Recent Advances in Management and Control of Infections in Eastern Africa. Tukei, P.M., Koech.D.K., Kinoti, S.N. (Eds.).

HESS, J.H. (1930). Splenic puncture as a diagnostic procedure in infancy and childhood. ***Ann. Intern. Med. 4***: 467-483.

HOLZWARTH, G., MCKEE, C.B., STEIGER, S. and CRATER, G. (1987). Transient orientation of linear DNA molecules during pulsed field gel electrophoresis. ***Nucleic Acids Research 15***: 10031-10044.

HOOGSTRAAL, H. and HEYNEMAN, D. (1969). Leishmaniasis in the Sudanese Republic. V. Infection in man with Sudanese kala-azar. ***Am. J. Trop. Med. Hyg. 18***: 1170-1182.

HOMMEL, M. (1978). The genus ***Leishmania***: Biology of the parasites and clinical aspects. ***Bull. Inst. Past. 75***: 5-102.

HONIGBERG, B.M., BALAMUTH, W., BOVEE, E.C., CORLISS, J.O., GOJDICS, M., HALL, R.P., KUDO, R.R., LEVINE, N.D., LOEBLICH, A.R., WASER, J. and WENRICH, D.H. (1964). A revised clasification of the phylum Protozoa. ***J. Protozool. 11***: 7-20.

JACKSON, P.R., LAWRIE, J.M., STITELER, J.M., HAWKINS, D.N., WOHLHIETER, J.A. and ROWTON, E.D. (1986). Detection and characterization of ***Leishmania*** species and strains from mammals and vectors by hybridization and restriction endonuclease digestion of kinetoplast DNA. ***Veterin. Parasit. 20***: 195-215.

JACKSON, P.R., WOHLHIETER, A., JACKSON, J.E., SAYLES, P., DIGGS, C.L. and HOCKMEYER, W.T. (1984). Restriction endonuclease analysis of leishmania kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. ***Am. J. Trop. Med. Hyg. 33*** (5): 808-819.

JONES, H.I. (1979). Cutaneous leishmaniasis in western Australia. ***Med. J. Australia 2***: 495-496.

KADDU, J.B. (1986). ***Leishmania*** in Kenyan Phlebotomine sandflies. III. Advances in the investigations of vectorial capacity and vector-parasite relationships of various species of sandflies in Kenya. ***Insect Sci. Applic.*** 7: 207-212.

KADDU, J.B. and NYAMORI, M.P. (1988). ***Leishmania*** in Kenyan phlebotomine sandflies - VI: Interactions of ***Leishmania*** and bacteria and fungi isolated from sandflies. ***J. Med. Ent.*** (in press).

KAGER, P.A. (1983). The pancytopenia of visceral leishmaniasis: literature review. In: ***Clinical Aspects of kala-azar in Kenya***. Kager and Rees pp. 91-104.

KAGER, P.A. and REES, P.H. (1983). ICG Printing, Dordrecht, Netherlands. ***Clinical Aspects of Kala-azar in Kenya***.

KAGER, P.A., REES, P.H., WELLDE, B.T., HOCKMEYER, W.T. and LYERLY, W.H. (1983). Allopurinol in the treatment of visceral leishmaniasis. In: ***Clinical Aspects of kal-azar in Kenya***. Kager and Rees; pp. 141-147.

KEMP, D.J., CORCORAN, L.M., COPPEL, R.L., STAHL, H.L., BIANCO, A.E., BROWN, G.V. and ANDERS, R.F. (1985). Size variation in chromosomes from independent cultured isolates of ***Plasmodium falciparum***. ***Nature 315***: 347-350.

KENNEDY, W.P.K. (1984). Novel identification of differences in the kinetoplast DNA of ***Leishmania*** isolates by recombinant DNA techniques and in situ hybridization. ***Mol. Biochem. Parasitol. 12***: 313-325.

KILLICK-KENDRICK, R. (1978). Recent advances and outstanding problems in the biology of Phlebotominae sandflies. ***Acta Tropica 35***: 297-313.

KIRK, R. (1949). The differentiation and nomenclature of ***Leishmania***. ***Parasit. 39***: 263-273.

KIRK, R. and LEWIS, D.J. (1955). Studies in leishmaniasis in the Anglo-Egypt Sudan. Phlebotomus in relation to leishmaniasis in the Sudan. ***Trans. R. Soc. Trop. Med. Hyg. 49***: 229-2e40.

KREUTZER, R.D., SEMKO, M.E., HENDRICKS, L.D. and WRIGHT, N. (1983). Identification of leishmania spp. by multiple isozyme analysis. ***Am. J. Trop. Med. Hyg. 32 (4)***: 703-715.

KUNGU, A., MUTINGA, M.J. and NGOKA, J.M. (1972). Cutaneous leishmaniasis in Kenya. ***East. Afr. Med. J. 49***: 458

KUDO, R.R. (1966). Protozoology. Thomas Springfield, III.

KUKLA, B.A., MAJIWA, P.A.O., YOUNG, J.R., MOLOO, S.K. and OLE MOI YOI, O. (1987). Use of species-specific DNA probes for detection and identification of trypanosome infection in tsetse flies. ***Parasitol. 95***: 1-16.

KUSAOIMI, N.T. (1979). An outbreak of cutaneous leishmaniasis in the countryside. Ann. College Med., Mosul 10: 63-69.

LAINSON, R. (1982). Leishmaniasis parasites of mammals in relation to human diseases. ***Symp. Zool. Soc. Lond. 50***: 137-179.

LAINSON, R. (1983). The American leishmaniases: some observations on their ecology and epidemiology. ***Trans. R. Soc. Trop. Med. Hyg.*** **77**: 569-596.

LAINSON, R. and SHAW, J.J. (1978). Epidemiology and Ecology of leishmaniasis in Latin-America. ***Nature, 273***: 595-600.

LAINSON, R. and SHAW, J.J. (1979). The role of animals in the epidemiology of South American leishmaniasis. In: Biology of the kinetoplastida, Vol. 2 (Edited by Lumsden W.H.R. and Evans D.A..), pp. 1-116. Academic Press, London.

LAINSON, R. and SHAW, J.J. (1987). In: The Leishmaniases in Biology and Medicine. Vol. 1 (Peters, W. and Killick-Kendrick, R. eds.), pp 1-20, Academic Press.

LAINSON, R., SHAW, J.J. and POVOA, M. (1981). The importance of edentates (sloths and anteaters) as primary reservoirs of ***L. braziliensis guyanensis***, causative agent of "pian bois" in north Brazil. ***Trans. R. Soc. Med. Hyg.*** **75**: 611-612. UI:82108685

LANGSLEY, G., SIBILLI, L., MATTEI, D., FALANGA, P. and MERCEREAU-PUIJALON, O. (1987). Karyotype comparison between ***Plasmodium chabaudi*** and ***Plasmodium falciparum***: Analysis of a ***Plasmodium chabaudi*** cDNA containing sequences highly repetitive in ***Plasmodium falciparum***. ***Nucleic Acids Research 15***: 2203-2211.

LARSON, J.J., NICHOLSON, A.W. and SIEGEL, A. (1987). Field inversion of large DNA fragments using an inexpensive unit. ***BioTechniques 5***: 228-231.

LAVERAL, A. and MESNIL, F. (1903). Sur un protozoaire nouveau (***Piroplasma donovani*** Lav. et Mesn.), parasite d'une fievre de l'Inde. ***Comptes Rendu Hebdonadaine des Seances de l' academie des Sciences 137***: 957-961.

LAWRIE, J.M., JACKSON, P.R., STITELER, J.M. and HOCKMEYER, W.T. (1985). Identification of pathogenic ***Leishmania*** promastigotes by DNA : DNA hybridi-zation with kinetoplast DNA cloned in ***E. coli*** plasmids. ***Am. J. Trop. Med. Hyg. 34***: 257-265.

LE BLANCQ, S.M., BELEHU, A. and PETERS, W. (1986a). ***Leishmania*** in the Old World: 3. The distribution of ***L. aethiopica*** zymodemes. ***Trans. R. Soc. Trop. Med. Hyg. 80***: 360-366.

LE BLANCQ, S.M., CIBULSKI, R.E. and PETERS, W. (1986b). ***Leishmania*** in the Old World: 5. Numerical analysis of isoenzyme data. ***Trans. R. Soc. Trop. Med. Hyg. 80***: 517-524.

LE BLANCQ, S.M., SCHNUR, L.F. and PETERS, W. (1985). The geographical and hostal distribution of ***L. major*** zymodemes. ***Trans. R. Soc. Trop. Med. Hyg. 80***: 99-112.

LEE, J.J., HUNTER, S.H. and BOVEE, E.C. (1985). An illustrated guide to the protozoa. Society of Protozoologists.

LEISHMAN, W.B. (1903). On the possibility of the occurence of trypanosomiasis in India. ***Brit. Med. J. 1***: 1252-1254.

LEWIS, D.J. (1971). Phlebotomid sandflies. ***Bull. Wld. Hlth. Org. 44***: 535-551.

LOPEZ, U.G. and WIRTH, D.F. (1986). Identification of visceral ***Leishmania*** species with cloned sequences of kinetoplast DNA. ***Mol. Biochem. Parasitol. 20***: 77-84.

LUHE, M. (1906). Die im Blute schmarotzenden Protozoen und ihre nachsten Verwandten. In: ***Handbuch der Tropenkrankhelten***. (Mense, C., Ed.). J.A. Barth, Leipzig, 3; p.203.

MAJIWA, P.A.O. (1989). Recombinant DNA Probes as Tools for Epidemiological Studies of Parasitic Diseases. ***Discovery and Innovation 1*** (2): 35-40.

MAJIWA, P.A.O., MASAKE, R.A., NANTULYA, V.M., HAMERS, R. and MATTHYSSEN, G. (1985). ***Trypanosoma (Nannomonas) congolense***: identification of two karyotypic groups. ***Embo J. 4***(12): 3307-3313.

MAJIWA, P.A.O. and WEBSTER, P. (1987). A repetitive deoxyribonucleic acid sequence distinguishes ***Trypanosoma simiae*** from ***T. congolense***. ***Parasitol. 95***: 543-558.

MAJIWA, P.A.O., YOUNG, J.R., HAMMERS, R. and MATTHYSSENS, G. (1986). Minichromosomal variable surface glycoprotein genes and molecular karyotypes of ***Trypanosoma (Nannomonas) congolense***. ***Gene 41***: 183-192.

MANSON-BAHR, P.E.C. and HEISCH, R.B. (1961). Transient infection of man with a ***Leishmania*** (***L. adleri***) of lizards. ***Ann. Trop. Med. Parasitol. 55***: 381-382.

MANSON-BAHR, P.E.C., SOUTHGATE, B.A. and HARVEY, A.E. (1963). Development of kala-azar in man after inoculation with ***Leishmania*** from a Kenyan sandfly. ***Brit. Med. J. Hyg., 67***: 79-84.

MANSON-BAHR, P.E.C. and SOUTHGATE, B.A. (1964). Recent research on kala-azar in East Africa. ***J. Trop. Med. Hyg. 67***: 79-83.

MARSDEN, P.D. (1979). Current concepts in parasitology. ***New Eng. J. Med. 300***: 350-352.

MASAKE, R.A., NYAMBATI, V M., NANTULYA, V.M., MAJIWA, P.A.O., MOLOO, S.K. and MUSOKE, A.J. (1988). The chromosome profiles of ***Trypanosoma congolense*** isolates from Kilifi, Kenya and their relationship to serodeme identity. ***Mol. Biochem. Parasitol. 30***: 105-112.

MASSAMBA, N.N., MUTINGA, M.J. and ODERO, B.N. (1991). Characterization of ***Leishmania*** parasites from Kenya using cellulose acetate electrophoresis. ***Insect Sci. Applic.*** (in press).

MASSAMBA, N.N. and WILLIAMS, R.O. (1984). Distinction of Africa trypanosome species using nucleic acid hybridization. ***Parasitol. 88***: 55-65.

MAUEL, J. and BEHIN, R. (1982). Leishmaniasis: Immunity, immunopathology and immunodiagnosis. Immunology of parasitic infections. Ed. Cohen, S. and Warren, R.S., 2nd edition, Blackwell Scientific Publications. pp. 299-355.

MAYBRINK, W., DA COSTA, C.A., MAGALHAES, P.A., MELO, M.W., DIAS, M., OLIVEIRA LIMA, A., MICHALICK, M.S. and WILLIAMS, P. (1979). A field trial of a vaccine against American dermal leishmaniasis. ***Trans. R. Soc. Trop. Med. Hyg. 73***: 385-387.

MBUGUA, G.G. and ARAP SIONGOK, T.K. Visceral leishmaniasis outbreak in Kitui District. Abstract 63. 2nd Annual Medical Scientific Conference of Kenya Medical Research Institute and Kenya Trypanosomiasis Research Institute, Nairobi, 3-6 Febr. 1981.

McKINNON, J.A. (1962a). Kala-azar in the upper Rift Valley of Kenya. Part I. Background and discovery of the disease. ***J. Trop. Med. Hyg. 65***: 51-63.

McKINNON, J.A. (1962b). Kala-azar in the Upper Rift Valley of Kenya. Part II. Epidemiological factors. ***J. Trop. Med. Hyg. 65: 82-90.***

McKINNON, J.A. and FENDALL, N.R.E. (1956). Kala-azar in the Baringo district of Kenya: Progress report. ***J. Trop. Med. 59:*** 208-212.

McMAHON PRATT, D. and DAVID, J.R. (1981). Monoclonal antibodies that distinguish between New World species of ***Leishmania***. ***Nature 291***: 581-583.

McMAHON PRATT, D. and DAVID, J.R. (1982). In: M.L. Chance and B.C. Walton, eds. Biochemical Characterization of ***Leishmania***.

MEBRAHTU, Y., LAWYER, P., GITHURE, J., KAGER, P., LEEUWENBURG, J., PERKINS, P., OSTER, C. and HENDRICKS, L. (1988). Indigenous human cutaneous leishmaniasis caused by ***Leishmania tropica*** in Kenya. ***Am. J. Trop. Med. Hyg. 39***: 267-273.

MEBRAHTU Y., LAWYER, P., GITHURE, J., WERE, J.B., MUIGAI, R., HENDRICKS, L., LEEUWENBURG, J., KOECH, D. and ROBERTS, C. (1989). Visceral leishmaniasis unresponsive to pentostam caused by ***Leishmania tropica*** in Kenya. ***Am. J. Trop. Med. Hyg. 41***(3): 289-294.

MEBRAHTU, Y. LAWYER, P., HENDRICK, L., OSTER, C., PERKINS, P., KOECH, D., PAMBA, H. and ROBERTS, C. (1990). 11th Annual Medical Scientific Conference at Kemri, Nairobi. Theme: Recent Advances in Medical Research with a symposium on the role of man in disease control.

MINTER, D.M., WIJER, D.J.B., HEISCH, R.B. and MANSON-BAHR, P.E.C. (1962). ***Phlebotomus martini***, a probable vector of kala-azar in Kenya. ***Brit. Med. J. 2***: 835

MOLYNEUX, D.H. and ASHFORD, R.W. (1983). ***The Biology of Trypanosoma and Leishmania, Parasites of Man and Domestic Animals.*** Taylor and Francis, London.

MUIGAI, R., GITHURE, J.I., GACHIHI, G.S., WERE, J.B.O., LEEUWENBURG, J. and PERKINS, P.V. (1987). Cutaneous leishmaniasis caused by ***Leishmania major*** in Baringo District, Kenya. ***Trans. Roy. Trop. Med. Hyg 81***: 600-602.

MUTERO, C.M., MUTINGA, M.J. and OMOGO, D.M. (1988). Development of ***Leishmania*** spp. in mosquitoes - 1. Experimental infection of ***Aedes aegypti*** with ***Leishmania donovani*** promastigotes. ***Insect Sci. Applic. 9***: 453-456.

MUTERO, C.M., MUTINGA, M.J. and MUSYOKI, R. (1989). Isolation of leishmanial parasites from a wild caught ***Anopheles gambiae*** mosquito in Kenya. ***Intern. J. Parasit. 20***: 1-4.

MUTINGA, M.J. (1971). ***Phlebotomus longipes,*** a vector of cutaneous leishmaniasis in Kenya. ***Trans. Roy. Soc. Trop. Med. Hyg. 65***: 106

MUTINGA, M.J. (1975a). Phlebotomus fauna in cutaneous leishmaniasis focus of Mt. Elgon, Kenya. ***E. Afr. Med. J. 52***: 340-347.

MUTINGA, M.J. (1975b). The animal reservoir of cutaneous leishmaniasis on Mt. Elgon, Kenya. ***E. Afr. Med. J. 52***: 142-150.

MUTINGA, M.J. (1981). An efficient trap for sandflies (Diptera: Phlebotomidae). ***Insect Sci. Applic. 1***: 207-210.

MUTINGA, M.J. (1985). Leishmaniasis in Kenya. ***Medicus 4(2)***: 11-22.

MUTINGA, M.J. (1986). Epidemiology of leishmaniases in Kenya. Advances in research on vectors and animal reservoirs, and possible control measures. ***Insect Sci. Applic. 7***: 199-206.

MUTINGA, M.J. (1986a). Leishmaniases. ***Insect Sci. Applic. 7*** (3): 421-427.

MUTINGA, M.J., KADDU, J.B. and KYAI, F.M. (1983). Studies on vectors of ***L. major*** in Kenya. Proceedings of the 58th Annual Meeting of American Society of Parasitologists. San Antonio, Texas. U.S.A. pp. 57.

MUTINGA, M.J. and KYAI, F.M. (1985). A new record of ***Sergentomyia garnami*** (Heisch, Guggisberg and Teesdale, 1956) in Kenya. ***Trans. R. Soc. Trop. Med. Hyg. 79***: 137-138.

MUTINGA, M.J., KYAI, F.M. and OMOGO, D.M. (1986). Investigations on the epidemiology of leishmaniases in Kenya-I. Studies on vectors of ***Leishmania major*** in Marigat, Baringo District, Kenya. ***Insect Sci. Applic. 7***: 181-189.

MUTINGA, M.J., MUTERO, C.M., NGINDU, A. and AMIMO, F. (1988). The isolation of leishmanial parasites from domestic goats and wild hosts and possible role of goats as reservoirs of leishmaniasis. ***Insect Sci. Applic. 9***: 347-349.

MUTINGA, M.J., KIHARA, S.M., LODHDING, A., MUTERO, C.M., NGATIA, T.A. and KARANU, F. (1989). Leishmaniasis in Kenya. Description of leishmaniasis of a domestic goat from Trans mara, Narok District, Kenya. ***Trop. Med. Parasitol. 40***: 91-96.

MUTINGA, M.J. and NGOKA, J.M. (1970). Cutaneous leishmaniasis in Kenya. ***J. Parasitol. 56***: 452-453.

MUTINGA, M.J. and NGOKA, J.M. (1978a). Visceral leishmaniasis in Kenya. The onset of an epidemic outbreak in the Machakos District of Kenya. ***E. Afr. Med. J. 55***: 328-331.

MUTINGA, M.J. and NGOKA, J.M. (1978b). Incrimination of the vector of visceral leishmaniasis in Kenya. ***E. Afr. Med. J. 55***: 337-340.

MUTINGA, M.J. and NGOKA, J.M. (1983). Investigations of animal reservoir of visceral leishmaniasis and the isolation of ***Leishmania major*** in Marigat, Baringo District, Kenya. ***Insect Sci. Applic. 4***: 237-240.

MUTINGA, M.J., NGOKA, J.M. and ODHIAMBO, T.R. (1984). Epidemiological investigations of visceral leishmaniasis in the West Pokot District, Kenya. ***Insect Sci. Applic. 5***: 521-525.

MUTINGA, M.J., NGOKA, J.M., SCHNUR, L.F. and CHANCE, M.L. (1980). The isolation and identification of leishmanial parasites from domestic dogs in the Machakos District of Kenya, and the possible role of dogs as reservoirs of kala-azar in East Africa. ***Ann. Trop. Med. Parasit. 74***: 139-144.

MUTINGA, M.J. and ODHIAMBO, T.R. (1982). Studies on infection rates of human baited anthropophilic sandflies in Machakos District, Kenya. ***Insect Sci. Applic. 3***: 211-214.

NAIK, K.G., HIRA, P.R., BHAGWANDEEN, S.B. and EGERE, J.U. (1976). Kala-azar in Zambia: first report of two cases. ***Trans. Roy. Soc. Trop. Med. Hyg. 70***: 328-332.

NGOKA, J.M. and MUTINGA, M.J. (1978). Visceral leishmaniasis animal reservoir in Kenya. ***E. Afr. Med. J. 55***: 332-336.

NICOLLE, C. (1908). Sur trois cas d' infection spenique infantile a corps de Leishman observes en Tunisie. ***Archives de l'Institut Pasteur, Tunis, 3***: 1-26.

NUERNBERGER, S., RAMOS, P.C.V. and CUSTODIO, R. (1975). Visceral leishmaniasis in Honduras. Report of three proven cases and a suspected case. ***Am. J. Trop. Med. Hyg. 24***: 917-920.

NUWAYRI-SALTI, N. and KHANSA, H.F. (1985). Direct non-insect-vector transmission of ***Leishmania donovani*** in mice. ***Internat. J. Parasitol. 15***(5): 497-500.

NYAKUNDI, P.M., MUIGAI, R., WERE, J.B.O., OSTER, C.N. and GACHIHI, G.S. (1988). Congenital visceral leishmaniasis: Case Report, Clinical Research Centre. ***Trans. Roy. Soc. Trop. Med. Hyg. 82***(4): 564

OKANO, K., SHIMADA, M., GO, S., MIYANO, Y., TANAKA, M., WATANABE, Y., TAGAMI, E., ASAI, T. and INOKI, S. (1977). A probable case of leishmaniasis (espundia) acquired in Japan. ***Southeast Asian J. Trop. Med. Pub. Hlth., 8***: 546-551.

OKOT-KOTBER, B.M., MUTINGA, M.J. and KADDU, J.B. (1989). Biochemical characterization of ***Leishmania*** spp. isolated from man and wild animals in Kenya. ***Intern. J. Parasitol. 19*** (6): 657-663.

PEREZ, H., LABRADOR, F. and TORREALBA, J.W. (1979a). Variation in response of five strains of mice to ***Leishmania mexicana. Inter. J. Parasitol. 9***: 27-32.

PEREZ, H., MALARE, I. and ARRENDONDO, B. (1979b). The effects of protein malnutrition on the course of ***Leishmania mexicana*** infection in C57B1/6 mice: nutrition and susceptibility to leishmaniasis. ***Clin. Exp. Immunol. 38***: 453-460.

PERKINS, P.V., GITHURE, J.I., MEBRAHTU, Y., KIILU, G., ANJILI, C., NGUMBI, P.S., NZOVU, J., OSTER, C.N., WHITMIRE, R.E., LEEWENBURG, J., HENDRICKS, L.D. and KOECH, D.K. (1988). Isolation of ***Leishmania donovani*** from ***Phlebotomus martini*** in Baringo District, Kenya. ***Trans. Roy. Trop. Med. Hyg. 82*** (5): 695-700.

PETERS, W. (1981). Technological advances in protozoal taxonomy and systematics. In: Parasitological Topics: a Presentation Volume to P.C.C. Garnham FRS on the occasion of his 80th Birthday (Edited by Canning E.V.), pp. 200-209. Society of Protozoologists Special Publication No.1.

PETERS, W., CHANCE, M.L., MUTINGA, M.J., NGOKA, J.M. and SCHNUR, L.F. (1977). The identification of human and animal isolates of ***Leishmania*** from Kenya. ***Ann. Trop. Med. Parasit. 71***: 501-502.

PETERS, W. and KILLICK-KENDRICK, R. (1987). Biology and epidemiology. In: Peters and Killick-Kendrick (eds). The leishmaniasis in Biology and Medicine. Vol 1, Academic Press.

PIFANO, F., ORITZ, I. and ALVAREZ, A.(1960). La ecologia en condiciones naturales de laboratorio, de Guatopo, Estado Miranda, Venezuela. ***Arch. Venez. Med. Trop. Parasitol. Med. 3***: 63-71.

POST, R.J. and CRAMPTON, J.M. (1987). Probing the unknown. ***Parasitol. Today 3***: 380-383.

PRICE, S.M. and SILVERS, D.N. (1977). New World leishmaniasis. ***Arch. Dermatol. 113***: 1415-1416.

READY, P.D., SMITH, D.F. and KILLICK-KENDRICK, R. (1988). DNA hybridizations on squash-blotted sandflies to identify both ***Phlebotomus papatasi*** and infecting ***Leishmania major***. ***Med. Vet. Entomol. 2***: 109-116.

REES, P.H., ARAP SIONGOK, T.K., MNGOLA, E.N., ODUORI, M.L. and SHAH, S.D. (1977). Visceral leishmaniasis: a new Kenyan focus. ***E. Afr. Med. J. 54***: 591 (abstract).

RIVKIN, M.I. (1982). In: Application of Biochemical Micromethods in the Investigation of Tropical Disease Pathogens. Edited by F. Michal. p.165-173.

ROSS, R. (1903). Further notes on Leishman's bodies. ***Brit. Med. J. 2***: 1401.

RUTHERFORD, G.S. and UYS, C.J. (1978). Cutaneous leishmaniasis in southern Africa: a case report. ***S. African Med. J. 53***: 716-718.

SACKS, D.L. and PERKINS, P.V. (1984). Identification of an infective stage of ***Leishmania*** promastigotes. ***Science 223***: 1417-1419.

SACKS, D.L. and PERKINS, P.V. (1985). Development of infective stage ***Leishmania*** promastigotes within phlebotomine sandflies. ***Am. J. Trop. Med. Hyg. 34***: 456-459.

SCHALM, O.W. (1979). Uncommon hematologic disorders: spirochetosis, trypanosomiasis, leishmaniasis, Pelger-huet anomaly. ***Canine Practice 6***: 46-49.

SCHLEIN, Y. (1986). Sandfly diet and ***Leishmania***. ***Parasitol. Today 2***: 175-177.

SCHLEIN, Y., POLACHECK, I. and YUVAL, B. (1985). Mycoses, bacterial infections and antibacterial activity in sandflies (Psychodidae) and their possible role in the transmission of leishmaniasis. ***Parasitol. 90***: 57-66.

SCHNUR, L.F. (1982). The immunological identification and characterization of leishmanial stocks and strains, with special reference to excreted factor serotyping, pp.25-47. In: ***M.l. Chance and B.C. Walton, eds., Biochemical Characterization of Leishmania***. UNDP/WORLD BANK/ WHO Special Programme for Research and Training in Tropical Diseases, Geneva, Switzerland.

SCHNUR, L.F. and CHANCE, M.L. (1976). The characterization of African leishmanial strains: a comparison of differentiating methods. ***Trans. R. Soc. Trop. Med. Hyg. 70***: 14

SCHNUR, L.F. and JACOBSON, R.L. (1989). Surface reaction of ***Leishmania***. IV. Variation in the surface membrane carbohydrates of different strains of ***Leishmania major***. ***Ann. Trop. Med. Parasitol. 83***(5): 455-463.

SCHNUR, L.F. and ZUCKERMAN, A. (1977). ***Leishmania*** excreted factor (EF) serotypes in Sudan, Kenya and Ethiopia. ***Ann. Trop. Med. Parasitol. 71***: 273-294.

SCHNUR, L.F., ZUCKERMAN, A. and GREENBLATT, C.L. (1972). Leishmanial serotypes as distinguished by the gel diffusion factors excreted ***in vitro*** and ***in vivo***. ***Israel J. Med. Sci. 8***: 932- 942.

SCHOLLER, J.K., REED, S.G. and STUART, K. (1986). Molecular karyotype of species and subspecies of ***Leishmania***. ***Mol. Biochem. Parasitol. 20***: 279-293.

SCHWARTZ, D.C. and CANTOR, C.R. (1984). Separation of yeast chromosomes-sized DNAs by electrophoresis. ***Cell 37***: 67-75.

SCHWARTZ, D.C., SAFFRAN, W., HAAS, R. GOLDBERG, M., and CANTOR, C.R. (1983). New techniques for purifying large DNAs and studying their properties and packaging. Cold Spring Harbour. ***Symp. Quant. Biol. 47***: 189-195.

SEAL, S.C. (1977). Return of kala-azar. ***Indian J. Pub. Health 21***: 109-110.

SERWER, P. (1980). Electrophoresis of duplex deoxyribonucleic acid in multiple-concentration agarose gels: Fractionation of molecules with molecular weights between 2X106 and 110X106. ***Biochemistry 19***: 3001-3004.

SHEPPARD, M., THOMPSON, J.K., ANDERS, R.F., KEMP, D.J. and LEW, A.M. (1989). Molecular karyotyping of the rodent malarias ***Plasmodium chabaudi, Plasmodium berghei*** and ***Plasmodium vinckei. Mol. Biochem. Parasitol. 34***: 45-52.

SHORTT, H.E. (1945-1946). Recent research on kala-azar in India. ***Trans. Roy. Soc. Trop. Med. Hyg. 34***: 13-31.

SMITH, C.L., WARBURTON, P.E., GAAL, A. and CANTOR, C.R. (1987). Analysis of genome organization and rearrangements by Pulsed Field Gradient gel Electrophoresis. In: Genes and Proteins. Editors: K. Jeyaseelan, M.C.M Chung and O.L. Kon; pp. 89.

SOUTHGATE, B.A. and ORIEDO, B.V.E. (1962). Studies on the epidemiology of East African leishmaniasis. I. The circumstancial epidemiology of kala-azar in the Kitui District of Kenya. ***Trans. R. Soc. Trop. Med. Hyg. 56***: 30-47.

SPITHILL, W. and GRUMONT, R.J. (1984). Identification of species, strains and clones of leishmania by characterization of kinetoplast DNA minicircles. ***Mol. Biochem. Parasitol. 12***: 217-236.

SPITHILL, T.W. and SAMARAS, N. (1985). The molecular karyotype of ***Leishmania major*** and mapping of alpha and beta tubulin gene families to multiple unlinked chromosomal loci. ***Nucleic Acids Research 13***: 4155-4169.

STEWART, C.D. and PILCHER, J.F. (1945). American leishmaniasis: report of an autochthonous case. ***Arch. Dermatol 51***: 124-128.

STEWART, W., FURST, A. and AVDALOVIC, N. (1988). Transverse alternating field electrophoresis. ***BioTechniques 6*** (1): 68-73.

SWAMINATHAN, C.S., SHORTT, H.E. and ANDERSON, L.A.P. (1942). Transmission of Indian kala-azar to man by the bites of ***Phlebotomus argentipes***. ***Indian J. Med. Res. 30***: 473-477.

UNDP/WORLD BANK/WHO Special Program for Research and Training in Tropical Diseases (1981). Report of Third SWG on leishmaniases. TDR/LEISH-SWG (3) 81.3.

UNDP/WORLD BANK/WHO Special Program for Research and Training in Tropical Diseases (1990). Tropical diseases in media spotlight. No. 31 March; pp. 3.

VAN DER PLOEG, L.H.T., CORNELISSEN, A.W.C.A., BARRY, J.D. and BORST, P. (1984a). Chromosomes of kinetoplastida. ***EMBO J. 3***: 3109-3115.

VAN DER PLOEG, L.H.T., CORNELISSEN, A.W.C.A., MICHELS, P.A.M. and BORST, P. (1984b). Chromosome rearrangements in ***Trypanosoma brucei***. ***Cell 39***: 213-221.

VAN DER PLOEG, L.H.T.,SCHWARTZ, D.C., CANTOR, C.R. and BORST, P. (1984c). Antigenic variation in ***Trypanosoma brucei*** analyzed by electrophoretic separation of chromosome-sized DNA molecules. ***Cell 37***: 77-84.

VAN DER PLOEG, L.H.T., SMITS, M., PONNUDURAI, T., VERMEULEN, A., MEUWISSEN, J.H.E. and LANGSLEY, G. (1985). Chromosome-sized DNA molecules of ***Plasmodium falciparum***. ***Science 229***: 658-661.

VAN EYS, G.J.J.M., SCHOONE, G.J., LIGTHART, G.S., LAARMAN, J.J. and TERPSTRA, W.J. (1987). Detection with non-radioactive probes. ***Parasitol. Res. 73***: 199-202.

VAN EYS, G.J.J.M., SCHOONE, G.J., LIGTHART, G.S., ALVAR, J., EVANS, D.A. and TERPSTRA, W.J. (1989). Identification of 'Old World' ***Leishmania*** by DNA recombinant probes. ***Mol. Biochem. Parasitol. 34***: 53-62.

VIANNA, G. (1911). Sobre uma nova especie de ***Leishmania*** (nota preliminar). ***Brasil- Medico 25***: 411.

WARD, R.D. (1977). The colonization of Lutzomyia flaviscutellata (Diptera : Psychodidae), a vector of ***Leishmania mexicana amazonensis*** in Brazil. ***J. Med. Entomol. 14***: 469-476.

WASUNNA, A. (1977). The visceral leishmaniasis survey. April 1977 (mimeographed report). Dept. of Community Health, University of Nairobi, 1977 cited in Oomen JMV. Monitoring health in African dams. Rotterdam, thesis 1981.

WENYON, C.M. (1926). Protozoology. Bailliere, Tindall and cox, London, pp. 396-400.

WERE, J., MUIGAI, R., WASUNNA, K., RASHIDI, J.R. and OSTER, C.N. (1986). Chemotherapy of leishmaniasis. ***Insect Sci. Applic.*** **7**(3): 421-427.

WIJERS, D.J.B. (1963). Studies on the vector of kala-azar in Kenya. II. Epidemiological evidence. ***Ann. Trop. Med. Parasit. 47***: 7-18.

WIJERS, D.J.B. (1971). A ten years' study of kala-azar in Tharaka (Meru District, Kenya). Part I: incidence studies from the records at Marimanti. ***E. Afr. Med. J. 48***: 533-550.

WIRTH, D.F. and PRATT, D.M. (1982). Rapid identification of ***Leishmania*** species by specific hybridization of kinetoplast DNA in cutaneous lesions. ***Proc. Natl. Acad. Sci. 79***: 6999-7003.

WORLD HEALTH ORGANIZATION (1984a). The leishmaniases. WHO Technical Report 704, pp.10-14.

WORLD HEALTH ORGANIZATION (1984b). WHO Technical Report Series 710, pp.140.

WORLD HEALTH ORGANIZATION (1990). Control of leishmaniases. WHO Technical Report Series 793, pp. 9-10.

WRIGHT, T.H. (1903). Protozoa in a case of tropical ulcer ("Delhi Sore"). ***J. Med. Res. 10***: 472-482.

WRIGHT, F.J. (1943). Case of kala-azar. ***E. Afr. Med. J. 20***: 19-20.

ZAHAR, A.R. (1981). Studies on leishmaniasis vectors/ reservoirs and their control in the Old World: Part V. Tropical Africa. World Health Organization /VBC/81. 825, pp. 198.

ZUCKERMAN, A. (1975). Current status of the immunology of the blood and tissue protozoa. I. ***Leishmania***. ***Exp. Parasitol. 38***: 370-400.

ZUCKERMAN, A. and LAINSON, R. (1977). ***Leishmania***. In: Parasitic Protozoa, Vol. 1 (Edited by Kreier, J.P.) pp.57-133. Academic Press, New York.