CHARACTERIZATION OF KENYAN ISOLATES OF *Leishmania* BY ISOENZYME ELECTROPHORESIS AND INFECTIVITY TO MICE.

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other University.

Signed..... Date..12/11/93.....

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DEDICATION

This thesis is dedicated to my husband Muigai,my two sons Mwaura and Kimani and my daughter Wambui for their patience and encouragement during the hard times of the study.

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SUMMARY

In this study, *Leishmania major* and *Leishmania donovani* strains from Kenya were cloned by limiting dilution. Population homogeneity of the strains was determined by determining the infectivity of the clones in Balb/c mice and their isoenzyme profiles.

Characterization of *L. donovani* isolates from kala-azar endemic areas in Kenya was done by cellulose acetate electrophoresis to determine strain variation.

High temperature adaptation of *L. major* strain was done by repeated intraperitoneal passages in Balb/c mice to determine whether this parasite can visceralize without causing a cutaneous lesion.

Cloning of *L. major* strain (NLB 144) and *L. donovani* strain (NLB 065), was done by limiting dilution. The obtained clones were used to infect Balb/c mice. Results demonstrated that *L. major* strain is not homogenous but consists of a genetically mixed population. This was confirmed by finding of clones, that although had the same zymodeme, showed a difference in infectivity in mice. One clone was very virulent and resulted in severe ulceration of the nasal area of mice. *L. donovani* clones also showed population homogeneity with reference to isoenzymes but a difference in infectivity to mice. Only clone-1 was infective to Balb/c mice. These results are of great

significance to leishmaniasis vaccine production. Presence of clonal homogeneity of a strain suggests that one type of vaccine can be used in the treatment and control of leishmaniasis.

Cellulose acetate electrophoresis of 15 *L. donovani* isolates from Baringo, Turkana and Machakos Districts in Kenya revealed low levels of variation in the parasite genome. Only one isolate, NLB 659 from Baringo District showed a different banding pattern for isocitrate dehydrogenase (ICD). This suggests that there is no intraspecific variation of *L. donovani* strains in Kenya or if it occurs, its in very low level .This is very important in the treatment of visceral leishmaniasis because *L. donovani* has been known to cause both kala-azar and post- kala-azar dermal leishmaniasis.

Intraperitoneal passaging of *L. major* strain (NLB 144) resulted in visceralization of the parasite. The incubation period became shorter with each successive passage. The parasite behaved like viscerotropic *L.donovani* since no conspicuous cutaneous lesion was formed at the site of inoculation. Isoenzyme electrophoresis of this viscerotropic *L. major* showed enzyme profiles similar to those of the original stock of *L.major* except for the enzymes glucose phophate isomerase (GPI) and glucose 6 phosphate dehydrogenase (G6PD). 3-4 months post-infection, some parasites

disseminated to the footpads and caused lesions.

This viscerotropism of *L. major* in Balb/c mice emphasizes the need to characterize *Leishmania* parasites particularly those isolated from rodents before making conclusions of their identity.

CHAPTER ONE.

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Global importance of leishmaniases and the need to characterize *Leishmania* parasites

Leishmaniasis is one of the six major diseases recognised by WHO since it affects millions of people in the tropics and sub-tropics. It is caused by a protozoan parasite belonging to the genus *Leishmania*. The other five diseases are malaria, schistosomiasis, trypanosomiasis, filariasis and leprosy. All these six diseases have endemic foci in Kenya and alot of research is being carried out by various institutions in the country.

Leishmania parasites are restricted to the mononuclear phagocytes (macrophages) of the skin or viscera. This difference in localisation of the parasites results in the different clinical manifestation of the disease. The host macrophages engulf these parasites by phagocytosis but they are not destroyed by the lysosomal enzymes released by the macrophages, instead they multiply and destroy the macrophages to infect others. In Kenya there are only two forms of leishmaniasis, namely, cutaneous leishmaniasis caused by *L. major* (Muigai *et al*, 1987), *Leishmania aethiopica* (Kungu *et al*, 1972), and

Leishmania tropica (Mebrahtu *et al*, 1987) and visceral leishmaniasis caused by *L. donovani* (Bryceson *et al*, 1985). The two forms of disease are endemic in Kenya and are mostly found in the dry semi-arid regions of the country, for example, Baringo, Machakos, Laikipia and Turkana districts.

The inability to differentiate easily between species and strains of *Leishmania* has been a considerable hindrance to research into many aspects of leishmaniasis, especially its epidemiology (Kilgour *et al*, 1974). Consequently their classification has been based largely on clinical manifestation in man and other animals, in conjunction with geographical distribution.

A variety of serological tests have been used to distinguish between the different *Leishmania* that cause the three forms of leishmaniasis, for example, excreted factor serotypes (Schnur and Zuckerman, 1977) and monoclonal antibodies (Schotelius 1982; Jaffe *et al*, 1984). More recently, biochemical techniques, for example, polymerase chain reaction and isoenzyme electrophoresis have been used with success to characterize different species of *Leishmania*.

Variant forms of enzymes as revealed by electrophoretic techniques often occur in parasite populations. Enzyme polymorphism has been found in all parasitic protozoa including *Amoeba*, *Trypanosoma*, *Leishmania*, *Coccidia* and *Plasmodium* (Godfrey, 1978;

Gibson and Gashumba, 1983; Al-Taqi and Evans, 1978). Such isoenzyme forms are important for characterization of parasites of the same or different species as they reveal the genetic diversity of such parasites. They can show the heterogenous nature of certain isolates thus enabling us to separate them into different strains from which distinct clones can be obtained. Isoenzymes can act as genetic markers of different strains or varieties of parasite species.

Biological, biochemical and immunological studies have shown the population heterogeneity of clones isolated from parasites of the genera *Trypanosoma* and *Leishmania* (Morel *et al*, 1980; Engel *et al*, 1980, Handman *et al*, 1983.

The ability to clone individual *Leishmania* species can be important in selecting parasites from populations with different biochemical and immunological properties such as drug resistance and loss of virulence. Various cloned populations can then be compared with both the original parent (wild type) stock and other clones exhibiting different properties. This allows an understanding at both the biochemical and genetic levels. Considerable work has been done on cloning *T. cruzi* and several *Trypanosomatidae* (Keppel and Janovy, 1980, Tanuri *et al*, 1981).

The use of murine models offers some obvious advantage in studies designed to investigate

immunological mechanisms associated with human leishmaniasis. Apart from being immunologically well defined, mice can serve as models for most of the *Leishmania* species infective to man. In addition, the availability of many inbred strains of mice facilitates genetic studies and allows a comparison of the infection in animals of varying susceptibility to the parasite (Zuckerman, 1975).

In the present study, population heterogeneity of two *Leishmania* strains from Kenya, *L.major* and *L.donovani* will be determined. The two strains will be cloned by limiting dilution and the obtained clones used to infect Balb/c mice to determine the differences in virulence. Isoenzyme electrophoresis of the cloned and uncloned strains will be carried out to determine the genetic variation at enzyme level.

Intraspecific variation of *L.donovani* isolates from kala-azar patients in Baringo. Machakos and Turkana Districts in Kenya will be determined by isoenzyme electrophoresis.

Leishmania major causes cutaneous leishmaniasis in Kenya . In this study, experimental adaptation of this parasite to viscerotropism will be carried out by several intraperitoneal passages in Balb/c mice. Isoenzyme electrophoresis of the passaged parasites will be carried out to determine if there exists enzyme variation due to high temperature adaptation.

1.2 LITERATURE REVIEW

1.2.1 Biochemical characterization of *Leishmania* by isoenzyme electrophoresis.

Leishmaniasis is one of the most widespread and debilitating protozoa diseasesof man, (Scott and Farell, 1982). A third of the world's population, about 1,600 million people are at risk of infection with leishmaniasis. Precise prevalence and incidence data are unknown. However, an approximate incident rate of 40,000 new cases of leishmaniasis per year has been reported, (Modabber, 1987; Walsh and Werrens, 1979).

Human leishmaniasis occurs in three different forms, namely, visceral leishmaniasis, cutaneous leishmaniasis and mucocutaneous leishmaniasis, (Winslow, 1971; Manson -Bahr and Winslow, 1971). The clinical separation is to some degree artificial since all forms of the disease begin in the skin (Napier,1946; Garnham and Humphrey, 1969). Adler (1964) and Garnham and Humphrey (1969), have emphasized that the strict separation between cutaneous and visceral leishmaniasis may be misleading in that the primary lesions of kala-azar may present as Oriental sore and that some strains with the capacity to visceralize may fail to do so causing only dermal lesions.

The classification of *Leishmania* parasites has been based primarily on comparison of extrinsic characters. Since the early 80's, attempts have been made to

evaluate the intrinsic characters which make the same organisms to show different behaviour in different hosts and also in culture. A variety of serological and biochemical tests have been used to distinguish between the different *Leishmania* parasites that cause the three forms of leishmaniasis. The tests include, excreted factor serotypes (EF), monoclonal antibodies, DNA hybridization techniques, isoenzyme electrophoresis and polymerase chain reaction (PCR). These methods are important because clinical and epidemiological data may lead to equivocal taxon designation. The imprecise nature of conclusion drawn from clinical histories is illustrated by the discovery of *L. tropica* visceralizing in India and Kenya (Schnur *et al*, 1981; Mebrahtu *et al*, 1989), *L.donovani chagasi* causing active cutaneous leishmaniasis in Brazil (Oliveira *et al*, 1982), *L.donovani* causing post-kala-azar dermal leishmaniasis in Kenya (Rashid *et al*, 1986). *Leishmania infantum* which causes infantile visceral leishmaniasis has been isolated from cutaneous lesions (Rioux *et al*, 1986). *L.mexicana amazonensis* has been isolated from the bone marrow of a patient having American visceral leishmaniasis (Barral Neto *et al*, 1986). This parasite is thought to cause human cutaneous leishmaniasis which most often progresses into diffuse cutaneous leishmaniasis (Lainson, 1983).

In Kenya cases of human cutaneous leishmaniasis

caused by *L. major, L.aethiopica* and *L.tropica* occur (Mebrahtu *et al*, 1987, Muigai *et al*, 1987, Kungu *et al*, 1972). Mebrahtu *et al*, (1989), reported that *L.tropica* visceralized in humans in Kenya. This parasite is known to cause anthroponotic cutaneous leishmaniasis in Algeria and Tunisia (WHO 1984).

All these findings emphasize the need to characterize *Leishmania* spp in Kenya because the disease they cause differ in different hosts. In addition, more than one *Leishmania* species occur in the same endemic area, for example *L.major, L.donovani* and *L.adleri* are found in Baringo District Kenya, (Muigai *et al*, 1987; Beach *et al*, 1984). The data which have been reported on enzyme profiles of leishmanial groups have been obtained from small numbers of isolates. Among known species isolates, there is a certain amount of naturally occuring enzyme polymorphism. This type of population polymorphism is expected and has been noted in all groups of organisms studied by electrophoresis, (Myers, 1978).

Variation in the electrophoretic mobility of enzymes has been used increasingly in the differentiation of leishmanial strains. Variation in the electrophoretic mobility of phosphoglucomutase (PGM) using 10 leishmanial strains have shown six electrophoretic variant types of PGM, (Brazil, 1978). This variation is a suitable character for

differentiating leishmanial strains and can be used in furthering their characterization. Visceral and cutaneous leishmaniasis have been widely reported in the Aethiopian Zoogeographical Region of Africa. *Leishmania* parasites have been isolated from a wide range of clinical types, animal reservoirs and sandfly vectors in the area. This wide variation in the epidemiology of leishmaniasis has led to widespread speculation regarding the identity of the responsible parasites. Biochemical and serological methods allow the determination of intrinsic characters, which because they are closely related to the genome of an organism are clearly superior to circumstantial characters derived from epidemiological data (Lumsden, 1974). Chance *et al*, (1978) characterized several strains of African *Leishmania* using three methods, DNA bouyant density, excreted factor serotypes and isoenzyme variants. Results showed that there is apparent identity in terms of the characters of all the strains isolated from human cases of visceral leishmaniasis, even though they originated from a number of different geographical areas, in which some cases show marked differences in the epidemiology of visceral leishmaniasis. There is therefore no need to draw distinction between the parasite causing visceral leishmaniasis in the Sudan or Kenya as suggested by some authors, for example, Bray (1974), nor is there any indication of differences

between the anthroponotic focus south of Tana River and the more diffuse endemic foci of the north-west region of Kenya, (Chance *et al*, 1978).

Chance *et al*, (1978) discussed the significant contributions that electrophoretic studies made in clarifying the taxonomy of *Leishmania*. Papers by Kilgour *et al*, (1974), Gardener *et al*, (1974) and Chance *et al*, (1974), Lumsden, (1974), AL-Taqi and Evans, (1978) and Chance *et al*, (1978), have reported biochemical variation at both inter and intraspecific levels. They also noted the need for additional, more comprehensive biochemical studies of these intrinsic taxonomic characters, which could be used in conjuction with the already available extrinsic ones. Kreutzer and Christensen, (1980) using the method of cellulose acetate electrophoresis, characterised six strains of *Leishmania*. These were *L. braziliensis, L. hertigi , L. mexicana, L. donovani, L. tropica* and *L. adleri* using 14 enzymes. Each of the species had a distinct isoenzyme pattern for aspartate aminotransferase (ASAT), glucose 6 phosphate dehydrogenase (G6PD), 6 phosphogluconate dehydrogenase (6PGD) and fructokinase (FK). Two or more species had identical migrating bands. However, by using a combination of the other 10 enzymes, it was not possible to separate any of the six species. The Panama *L. braziliensis* strain showed a different banding

pattern for MDH isoenzyme which distinguished it from the other strains.

Similar high levels of interspecific isoenzyme variability and comparatively low levels of intraspecific variability have been reported in *Leishmania* from the Aethiopian Zoogeographical Regions of Africa, (Chance *et al*, 1978) and from Kuwait, (AL-Taqi and Evans, 1978) as well as studies on *Drosophilla* (Ayala, 1975) and mosquitoes (Bullini and Coluzzi, 1973).

The failure of classical biological parameters to provide valid criteria for the reliable identification of *Leishmania* has meant that many problems of medical and especially epidemiological importance have remained unanswered. The refinement of serological methods used for the differentiation of *Leishmania* has assisted in the development of a more logical approach to their classification, (Bray, 1970).

A number of stocks of *Leishmania* isolated from various hosts and localities in India have been identified by characterization of a number of their isoenzymes using the method of Chance *et al*, (1978) and serotype procedure of Schnur and Zuckerman (1977). Results have shown that variants of both *L.tropica* and *L. major* are present in the West of India. However, the existence of stocks resembling *L.tropica* in India and elsewhere from patients with kala-azar necessitates a

close study of a wider range of isolates from various geographical areas to ascertain whether indeed this parasite can be responsible for visceral, as well as cutaneous leishmaniasis.

Isoenzyme electrophoresis has been used to characterize *Leishmania* species from Kuwait where cutaneous leishmaniasis is endemic (Selim and Kandil, 1972, Al-Taqi and Evans, (1978). *Leishmania* stocks isolated from skin lesions of 26 patients were compared amongst themselves and with *Leishmania* stocks collected from other parts of the world (Old and New) using seven enzymes; alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), glucose phosphate isomerase (GPI), glucose 6 phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), malic enzyme (ME) and phosphoglucomutase (PGM).

The pattern obtained with 15 Kuwait stocks were identical with *L. major* identified on clinical and geographical characteristics, 7 stocks were identical with *L.tropica* similarly identified. Three stocks had isoenzyme patterns different from each other and from other *Leishmania* examined in the study. One stock gave isoenzyme banding pattern identical with those of *L.donovani* isolated from Sudan, suggesting the possibility of *L.donovani* causing cutaneous leishmaniasis.

It has been noted that in Iraq, children with

visceral leishmaniasis show marked difference in the severity of the disease as well as their response to chemotherapy (Taj- Eldin and Alouis, 1954; Taj- Eldin and Al- Hassimi, 1961; Nouri and Al-Jeboori, 1973). These differences are either due to presence of *L.donovani* "strains" of different virulence or to different host response. As a result of this, isolates of *L.donovani* from children with different clinical pictures (one mild and one severe) were studied and the behaviour of the parasites in hamsters and mice. Evidence from this work suggests the possibility of more than one strain of *L.donovani* in Iraq. In Kenya, we encounter cases of human cutaneous leishmaniasis caused by *L.donovani* (post-kala-azar dermal leishmaniasis), *L.major*, *L.aethiopica* and *L.tropica* (Beach *et al*, 1984; Chulay *et al*, 1983; Mebrahtu *et al*, 1988).

Muigai *et al*, (1987) reported the first case of cutaneous leishmaniasis due to *L.major* from a school girl from Baringo District. Cellulose acetate electrophoresis of the isolates from the lesions showed similar enzyme profiles as the *L. major* strain from a rodent and a sandfly caught in that area. The rodent and sandfly strains from the same area were earlier shown to have enzyme profiles identical with *L. major* reference strain, (Beach *et al* 1984, Githure *et al*, 1986).

In Kenya, *L.donovani* has been found to cause cutaneous leishmaniasis (Post kala-azar dermal

leishmaniasis) in some patients, (Rashid *et al*, 1986). This usually occurs after unsuccessful treatment of kala-azar. Cellulose acetate electrophoresis of the enzyme glucose phosphate isomerase (GPI) have been used to identify isolates from 10 cases of human cutaneous leishmaniasis (Kreutzer and Christensen, 1980). Two isolates were identified as *L.donovani* (post-kala-azar dermal leishmaniasis), one as *L.major* and six as *L.aethiopica*.

L. tropica which causes anthroponotic cutaneous leishmaniasis has been reported from Algeria and Tunisia, (WHO 1984) and was recently isolated from three American residents in Kenya, (Mebrahtu *et al*, 1987). Characterization of the Kenyan isolates of *Leishmania* from the three patients by isoenzyme electrophoresis using 7 enzymes resulted in enzyme profiles which were similar to those of *L.tropica* reference strains.

Indigenous human cutaneous leishmaniasis due to *L.tropica* has been reported in Kenya, (Mebrahtu *et al*, 1988). *Leishmania* isolates from three indigenous Kenyans and two Canadian expatriates were thought to be *L.aethiopica* based on geography and histopathology. Cellulose acetate electrophoresis of the isolates using 9 enzymes yielded isoenzyme profiles indistingushable from *L.tropica* reference strains (Mebrahtu *et al*, 1988). This finding of *L.tropica* among indigenous Kenyans as well as expatriates confirmed the earlier report of *L.*

tropica in Kenya (Mebrahtu *et al*, 1987).

L. tropica has been found to cause visceral leishmaniasis which is unresponsive to Pentostam in Kenya (Mebrahtu *et al*, 1989). Isoenzyme migration patterns of six *Leishmania* isolates from splenic aspirates of two patients which were unresponsive to treatment with sodium stibogluconate, were indistinguishable from those of two WHO reference strains of *L.tropica*. These were the first reported cases of visceral leishmaniasis caused by *L.tropica* in Africa.

Reports have also implicated *L.tropica* as a causative agent of visceral leishmaniasis in the Middle East and in the Indian subcontinent (Schnur *et al*, 1981; AL-Jeboori and Evans, 1980a). Schnur *et al*, (1981) characterized five leishmanial strains from visceral cases, four from Israel and one from India against three reference strains of *L.tropica*. All the five strains were indistingushable from the *L.tropica* reference strains biochemically and serologically.

All these results show that some species of *Leishmania* parasites are not restricted exclusively to the skin or viscera and that a given parasite may on occasion cause cutaneous leishmaniasis or visceral leishmaniasis. This shows the emphasis of using methods other than clinical and epidemiological in diagnosing *Leishmania*.

A remarkable degree of homogeneity has been found

among *L.major* stocks characterized by isoenzyme electrophoresis, (Le Blancq *et al*, 1986) . This enzyme homogeneity of *L.major* throughout its geographical and host range appears to be correlated with the close association between *L.major* and sandflies of the genus *Phlebotomus*. Considerable intraspecific variation has been found among *L.tropica* stocks, (Le Blancq *et al*, 1986).

This does not correlate with its epidemiological uniformity. *Leishmania tropica* appears to have only one vertebrate reservoir, man ,and one sandfly vector, *P. sergenti* whereas *L.major* has many vertebrate hosts and at least three albeit closely related vectors (Le Blancq *et al*, 1986). Hence the selective pressure operating on *Leishmania tropica* seems to be less than that operating on *L. major*. However, there is much greater variation in *L.tropica* than in *L.major* (Le Blanq *et al*, 1986). This variation cannot therefore be attributed to these selective pressures. An explanation for this apparent paradox may be that *L. tropica* simply has a greater genetic diversity than *L.major*.

The status of *L.aethiopica* as a seperate species has been supported by isoenzyme electrophoresis (Le Blancq *et al*, 1986). This parasite has been considered as a subspecies of *L. tropica* (Lainson, 1982). Isoenzyme profiles of 28 stocks of *L.aethiopica* when compared with those of reference strains of *L.aethiopica*

and *L.major* using 13 enzymes have shown some intraspecific variation although an overall low level of heterogeneity is indicated. This may correlate with the well stable ecological niche occupied by the parasite.

The distribution of *L.donovani* sensu lato zymodemes have shown a striking degree of homogeneity thereby indicating low levels of variation in the parasite genome (Le Blancq and Peters, 1986). This is in contrast to the diversity of habitat occupied throughout its range which whilst reflecting the involvement of other factors such as sandfly behaviour, also suggests the existence of differences in the parasite. Studies using immunological techniques for example, monoclonal antibodies have also indicated homogeneity within *L.donovani* sensu lato in the Old World (Schottelius, 1982; Jaffe *et al*, 1984).

Visceral leishmaniasis has low prevalence in several endemic areas in Kenya, for example in Masinga, prevalence rate is 0.30% (Ho *et al*, 1982). Mbugua and Siongok, (1981) reported a prevalence rate of 0.62% in Kitui District.

1.2.2 Cloning of *Leishmania* spp and the infectivity of the clones in laboratory animals.

Morel *et al*, (1980) using the technique of colony formation for the isolation of clones and schizodeme

analysis showed that the CL strain of *Trypanosoma cruzi* is heterogenous, being composed of a mixture of at least two subpopulations. Golberg & Pereira, (1983), continued these investigations and found different zymodemes among clones of the CL strain of *T.cruzi*.

Postan *et al*, (1983) used two single-cell isolate cloned stocks of the Sylvio - X10 strain of *T. cruzi*, recovered from an acute *T. cruzi* infection to infect inbred mice. Results obtained demonstrated pluripotential pathogenic nature of a *T. cruzi* strain due to genetic heterogeneity of the population of parasites that constitute the strains. These characteristics do not change during the long-term *in vitro* maintenance of the clones (Postan *et al*, 1986).

In the literature, evidence has accumulated concerning the occurence of naturally acquired mixed infections in *T. cruzi* and *Leishmania* and also the selection of populations dependent on the culture medium in which the organisms were grown (Safjanova,*et al*, 1980, Deane *et al*, 1984).

Pacheco *et al*, (1989), using the technique of colony formation described by Jaffe *et al*, (1984), demonstrated the existence of mixtures of subpopulations in the genus *Leishmania* such as occurs in *T. cruzi*. In addition, schizodeme analysis provided genotypic evidence in support of the clonal heterogeneity in stocks isolated from human beings and a sylvatic

reservoir.

Bongertz and Dvorak (1983), determined the antigenic constitution of two *T. cruzi* strains and six cloned stocks immunoelectrophoretically. Five to seven antigens common to all of the *T.cruzi* stocks were found. However, each stock also contained "strain specific antigens" and five of the six cloned stocks presented "clone specific antigens", therefore demonstrating that a *T.cruzi* strain is composed of an antigenically heterogenous population of organisms. These results also show that immunochemical properties can be used to classify flagellate species.

Postan *et al*, (1987) carried out comparative studies of the infection of Lewis rats with 4 clones of *T. cruzi* which had been previously used to infect inbred mice (Postan *et al*, 1983). Results observed showed quantitative and qualitative differences in parasitaemia levels and histopathological characteristics in the Lewis rats infected with the 4 clones. Myocardial fibrosis developed more rapidly in rats than had been reported in mice, (Postan *et al*, 1983).

Similarly, Handman *et al*, (1983), demonstrated that *Leishmania major* strain LRC-L137 consists of a genetically mixed population which was confirmed by the finding of clones that distributed in two closely related schizodemes and showed a difference in infectivity to mice. However, the kDNA of these clones

and of the parental strain show total sequence homology in the minicircles and can be distinguished only by the differences observed in their kDNA restriction profiles (Spithill and Grumont, 1984).

Cloning by limiting dilution of an isolate of *Leishmania major* (LRC-L137) that is infective to mice resulted in 7 stable clones only one of which was infective to Balb/c mice. Three of the non-infective clones that were examined for survival in Balb/c macrophages *in vitro* seemed to be killed more readily suggesting failure to establish in macrophages as the basis for non- infectivity *in vivo*. Two dimensional gel electrophoresis of the three non-infective clones and one infective clone showed similar patterns for all the *L.tropica* clones with minor differences. Schizodeme analysis also showed that the clones are closely related.

Boreham *et al*, (1987) using the technique of limiting dilution to obtain clones of *Giardia intestinalis* and drug sensitivity assays found that there is heterogeneity in the responses of these clones to antigiardial drugs. Clones of two stocks of *G.intestinalis* have been tested by the (^{3}H) thymidine uptake assay to determine their sensitivity to metronidazole, tinidazole, furazolidone and quinacrine. Results showed that each stock is not homogenous with reference to drug sensitivity, but is composed of

different populations of organisms. These results may in part account for treatment failures in human giardiasis (Mendelson, 1980; Davidson, 1984).

1.2.3 Importance of inbred laboratory animals in the study of leishmaniasis.

The use of inbred laboratory host lines in which to study leishmanial strains minimizes the diverse responses often associated with geneticaly or physiologically variable hosts (Bradley, 1977). Stauber (1966), stated that the source of parasites, quantity of inoculum and route of infection greatly affects the results of an infection.

Differences in susceptibility of various mouse strains to infection with *Leishmania* parasites was first described by Bradley and Kirkley (1977). Twenty five strains of inbred mice infected with *Leishmania donovani* showed some strains to be acutely susceptible and some strains acutely resistant to the infection. Similar studies have shown different strains of inbred mice which respond differently to infection with *L. major* (Behin *et al*, 1979, Preston and Dumonde, 1975).

Balb/c mice are susceptible to many species of *Leishmania* (Perez *et al*, 1979; De. Tolla *et al*, 1980 and Bradley, 1974). Scott and Farrell, (1982), found that infection of Balb/c mice with *L.tropica* leads to the development of massive primary lesions, cutaneous

metastatic lesions and in some, visceralization of the parasite. This susceptibility is not route dependent or dose dependent as parasite innocula of 10^3 to 10^7 given intradermally or intravenously led to the development of metastatic lesions and eventual death of the animals.

In contrast, C57B/6 mice considered genetically resistant to *L. tropica* (De. Tolla *et al*, 1980) developed healing infections following intradermal inoculation with 10^4, 10^5, or 10^6 *L. tropica* promastigotes. However intravenous inoculation of C57B/6 mice resulted in multiple paw, ear and facial lesions which showed no signs of ulceration or healing. This non-healing leishmanial infections similar to those of human diffuse cutaneous leishmaniasis (DCL) could be produced in both genetically susceptible (Balb/c) and resistant (C578/6) strain of mice.

Khairy and El-hashimi (1980), inoculated hamsters with 3 isolates of *L.donovani* from Iraqi kala-azar patients. High infection rates occured in two groups of hamsters inoculated with *L.donovani* from patients with severe clinical symptoms. Low infection rate occured in the third group of hamsters inoculated with *L.donovani* isolated from patients with mild clinical symptoms. These results suggested the possible existence of more than one strain of *L.donovani* in Iraq.

Stauber (1966) successfully differentiated 3 isolates of *L.donovani* from 3 geographical areas by

their virulence in groups of hamsters. Similarly, Mansour *et al*, (1970), showed differences in the virulence between three strains of *L. donovani* isolated from dogs and puppies in Sicily, Sudan and Kenya.

Different patterns of disease have been obtained in two inbred mouse strains infected with a clone of *L.mexicana amazonensis*. Balb/c mice and C578/6 mice were infected with the clone of *L.mexicana amazonensis* population which was obtained from the "Maria" strain. Progression of the infection and histopathological examination confirmed the extreme susceptibility of Balb/c mice and the resistant pattern of the C578/6 mice (Barral-Neto *et al*, 1987).

1.2.4 Passaging of *Leishmania* parasites in laboratory animals.

The passaging of *Leishmania* in animals, either in hamsters or mice is an important adjunct to any *in vitro* culturing technique. A sufficient number of primary isolates, especially members of the *L.brazielensis* complex do not grow well in any of the existing culture media. Also promastigotes which are extensively subpassaged *in vitro* can lose their virulence. By passaging *Leishmania* in animals, those stocks which are difficult to grow *in vitro* can be usually maintained and the virulence of all the stocks assured (Jaffe *et al*, 1984).

One of the criteria for separating parasites belonging to the *L.brazielensis* complex has been the general difficulty experienced in culturing these parasites (Walton *et al*, 1977; Zuckerman & Lainson, 1977; Lainson and Shaw, 1979). *L.brazielensis* is generally difficult to grow in both biphasic and liquid media (Hendricks *et al*, 1978). Generally biopsy set up for culturing is simultaneously injected into hamsters. This passaging from hamster to hamster can be used to maintain stocks which are difficult to culture *in vitro*. The culturing *in vitro* of some isolates of *L.donovani* have also been problematic (Hendricks *et al*, 1978). These stocks have been maintained by passaging from hamster to hamster.

The passaging of the promastigote stage *in vitro* for long periods of time can result in loss of virulence and the ability to re-establish the infection in a mammalian host (Schneider & Hertigi, 1966; Anomymous, 1968; Giannini, 1974; Dawidowicz *et al*, 1975; Handman *et al*., 1983). The reason for this loss of virulence are unknown and several mechanisms have been proposed. Schneider and Hertigi (1966) suggested that, loss of virulence during prolonged passaging may be correlated with the loss of an antigen associated with invasiveness. Changes in membrane glycosylation as detected by the binding of Ricin agglutinin have been correlated with the loss of pathogenicity of

L.braziliensis (Dawidowicz *et al*, 1975; Hernandez *et al*, 1980).

The isolation of cutaneous parasites for example *L.tropica*, *L.major* and *L.mexicana* by needle aspiration from cutaneous lesions in mice or hamsters is especially convenient since sufficient material can be obtained without killing the animal and grown out within a week.

Passaging of *Leishmania* promastigotes in animals can be used to obtain those stocks which have lost their virulence. The virulence of *L.enrietti* passaged over 300 times in culture has been re-established by passaging perinasally in guinea pigs and reisolation from the tissue at the site of injection after 5-7 days. This technique has also been used successfully for promastigotes of *L.b. braziliensis*, *L.m. mexicana*, *L.m. amazonensis*, *L. major* and *L. tropica* in Balb/c mice (Jaffe *et al*, 1984).

Passaging of *Leishmania* parasites in animals can be used to evaluate why different *Leishmania* spp are associated with cutaneous, mucocutaneous or visceral lesions. A lower optimal temperature for growth of certain *Leishmania* species has been proposed as the underlying mechanism for their preferential localization in the skin and certain exposed mucosal sites, which may normally maintain a temperature lower than the internal temperature of 37^{0} C (Marsden 1979).

However, only recently has evidence been obtained

that intracellular development of *Leishmania* spp amastigotes in mononuclear phagocytes may be markedly affected by temperature. Berman and Neva (1981) reported that *L.tropica*, an agent of cutaneous leishmaniasis multiplied more rapidly within human macrophages maintained in culture at 35^{0} C than at 37^{0} C. In parallel cultures, *L.donovani*, the pathogen for visceral leishmaniasis multiplied equally well in the host cells at either temperature. The authors postulated that these contrasting temperature requirements for intracellular growth of the two species of *Leishmania* could be related to the different somatic localization of the lesions produced by the two agents.

1. 3 OBJECTIVES

1.3.1 General objective.

To characterize Kenyan isolates of *Leishmania* by determining their isoenzyme profiles and infectivity to mice.

1.3.2 Specific objectives.

a. To clone *L.major* (NLB 144) and *L.donovani* (NLB 065) strains, determine their infectivity to mice and their isoenzyme profiles.

b. To determine the intraspecific variations of *L.donovani* isolates from kala-azar patients from Baringo Machakos and Turkana Districts in Kenya by isoenzyme

electrophoresis.

c. To adapt *Leishmania major* strain (NLB 144), which is a cutaneous parasite into the viscera of mice by several intraperitoneal passages. Determine by isoenzyme electrophoresis for any difference in enzyme profiles of the parasites that have adapted to viscerotropism.

1.4 JUSTIFICATION.

The classification of *Leishmania* has been based primarily on the comparison of extrinsic characters. Only recently, attempts have been made to evaluate the intrinsic characters. In the present study, isoenzyme electrophoresis technique and infectivity to mice will be used to characterize the different *Leishmania* species

In the present study, cloning of *L.major* strain (NLB 144) and *L.donovani* strain (NLB 065) will be done by limiting dilution. The homogeneous or heterogeneous nature of these strains will be assessed by determining the infectivity to mice and isoenzyme profiles of the obtained clones. Presence of similar clones (homogenous strain) will indicate that only one type of vaccine can be produced for the treatment and control of leishmaniasis.

Le Blancq and Peters, (1986), using isoenzyme electrophoresis, reported that *L.donovani* sensu lato parasites from across the Old World were almost similar genetically despite their great geographical

extremities. In the present study, intraspecific variations of *L. donovani* isolates from kala-azar patients from Machakos, Baringo and Turkana Districts in Kenya will be determined by isoenzyme electrophoresis. This will help evaluate for any genetic differences of this parasite from different parts of the country. This is important in the treatment of visceral leishmaniasis because it has been found that some patients become unresponsive to conventional doses of antimonials after repeated relapses (Bryceson *et al*, 1985a).

Leishmania major is restricted to macrophages of the skin where the body temperature is lower than in the viscera. However, this parasite has been found to visceralize in Balb/c mice, (Trotter *et al*, 1980). Janovy (1970), reported that some enzymes of the cutaneous *L.mexicana* are more thermolabile at higher temperatures than the same enzymes in viscerotropic *L.donovani*. This could therefore explain the cutaneous or viscerotropic nature of these parasites.

In the present study, intraperitoneal passaging of *L.major* in Balb/c mice will be carried out to determine whether this parasite can actually visceralize without causing a lesion on the skin. If this is so, then it could be possible that the parasite can also cause visceral as well as cutaneous leishmaniasis. This will also demonstrate that some species of *Leishmania*

parasites are not restricted exclusively to the skin or viscera.

CHAPTER TWO

CLONING OF LEISHMANIA MAJOR AND LEISHMANIA DONOVANI STRAINS

2.1 Introduction

Biological, biochemical and immunological studies have shown the population heterogeneity of clones isolated from parasites of the genera *Trypanosoma* and *Leishmania* (Morel *et al*, 1980; Handman *et al*, 1983; Bongertz and Dvorak 1983; Shurkhal *et al*, 1986). A potentially powerful approach to the study of the genetic basis of parasite virulence in leishmaniasis is the isolation of mutants of *Leishmania* species causing different disease types and the correlation of clinical manifestation with biochemical changes in these mutants. The possibility of this approach was suggested by the observation that promastigotes of *L. tropica* lose infectivity for otherwise susceptible mice upon prolonged cultivation *in vitro*. Prompted by this observation, Handman *et al*, (1983) cloned an isolate of *L. major* (LRC- L137) by limiting dilution that is infective to Balb/c mice. Seven stable clones were obtained only one of which was infective to Balb/c mice. Three of the non-infective clones that were examined for survival in Balb/c macrophages *in vitro* seemed to be killed more readily suggesting failure to establish in macrophages as the basis for

non infectivity *in vivo*.

However, all the seven clones were distributed in two closely related schizodemes. Therefore further characterization of these clones of *L. major* should allow a better understanding of the genetic basis of parasite virulence in cutaneous leishmaniasis.

Cell cloning techniques and schizodeme analysis have been used to detect mixtures of sub-populations in *Leishmania* parasites isolated from humans and a reservoir host. Presence of different schizodemes in one isolate from a sylvatic animal suggested the possibility of a mixed natural infection.

In the present study, cell cloning experiments of *Leishmania major* strain IPHL/KE/83=LRC-L447= NLB144 and *Leishmania donovani* strain MHOM/KE/82=LRC-L445= NLB065 will be carried out. The obtained clones will be characterized by their infectivity in mice and isoenzyme profiles to determine strain differences in the population in these parasites.

2.2 Materials and Methods

2.2.1 Cloning by limiting dilution

Nicole, Ncneal, Novy (NNN) medium was prepared with 7.5% defibrinated rabbit blood and 1 ml pipetted into each of the ambitubes under sterile conditions. This was allowed to solidify and stored at $4^{o}C$ until needed. *Leishmania major* promastigotes (NLB 144) and *Leishmania donovani* promastigotes (NLB 065) were

cultivated in ambitubes containing NNN medium with an overlay of 1 ml Schneider's Drosophila medium supplemented with 20% heat inactivated foetal bovine serum. These were allowed to multiply at 25^{o}C until they reached the late logarithmic phase. The parasites in the parent stock were counted using a haemocytometer and a parasite concentration of 1.2 x10^{6} parasites /ml was obtained. This was diluted using Schneider's medium to 15 parasites /ml. A drop of this diluted isolate was then transferred into the centre of the well of the cavity slide using a 1ml tuberculin syringe with 26 gauge needle. The drop was covered with a cover slip and observed under a microscope for the presence of only one parasite. Any drop with more than one parasite was discarded. The drop with one *Leishmania* promastigote was then transferred under sterile conditions into an ambitube containing NNN medium with an overlay of Schneider's supplemented with 20% heat inactivated foetal bovine serum, 250 ug/ml streptomycin, 250 u/ml penicillin and 500 ug/ml 5-fluorocytosine. The cultures were then incubated at 25^{o}C and examined daily for the growth of the promastigotes. The parasites were allowed to grow to late log phase for infecting Balb/c mice and preparing lysates for electrophoresis.

2.2.2 **Infectivity of *L. major* clones in Balb/c mice**

Eight clones of *L. major* were obtained. Four clones which were doing well in culture were selected for infecting Balb/c mice and designated LM-1, LM-2, LM-3, and LM-4 . The parasites were counted using a haemocytometer and a parasite concentration 1 x 10^7 parasites per ml was used. Each clone was inoculated subcutaneously on the nasal area into a group of 7 clean Balb/c mice using 1ml tuberculin syringe and 26 gauge needle. Each mouse in all the 4 groups was marked with picric acid for identification. Lesion development and progression was observed weekly for 12 weeks. After inoculating the mice, the remaining parasites for each clone were cultivated in Schneider's medium supplemented with 20% heat inactivated foetal bovine serum for electrophoresis.

2.2.3 **Infectivity of *L. donovani* clones in mice**

Nine clones of *L. donovani* were obtained. Four clones which were doing well in culture were selected for infecting Balb/c mice and designated LD-1, LD-2, LD-3 and LD-4. The parasites were counted using a haemocytometer. A parasite concentration of 1 x 10^7 parasites/ ml for each clone was used to inoculate intraperitoneally a group of 10 clean Balb/c mice. Mass cultivation of the remaining parasites after

inoculating the mice was carried out for each clone in Schneiders medium supplemented with 20% heat inactivated foetal bovine serum for electrophoresis.

2.2.4 **Examination of mice infected with** *L.major.* **clones**

The mice in each group were observed daily for lesion development. Once the lesions developed, their sizes were evaluated by measuring the length and width with the aid of calibrated calipers at one week interval for 8 weeks. Lesion area was calculated using the formular $\pi r_1 r_2$ as described by Wilson *et al*, (1979). One mouse from each group was sacrificed at the end of the 8 weeks and sections of liver and spleen cultured in Schneider's medium supplemented with 20% foetal bovine serum. The cultures were incubated at $25^{o}C$ and observed daily for presence of promastigotes.

2.2.5 **Mice inoculated with** *L. donovani* **clones**

Three mice from each group were sacrificed 63 days postinoculation. The liver and spleen were examined for signs of enlargement. Sections of the liver and spleen were then cultured in NNN medium with an overlay of Schneider's medium supplemented with 20% foetal bovine serum. Impression smears of the liver and spleen were made on clean microscope

slides. These were fixed with absolute methanol and stained with 10% giemsa. The smears were examined for the presence of amastigotes to determine the parasite load (Stauber, 1966).

2.2.6 **Mass cultivation of the various clones**

Mass cultivation of the 4 clones of *L. major* and 4 clones of *L. donovani* was carried out using Schneider,s Drosophila medium, supplemented with 20% foetal bovine serum in 75 cm^2 tissue culture flasks. The parasites were allowed to grow to late log phase with more media being added gradually until sufficient number of organisms were obtained.

2.2.6.1 **Preparation of the samples for electrophoresis**

After mass cultivation, the promastigotes were harvested in the late log phase by centrifugation. The promastigotes were centrifuged at 3600 RPM for 20 minutes at 4^{o} C in 50 ml centrifuge tubes. The supernatant was discarded and the pelleted organisms resuspended in about 5 ml of PBS. This was transferred into 15 ml centrifuge tube and centrifuged as before. The resulting pellet was again resuspended in 1 ml PBS and transferred into a 2 ml plastic centrifuge tube (nunc tube) and centrifuged as before. The supernatant was then

carefully removed using a finely drawn out Pasteur pipette. One drop of distilled water was added to the resulting lysate and mixed thoroughly using a vortex to ensure complete rupturing of the organism. This was stored in liquid nitrogen until needed.

2.2.6.2 **Extraction of water soluble enzymes**

The method of freeze-thaw lysis was used. The lysates were removed from the liquid nitrogen, allowed to thaw and then mixed using a vortex. Then they were frozen in liquid nitrogen in a container. This freeze-thaw method was repeated 3 times for each lysate.

2.2.6.3 **Making beads in liquid nitrogen**

The lysates were kept in ice so as to avoid denaturation of the enzymes. Liquid nitrogen was collected in a plastic container. Using a plastic beaker, small amounts of liquid nitrogen were poured into a small shallow wooden box. The lysate to be beaded was allowed to thaw and using thinly drawn out Pasteur pipette, this was withdrawn from the nunc tube and small drops allowed to fall sequentially into liqud nitrogen. As each drop entered the liquid nitrogen a solid bead formed. The formed beads were transferred quickly into pre-cooled labelled nunc tubes. The tubes were capped and transferred in

liquid nitrogen until needed for electrophoresis.

2.2.6.4: Cellulose acetate electrophoresis

Electrophoresis equipment was from Helena laboratories, Beaumont, Texas, USA.

Titan III cellulose acetate membrane (CAM) was first labeled with enzyme initials on the shiny side with a permanent marker and then pre-soaked for 5-20 minutes in soaking buffer. After removing the CAM from the soaking buffer, it was blotted once firmly using Titan blotting pads. The GAM was then aligned on the super Z^{R} alignment base. Small aliquots of the different isolates to be examined were put in a microtiter plate using a microdispenser. 0.25 ul of the aliquots were then transferred from the microtiter plate to the membrane (CAM) with a super Z^{R} applicator.

Each membrane was then placed on the pre-soaked paper wicks in the Zip Zone^{R} chamber containing soaking buffer. For maximum contact with the paper wicks, two clean glass slides were placed on top of the shiny side of the CAM. The chamber was then covered with a lid and connected to a power supply. The time of running the electrophoresis depended on the enzyme. Some enzymes like GP1 and ME migrate very fast and require a shorter time. Others like MDH, isocitrate dehydrogenase (ICD) and nucleoside

hydrolase (NH) require a longer time.

Preparation of the buffers (Table 1) staining components and noble agar were adopted from those of Kreutzer & Christensen (1980), Kreutzer *et al*, (1983).

Table 1.

Enzyme systems used in characterization of *Leishmania* isolates.

Enzyme	Abbrev	Ref No	Tank buffer	Staining buffer	Running time(min)
Malate dehydrogenase	MDH	Ec 1.1.1.37	5	A	30
Malic enzyme	ME	Ec 1.1.1.40	6	C	20
Isocitrate dehydrogenase	ICD	Ec 1.1.1.42	3	C	30
Glucose 6-phosphate dehydrogenase	G6PD	Ec 1.1.1.49	6	C	20
6-Phosphogloconate dehydrogenase	6-PGD	Ec 1.1.1.44	1	C	20
Nucleoside hydrolase	NH	Ec 3.2.2.2	3	B	30
Mannose phosphate isomerase	MPI	Ec 5.3.1.9	4	C	20
Glucose phosphate isomerase	GPI	Ec 5.3.1.9	2	A	20
Phosphoglucomutase	PGM	Ec 5.4.2.2	2	A	20

Footnote for Table 1

BUFFER SYSTEMS USED IN CELLULOSE ACETATE ELECTROPHORESIS

Tank buffers.

BUFFER 1. 0.1M Tris/0.0176M maleic acid/0.01M Na_2EDTA/0.01M magnesium acetate, PH 7.4, diluted 1:4 with distilled water.

BUFFER 2. 0.1M Tris/0.0176M maleic acid/0.01M Na_2EDTA/0.01M magnesium acetate, adjusted with NaOH, then diluted 1:7 with distilled water.

BUFFER 3. 0.008M Na_2PO_4/0.192 Na_2PO_4, PH 8.0 diluted 3:37 with distilled water.

BUFFER 4. 0.2M KH_2PO_4 adjusted to PH 7.0 with KOH, then diluted 3:37 with distilled water.

BUFFER 5. Tris barbital/Sodium barbital, PH 9.0, I= 0.075, 1mM magnesium acetate.

BUFFER 6. Tris barbital/ Sodium barbital, PH 9.0, I= 0.02, 1mM magnesium acetate, diluted 1:4 with distilled water.

Staining buffers.

BUFFER A. 0.3M Tris/HCL, PH 8.0

BUFFER B. 0.3M Tris/HCL, PH 7.0

BUFFER C. 0.3M Tris/HCL, PH 7.4

Plate 1a. Balb/c mouse inoculated subcutaneously on the nosal area with *L. major* clone -1.

Plate 1b. Balb/c mouse inoculated subcutaneously on the nosal area with *L. major* clone -2.

Plate 1c. Balb/c mouse inoculated subcutaneously on the nosal area with *L. major* clone -3.

Plate 1d. Balb/c mouse inoculated subcutaneously on the nosal area with *L. major* clone -4.

2.3 RESULTS

2.3.1 Infectivity of *L. major* clones in Balb/c mice.

2.3.1.1 Mice inoculated with LM-1

The mice developed small swellings with hair loss 28 days post inoculation. This was followed later by ulceration of the lesion and crust formation. The lesion size increased rapidly. The lesions were dry in the beginning with a thick crust but they later became wet with a lot of pus oozing from the sides. The crust was later lost in some mice leaving a deep crater-like ulcer. By day 95 postinoculation (PI), all the mice which had crater like ulcers developed a thick crust in that area. The lesions looked dry and the size increased throughout the 8 weeks of lesion measurement (Table 2). Maximum size of the lesions ranged from 73.86 mm^2 and 88.20 mm^2 and the pathology was similar in all the mice.

All the mice looked very weak at the end of the experiment. One mouse was sacrificed and sections of liver and spleen cultured in NNN medium with an overlay of Schneiders medium supplemented with 20% foetal bovine serum. The spleen culture was positive for *Leishmania* promastigotes within 4 days. However, culture of the liver remained negative for 14 days and was discarded. The spleen was also slightly enlarged.

Table 2

Lesion sizes (mm^2) of Balb/c mice inoculated subcutaneously on the nasal area with *L. major* clone LM-1

Mouse No	Weeks post-lesion development							
	1	2	3	4	5	6	7	8
1	28.73	38.47	45.34	56.05	70.85	65.00	76.94	80.08
2	29.69	39.02	54.73	62.88	72.35	81.67	86.55	77.72
3	23.32	45.78	54.08	58.74	69.36	74.62	76.16	79.29
4	20.02	42.99	48.99	51.50	67.17	75.39	71.59	73.86
5	16.97	34.71	44.75	52.78	58.06	70.10	79.29	84.09
6	21.23	41.83	55.39	61.48	67.89	67.89	73.86	74.62
7	16.97	36.83	42.41	56.72	62.18	70.85	81.67	88.20
Mean lesion sizes (mm^2)	22.42	39.95	49.38	57.16	66.84	72.22	78.00	79.69
Standard error (±) of mean	1.95	1.44	2.16	1.59	1.91	2.08	1.89	1.92

Table 3

Lesion sizes (mm^{2}) of Balb/c mice inoculated subcutaneously on the nasal area with *L.major* clone LM-2.

Mouse No	Weeks post-lesion development							
	1	2	3	4	5	6	7	8
1	23.75	28.26	35.77	37.37	44.75	31.16	27.79	23.32
2	17.34	26.41	35.24	36.83	48.37	47.15	45.34	40.13
3	24.62	30.19	48.99	51.50	48.99	47.76	45.34	32.15
4	20.42	27.79	40.69	45.34	47.76	39.57	37.37	32.15
5	25.50	25.06	46.54	50.24	54.73	49.17	42.41	41.83
6	25.50	33.68	39.57	41.83	58.74	47.76	46.54	42.41
Mean lesion sizes (mm^{2})	22.86	28.56	41.13	43.85	50.55	43.76	40.80	35.33
Standard error (±) of mean	1.35	1.24	2.29	2.56	2.11	2.88	2.93	3.05

2.3.1.2 **Mice inoculated with LM -2**

All the seven Balb/c mice in the group developed small nodules with slight hair loss at the site of inoculation, four weeks PI. The lesion sizes increased fairly rapidly with increased hair loss. The lesions later became severe ulcerating lesions with little swelling. By the fourth week of lesion measurement, the lesions in all the mice showed extensive ulceration and later crater-like ulcers were formed. The lesions were open and dry. They reached maximum size by the fifth week of lesion development and then the lesion started resolving. Maximum lesion sizes of the seven mice ranged from 44.75 mm^2 to 58.74 mm^2 (Table 3). All the mice looked healthy during the 12 weeks of observation.

One mouse was sacrificed at the end of 12 weeks and sections of liver and spleen cultured to detect for presence of visceralizing parasites. Culture of the spleen was positive for promastigotes within 6 days. Culture of the liver remained negative for 14 days and was discarded.

2.3.1.3 **Mice inoculated with LM -3**

All the mice developed large nodules and hair loss at the site of inoculation four weeks PI. The nodule size increased very rapidly and became severe ulcerating lesions. The ulceration was so gross that

Table 4

Lesion sizes (mm^2) of Balb/c mice inoculated subcutaneously on the nasal area *L. major* with LM-3

Mouse No	Weeks post-lesion development							
	1	2	3	4	5	6	7	8
1	34.19	60.79	67.89	83.28	86.55	94.99	93.27	94.99
2	41.26	51.50	67.17	79.29	77.72	80.08	84.09	91.56
3	39.57	52.14	60.79	72.35	73.10	80.87	70.85	78.50
4	28.73	49.61	58.14	70.10	74.62	82.47	70.10	84.09
5	42.99	53.43	65.72	80.87	81.67	68.63	78.50	-
6	30.18	35.77	47.15	61.48	62.88	67.17	82.47	83.28
7	40.69	48.37	60.10	66.44	68.63	79.29	78.50	87.37
Mean lesion sizes (mm^2)	36.80	50.23	61.08	73.40	75.02	79.07	79.68	86.63
Standard error (±) of mean.	2.16	2.83	2.69	3.05	2.99	3.52	3.03	2.43

it ate up the whole of the nasal area and down the sides of the mouth. The lesions in all the mice ulcerated by the sixth week PI and they all had a thick crust.

The lesions later became wet on the sides with a lot of pus oozing from under the thick crust. For those mice whose eyelids were reached by the ulceration, the eyes looked wet and could barely see. The lesion sizes increased throughout the 8 weeks of lesion measurement. They reached maximum size of between 78.5 mm^2 and 94.99 mm^2 (Table 4). One mouse died 91 days P1 and the remaining died by 103 days P1. Culture of sections of the spleen were positive for promastigotes within 4 days. The spleen was enlarged. Culture of the liver remained negative and was discarded.

2.3.1.4 **Mice inoculated with LM -4**

All the 7 mice in the group developed nodules and hair loss at the site of inoculation four weeks PI The nodule size increased fairly rapidly with increased hairloss and swelling until the fifth week of lesion measurement, when the lesion size started resolving, (Table 5).

Table 5

Lesion sizes (mm^2) of Balb/c mice inoculated subcutaneously on the nasal area with *L.major* LM-4.

Mouse No	Weeks post-lesion development							
	1	2	3	4	5	6	7	8
1	17.34	42.41	48.99	50.87	54.10	50.87	48.99	46.54
2	25.50	31.65	32.15	42.99	53.43	55.39	52.14	48.37
3	20.42	28.26	35.77	40.13	42.99	46.54	40.69	40.13
4	18.85	29.69	35.77	41.83	44.16	45.34	41.26	36.30
5	25.50	40.13	42.96	45.34	48.99	52.14	39.02	37.92
6	17.34	41.83	47.76	54.10	60.10	61.48	52.78	35.77
7	19.63	43.57	46.54	53.43	56.05	58.10	52.78	48.99
Mean lesion sizes (mm^2)	20.65	36.79	41.42	46.96	51.40	52.84	46.81	42.00
Standard error (±) of mean.	1.32	2.51	2.56	2.18	2.37	2.23	2.36	2.2

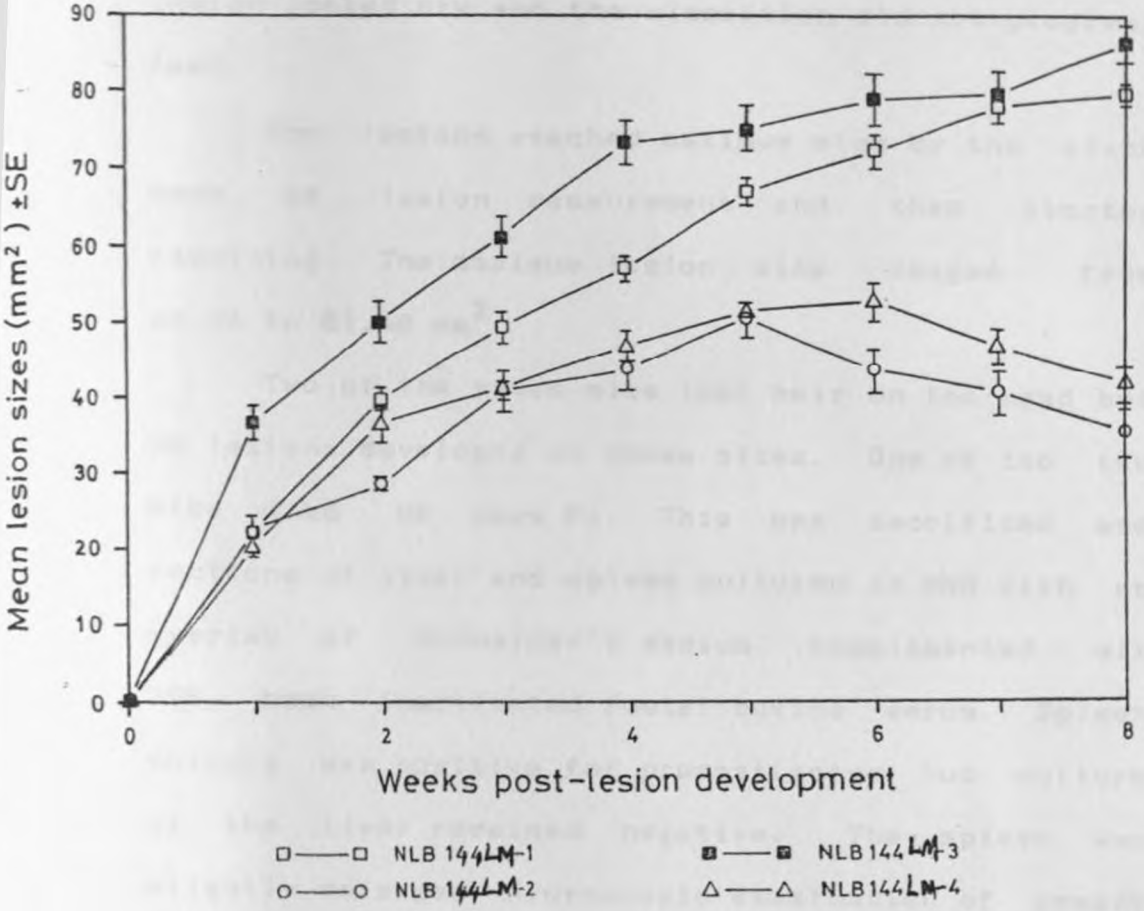


Fig. 1. Virulence of L. major clones in the nasal area of Balb/c mice.

The swollen lesion looked erythematous and did not ulcerate during the 8 weeks of lesion measurement except for one mouse where ulceration occurred and this was followed by crust formation. However, the lesion looked dry and the ulceration did not progress fast.

The lesions reached maximum size by the sixth week of lesion measurement and then started resolving. The maximum lesion size ranged from 45.34 to 61.48 mm^2.

Two of the seven mice lost hair on the head but no lesions developed on those sites. One of the two mice died 86 days P1. This was sacrificed and sections of liver and spleen cultured in NNN with an overlay of Schneider's medium supplemented with 20% heat inactivated foetal bovine serum. Spleen culture was positive for promastigotes but culture of the liver remained negative. The spleen was slightly enlarged. Microscopic examination of smears of liver and spleen were negative for amastigotes.

2.3.1.5 **Isoenzyme profiles of the four clones of** *L. major*

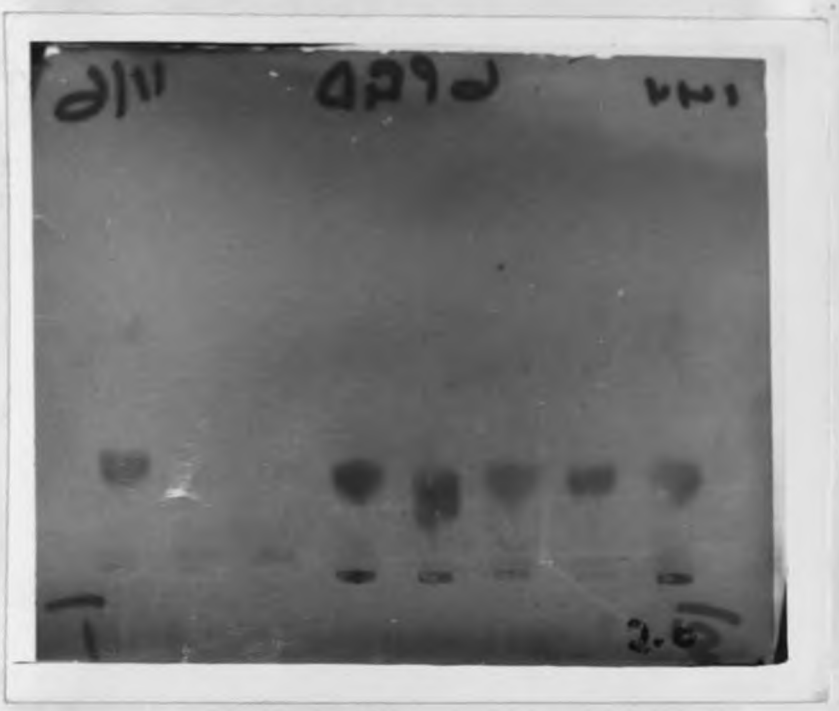
All the four clones of *L. major*, LM-1, LM-2, LM-3 and LM-4 showed similar isoenzyme profiles for

all the nine enzymes assayed. Isoenzyme banding of the clones was compared among themselves and with a *L. major* reference strain. All the clones showed no enzyme polymorphism for all the nine enzymes except in malate dehydrogenase. (Plates 2a-f).

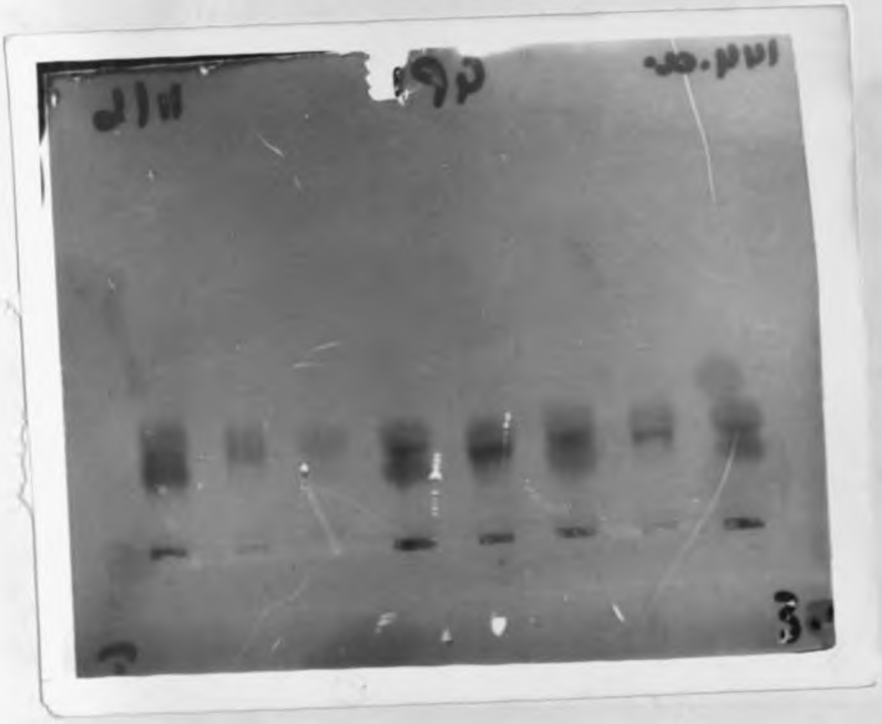
Plates 2a-f. Isoenzyme migration patterns of *L. major* clones with those of reference strains.

Table 6. Isolates number 1-8 in Plates 2a-f and Figures 2a-b.

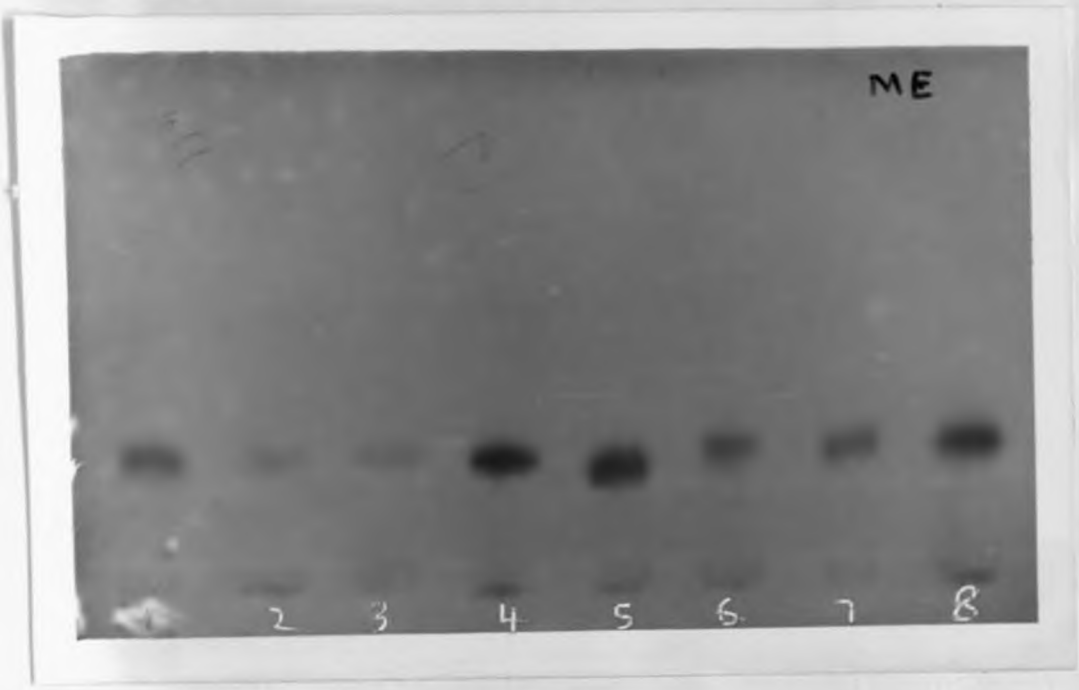
1. NLB-144 (reference strain).
2. NLB-144 LM-1
3. NLB-144 LM-2
4. NLB-314 (reference strain).
5. NLB-144 LM-3
6. NLB-144
7. NLB-144 LM-4
8. NLB-314



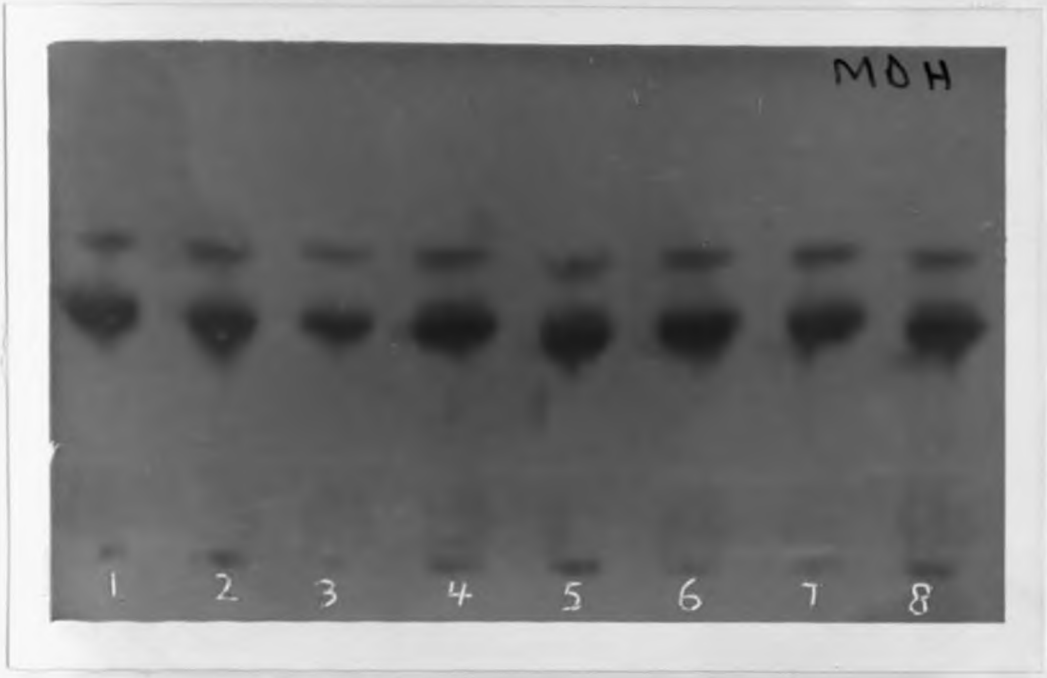
(2a) Zymogram pattern for 6PGD enzyme.



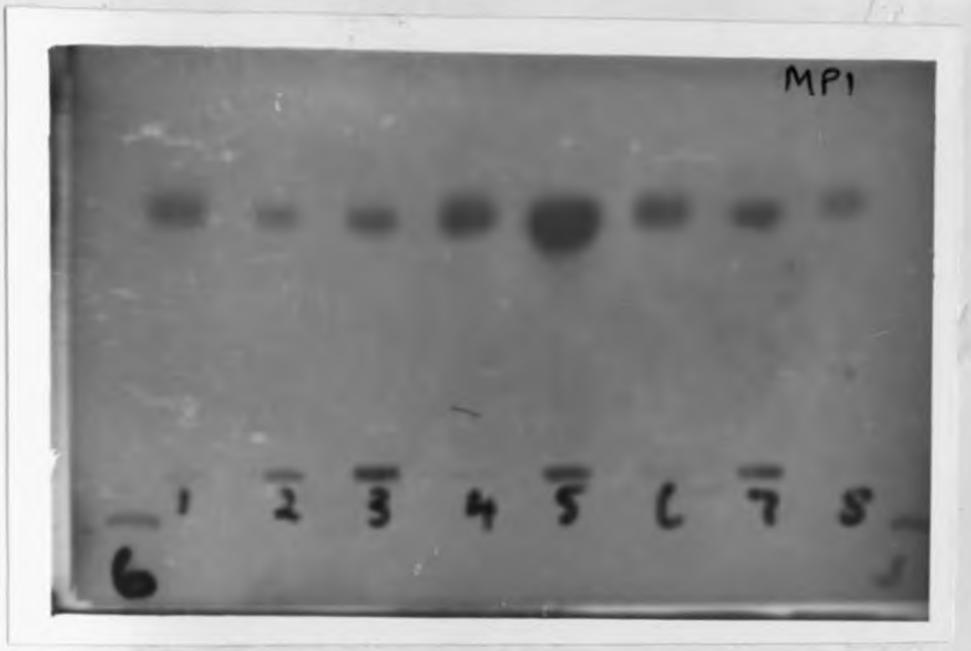
(2b). Zymogram pattern for GPI.



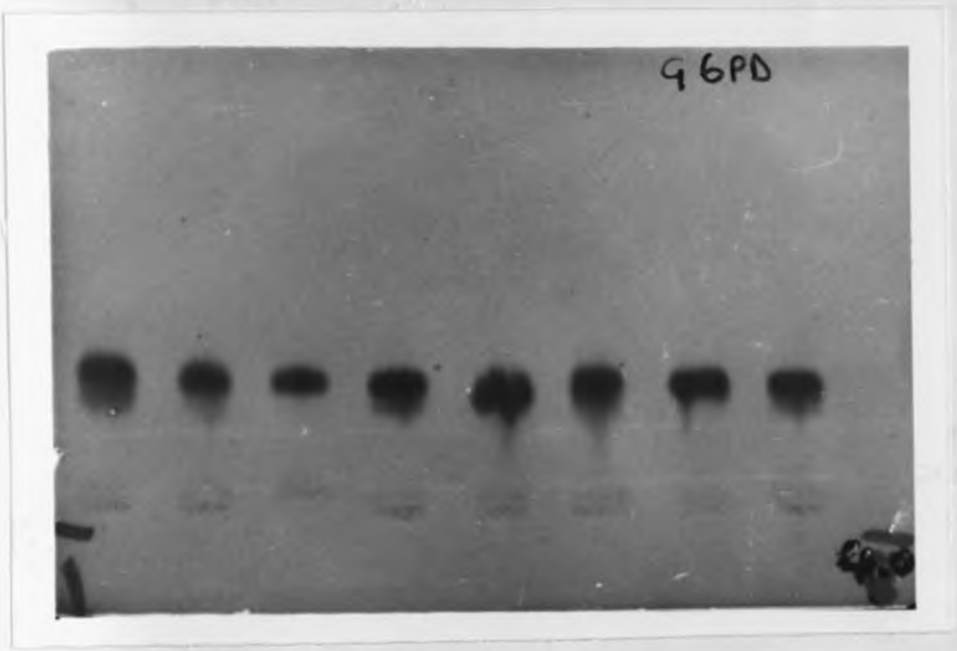
(2c). Zymogram pattern for ME.



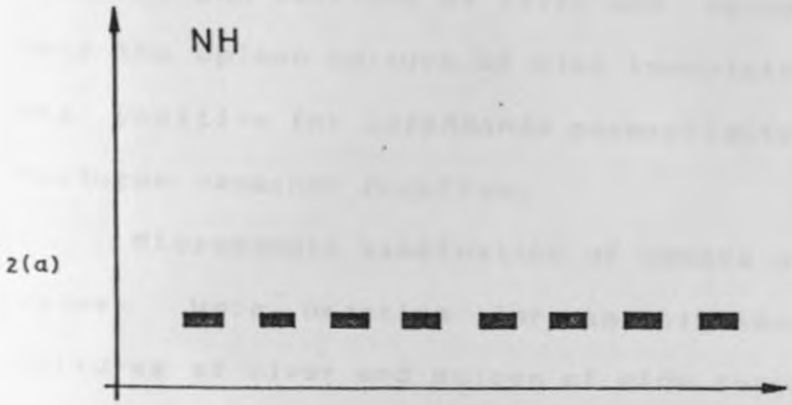
(2d).Zymogram pattern for MDH.



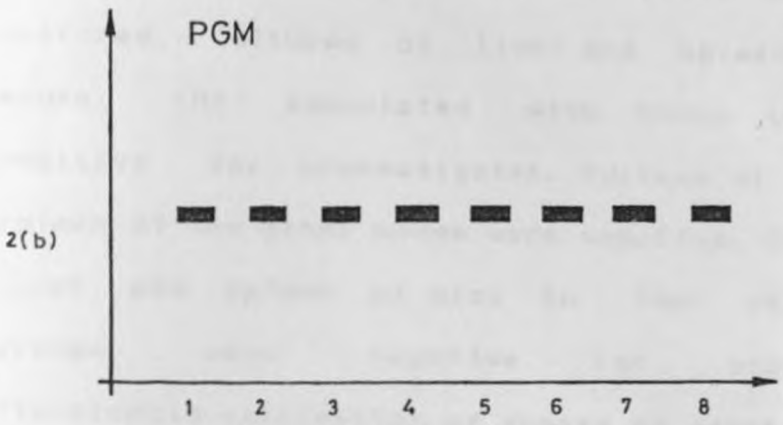
(2e). Zymogram pattern for MPI.



(2f). Zymogram pattern for G6PD.



2(a)



2(b)

Fig. 2a - b Diagramatic representations of zymogram patterns for enzymes NH and PGM from 4 L. major clones and two markers (L. major)

2.3.2 Infectivity of *L. donovani* clones in Balb/c mice

Three mice were sacrificed from each group at 63 days PI and sections of liver and spleen cultured. Only the spleen culture of mice inoculated with LD-4 was positive for *Leishmania* promastigotes . Liver cultures remained negative.

Microscopic examination of smears of liver and spleen were negative for amastigotes. However, cultures of liver and spleen of mice inoculated with clones LD-1, LD-2 and LD-3 were negative for promastigotes. Microscopic examination of smears of liver and spleen were negative for amastigotes.

Two mice were sacrificed from each group at 91 days PI and sections of liver and spleen cultured. Cultures of liver and spleen of one mouse, (M4) inoculated with clone LD-4 were positive for promastigotes. Culture of liver and spleen of the other mouse were negative. Cultures of liver and spleen of mice in the other three groups were negative for promastigotes. Microscopic examination of smears of liver and spleen were negative for amastigotes in all the four groups of mice.

Two mice were sacrificed from each group at 119 days PI. Cultures of sections of liver and spleen of mice inoculated with clones LD-1, LD-2 and LD-3 were

negative for promastigotes. However, in mice inoculated with LD-4 the spleen was slightly enlarged. Cultures of liver and spleen of one mouse, (M8) were positive for promastigotes. However, in the other mouse (M7), only the spleen culture was positive.

Microscopic examination of smears of liver and spleen were negative for amastigotes for all the groups.

Three mice were sacrificed from each group at 147 days PI. Mice inoculated with CL-4 had extremely enlarged spleens. Cultures of the spleen were positive for promastigotes within 4 days. Cultures of the liver were also positive.

Spleen and liver cultures of mice inoculated with the other clones were negative for promastigotes. Microscopic examination of smears of liver and spleen were negative for amastigotes. However, all the mice in the 4 groups looked healthy and active during the 147 days of observation.

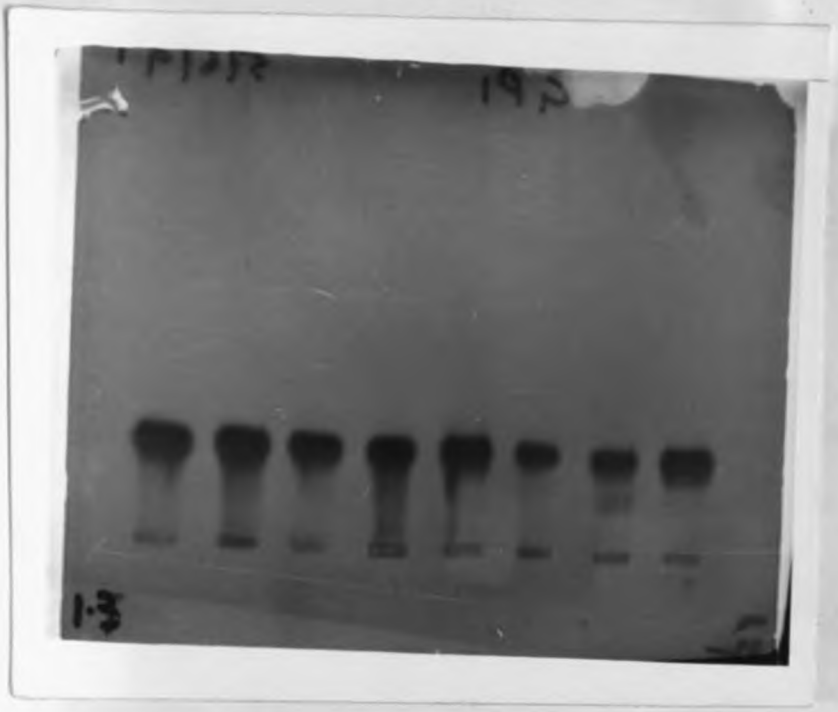
2.3.3 **Enzyme profiles of** *L. donovani* **clones**

All the four clones of *L. donovani*, that is, LD-1, LD-2, LD -3 and LD-4 showed isoenzyme profiles which were similar to the *L. donovani* reference strain. Enzyme variation was only seen in MDH enzyme in all the 4 clones and the reference strain (Plates 3a-f).

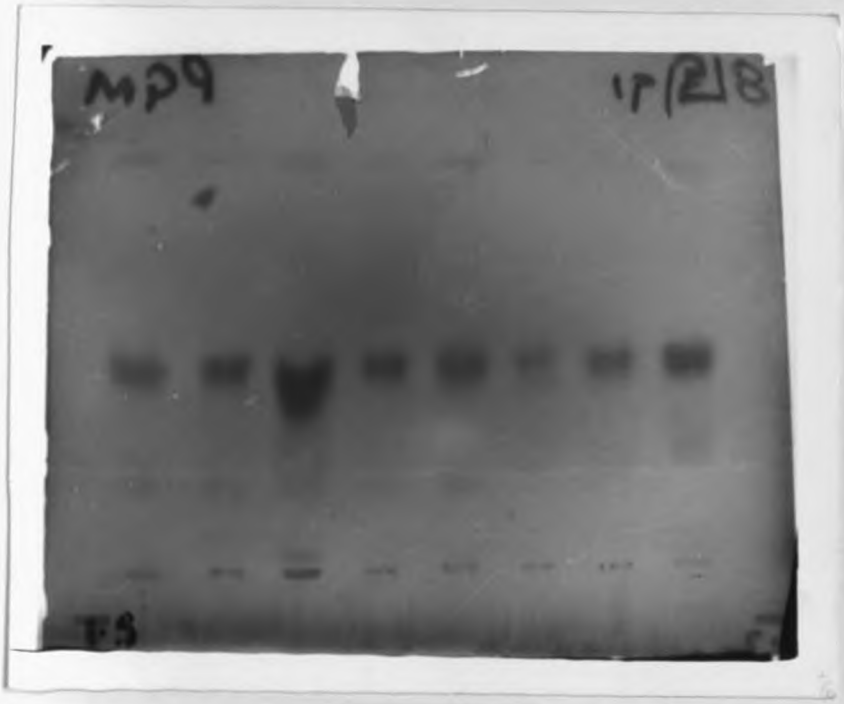
Plates 3a-f. Isoenzyme migration patterns of *L.donovani* clones with those of reference strains

Table 7. Isolates number 1-8 in Plates 3a-e and Figures 3a-b.

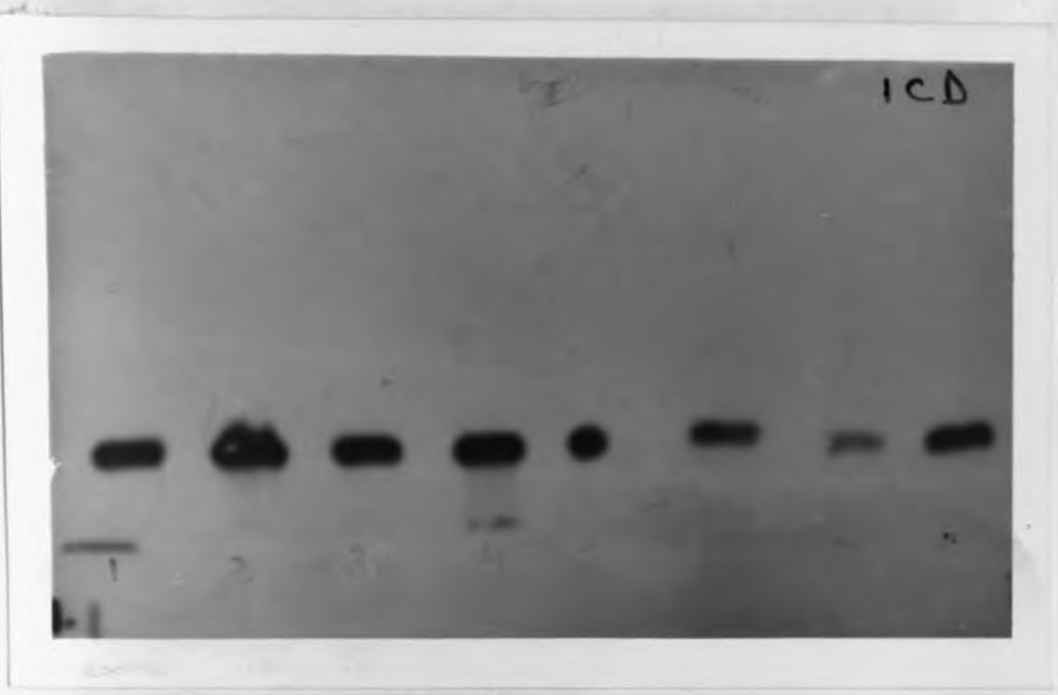
1. NLB-065 (reference strain).
2. NLB-065 LD-1
3. NLB-065 LD-2
4. NLB-061 (reference strain).
5. NLB-065 LD-3
6. NLB-065
7. NLB-065 LD-4
8. NLB-061



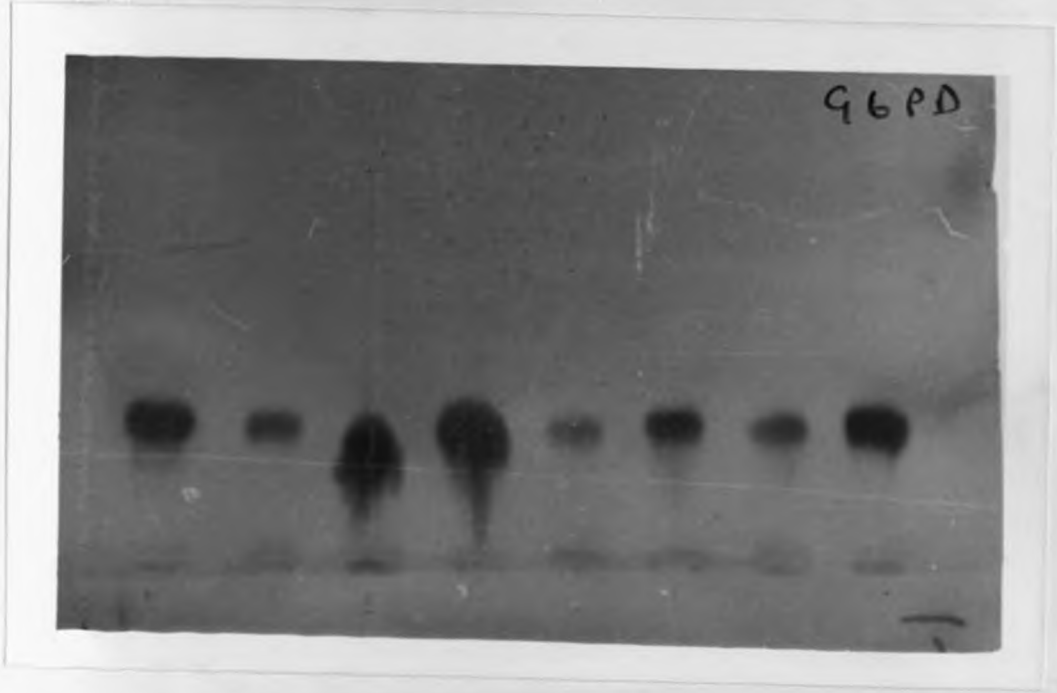
(3a). Zymogram pattern for GPI.



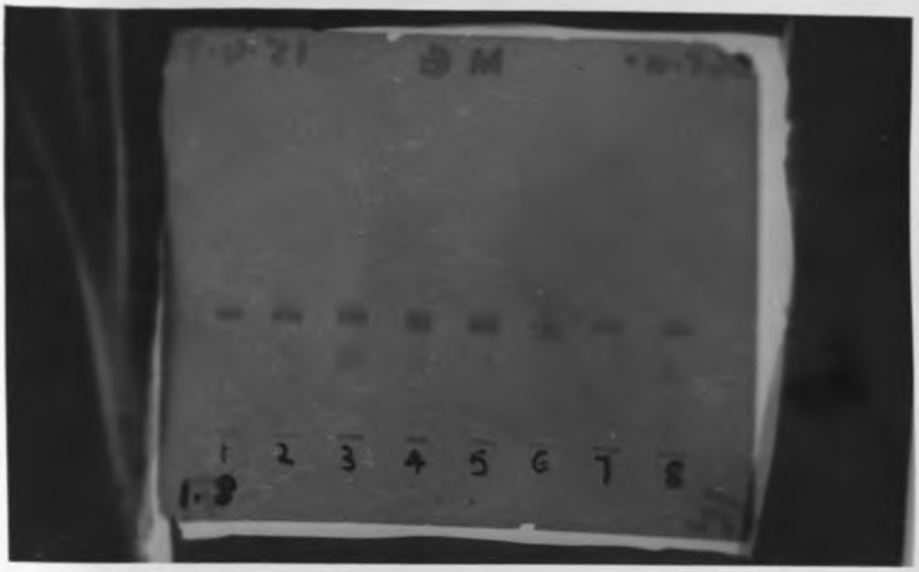
(3b). Zymogram pattern for PGM.



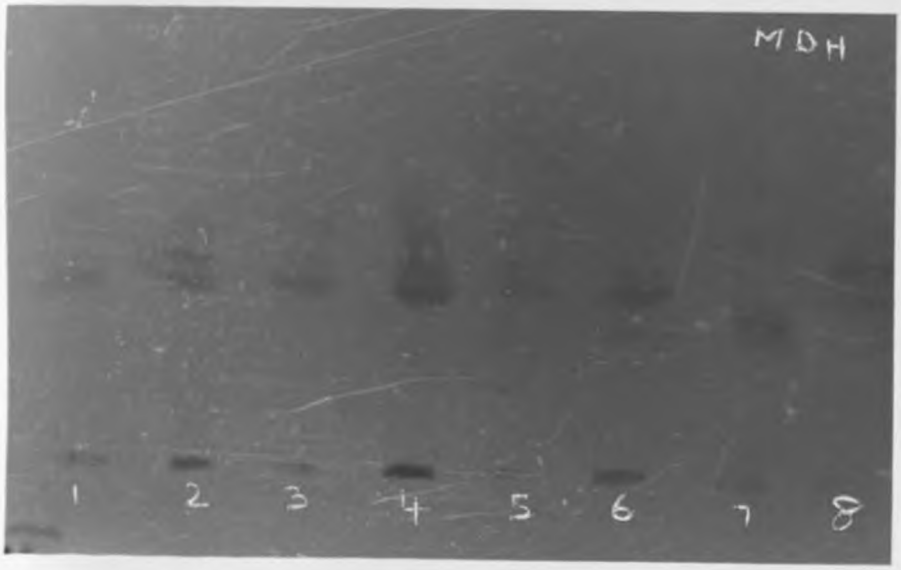
(3c). Zymogram pattern for ICD.



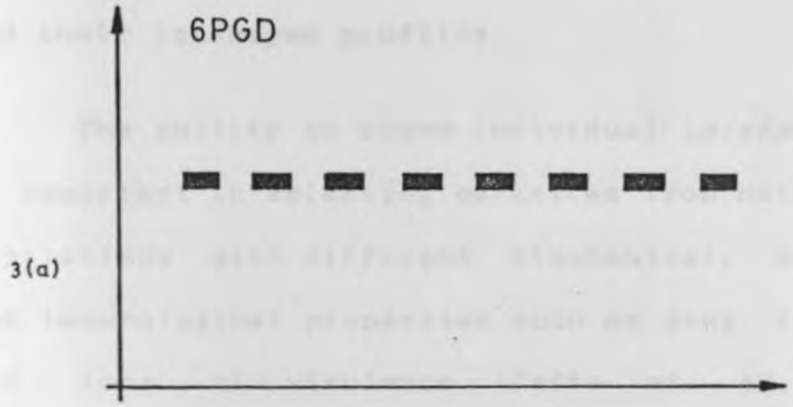
(3d). Zymogram pattern for G6PD



(3e). Zymogram pattern for ME.



(3f) Zymogram pattern for MDH



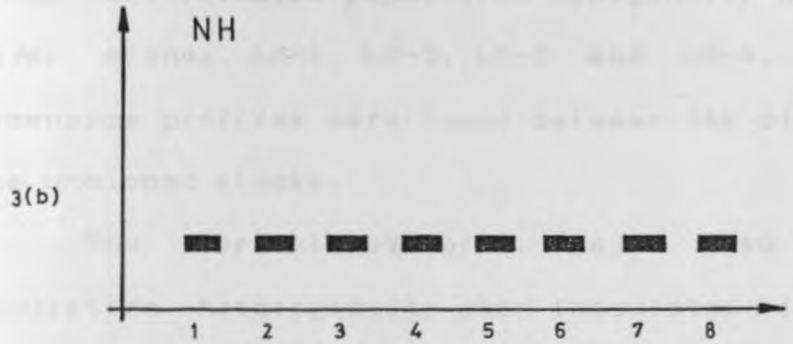


Fig. 3a - b Diagramatic representations of zymogram patterns for enzyme 6PGD and NH from 4 clones of L. donovani and two L. donovani markers

2.4 DISCUSSION

Virulence of *L. major* and *L. donovani* clones in mice and their isoenzyme profiles.

The ability to clone individual *Leishmania* can be important in selecting parasites from heterogenous populations with different biochemical, biological and immunological properties such as drug resistance and loss of virulence (Jaffe *et al*, 1984). Considerable work on cloning has been done with *Trypanosoma cruzi* and several other *Trypanosomatidae* (Keppel and Janovy, 1980, Tanuri *et al*, 1981).

Preliminary experiments carried out in this study have revealed population homogeneity of the *L. major* clones LM-1, LM-2, LM-3 and LM-4. Similar isoenzyme profiles were found between the cloned and the uncloned stocks.

The four clones of *L. major* also revealed population heterogeneity when inoculated in Balb/c mice. The clones, although distributed in the same zymodeme with uncloned parent strain showed a difference in infectivity to mice. The most virulent clone was LM-3. However, in all the four groups of mice, visceralization of the parasite took place and this terminated in the death of the rodents. Mice inoculated with the most virulent clones died earlier than those inoculated with milder clones.

This study may suggest that the pathological outcome of *L. major* clones in inbred Balb/c mice is clone-specific. This may imply that specific and restricted host-parasite interactions occur. These results may suggest that the degree of visceralization of the parasite and the time taken for the rodent to die is determined by the virulence of the given parasite.

Similar results were seen using *L. donovani* clones. The four clones LD-1, ID-2, LD-3 and LD-4 showed similar isoenzyme banding pattern with the uncloned stocks, therefore revealing population homogeneity. However, they showed a difference in infectivity to mice. Out of the four clones, only one, (LD-4) was infective to Balb/c mice. This was confirmed by finding promastigotes in cultures of liver and spleen. Cultures of liver and spleen of mice from the other three groups were negative for promastigotes.

The infectivity of LD-4 in Balb/c mice was not uniform although they all received the same concentration of parasites (1×10^6/ ml). One mouse from the group (M5) did not take up the infection. Both the cultures of liver and spleen were negative for promastigotes. In another mouse (M7), only the spleen culture was positive.

This difference in infectivity could be attributed to parasite-host relationship.

Handman *et al*, (1983) using an isolate of *L. major* (LRC-L137) also obtained clones which distributed in two very closely related schizodemes and a difference in infectivity for mice. Similar results have been obtained using clones of *T. cruzi* (Postan *et al*, 1987; Bongertz and Dvorak, 1983).

The results of isoenzyme profiles of *L. major* clones and *L. donovani* clones in this study and their infectivity in mice suggests that isoenzyme electrophoresis technique cannot be used alone to determine the genetic basis of parasite virulence in both cutaneous and visceral leishmaniasis. This emphasizes the need to use more than one technique in determining genetic differences in *Leishmania* parasites.

CHAPTER THREE

ISOENZYME ELECTROPHORESIS OF LEISHMANIA DONOVANI ISOLATES FROM BARINGO, TURKANA AND MACHAKOS DISTRICTS.

3.1 Introduction

Leishmania donovani is a viscerotropic protozoan parasite of canids, certain rodents and man. Infection may be cryptic, although in man, a specturm of diseases are observed ranging from visceral leishmaniasis to post-kala-azar dermal leishmanisis (PKDL).

The ability of this parasite to cause both visceral and PKDL leishmaniasis makes its classification difficult using extrinsic characters alone such as epidemiological features or clinical symptoms of the disease. This therefore emphasizes the need to use other techniques to evaluate the intrinsic characters.

Characterization of *L. donovani* has been carried out by Le Blancq and Peters (1986) using isoenzyme electrophoresis. Results have shown enzyme homogeneity of *L. donovani* isolates from the Old World. Studies using other characterization techniques have also shown homogeneity (Schotelius 1982, Jaffe *et al*, 1984).

The attempts to subdivide the classical viscerotropic *Leishmania* originate in the early

studies (Cestallni & Chalmers, 1919), before either the life-cycle or the geographical range of the parasite was known. Laveran & Mesnil (1903), described the parasite causing visceral leishmaniasis in adults in India as *L. donovani* sensu stricto. Nicolle (1908) describes the parasite causing visceral leishmaniasis in children in the Mediterranean region as *L. infantum*. However, work by Le Blancq and Peters (1986) using isolates from the Old World did not indicate divisions of these parasites into separate species. Serological work by Bray (1973) has also indicated that the visceral parasites causing visceral leishmaniasis in humans causes the subsequent post- kala-azar.

In the present study, isoenzyme electrophoresis of *Leishmania donovani* isolates from kala-azar patients in Kenya will be carried out, to determine for presence of strain difference in this parasite.

3.2 **Materials and Methods**

3.2.1 **Mass cultivation of the parasites**

All the 15 *L. donovani* isolates (see Table 8), were obtained from the Nairobi *Leishmania* bank at the Kenya Medical Research Institute where they were cryopreserved in liquid nitrogen. They were allowed to thaw and were subcultured in labelled ambitubes containing NNN with a 1 ml overlay of Schneider's

medium supplemented with 20% foetal bovine serum. The cultures were incubated at 25°C and examined daily for growth of the parasites.

The parasites were then transferred into 25 cm^2 tissue culture flasks and fresh medium added. They were allowed to multiply to stationary phase and then transferred into 75 cm^2 tissue culture flasks. More medium was added gradually as the parasites multiplied until sufficient number of promastigotes were obtained for electrophoresis.

3.2.2 **Preparation of the samples for electrophoresis.**

After mass cultivation, the parasites were prepared for electrophoresis and cellulose acetate electrophoresis carried out for nine enzymes as described in Chapter two, sections 2.2.6.1 to 2.2.6.4.

Table 8

Leishmania donovani isolates from the spleen of kala-azar patients from Baringo, Machakos and Turkana Districts, Kenya.

Baringo	Machakos	Turkana
NLB 656	NLB 610	NLB 645
NLB 657	NLB 616	NLB 652
NLB 658	NLB 578	NLB 653
NLB 659	NLB 762	NLB 654
NLB 660	NLB 771	NLB 655

3.3 RESULTS

3.3.1 Enzyme profiles of 15 *L. donovani* isolates

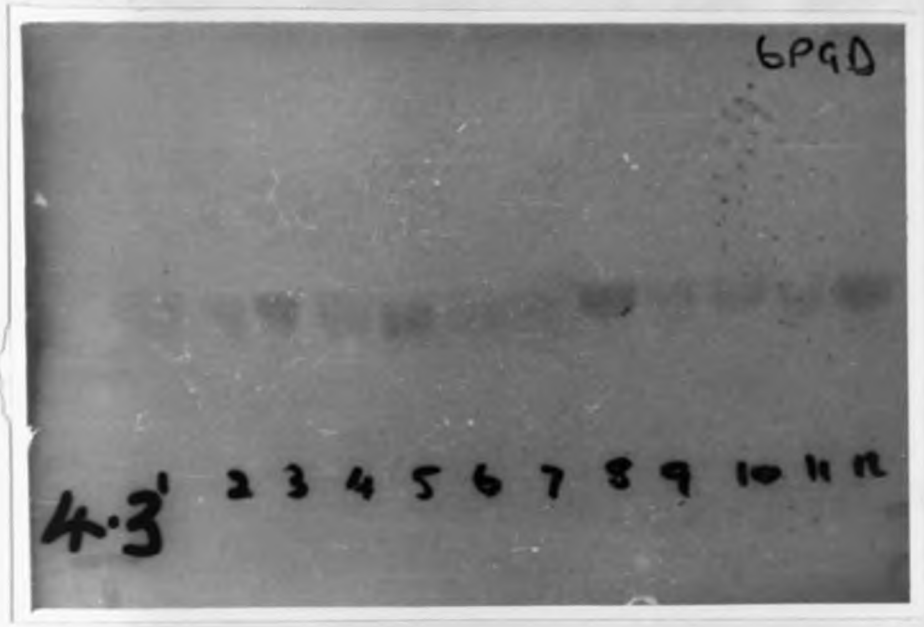
Enzyme profiles of all the 15 *L. donovani* isolates from kala-azar patients in Baringo, Machakos and Turkana showed low levels of variation in the parasite genome when compared among themselves and two *Leishmania donovani* reference strains MHOM/LE/82=LRC-L445= NLB 065 and MHOM/ET/67=LRC-L133= NLB 061,(Plates 4a- b, 5a-d, 6a-b and Figs 4a-d, 5a-d 6a-e). Only one isolate, NLB 659 (Fig 6a) from Baringo District showed a different banding pattern for ICD.

All the isolates showed no enzyme polymorphism for all the enzymes assayed except MDH.

Figs 4a-b. Isoenzyme profiles of *L.donovani* isolates from Machakos and Turkana Districts, Kenya.

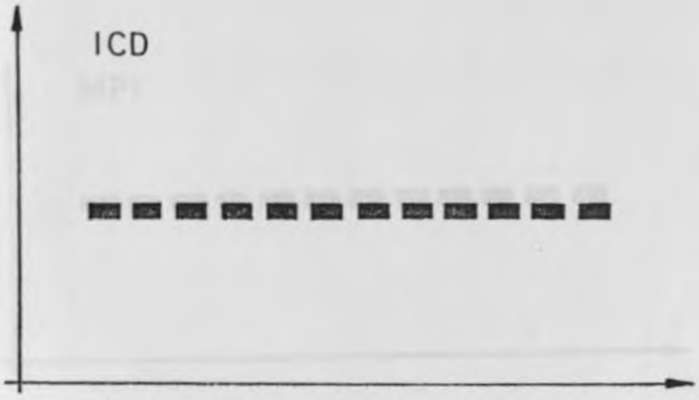
Table 9. *Leishmania donovani* isolates number 1-12 in Plates 4a-b and Figures 4a-d.

1. NLB-065	5. NLB-616	9. NLB-762
2. NLB-656	6. NLB-658	10. NLB-660
3. NLB-610	7. NLB-578	11. NLB-771
4. NLB-657	8. NLB-659	12. NLB-061



(4a). Zymogram pattern for 6PGD

(4b)Zymogram pattern for ME.



4(a)

4(b)

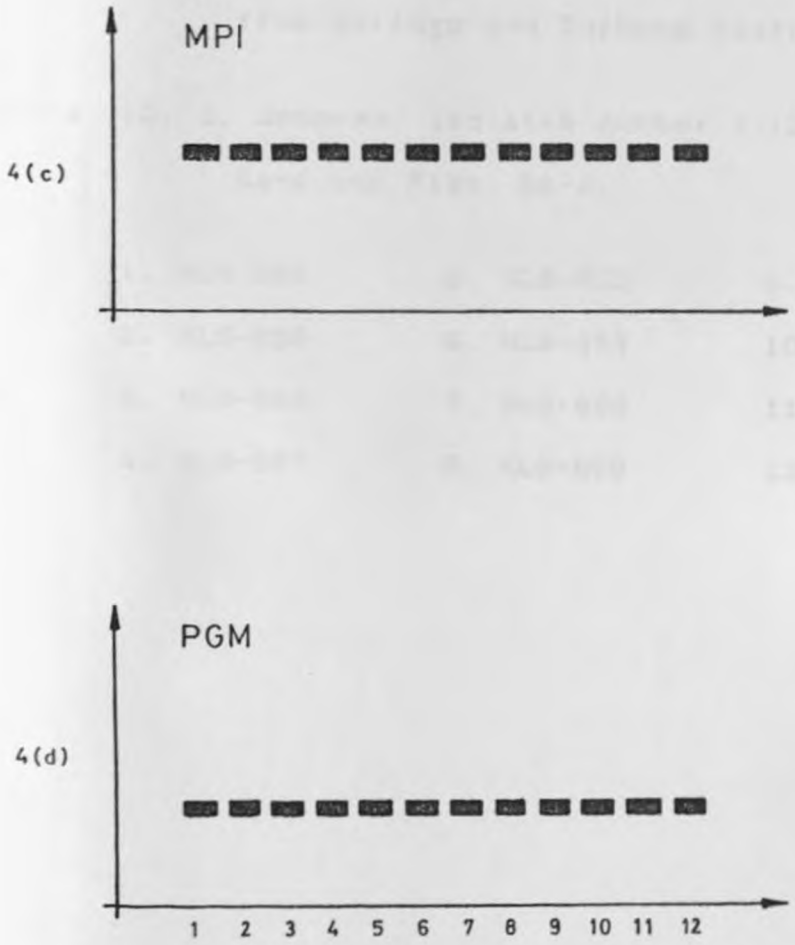
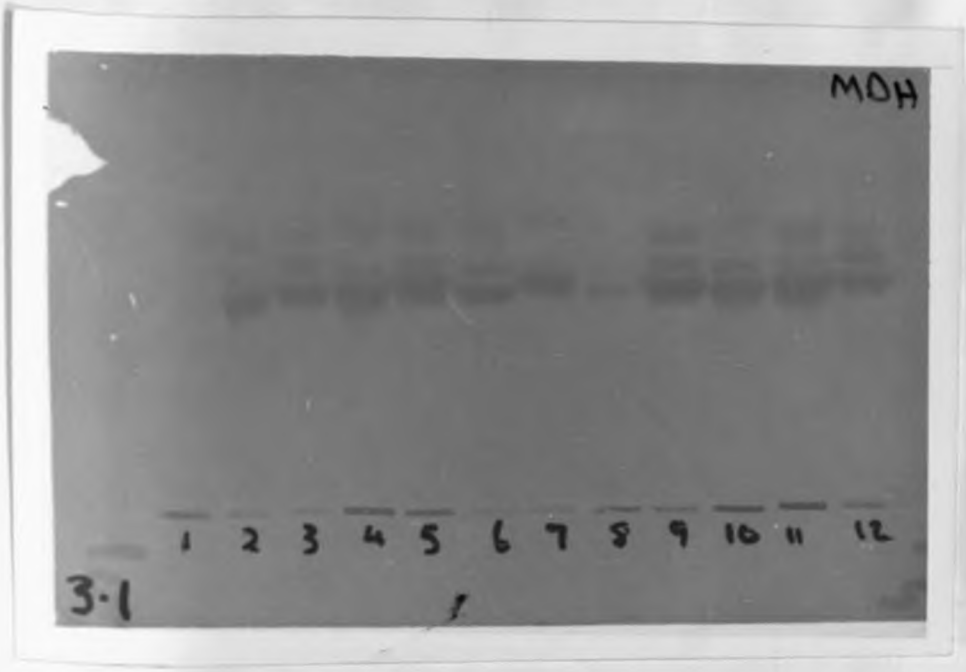


Fig. 4a - d Diagramatic representations of zymogram patterns for four enzymes from 12 L. donovani strains including reference strains

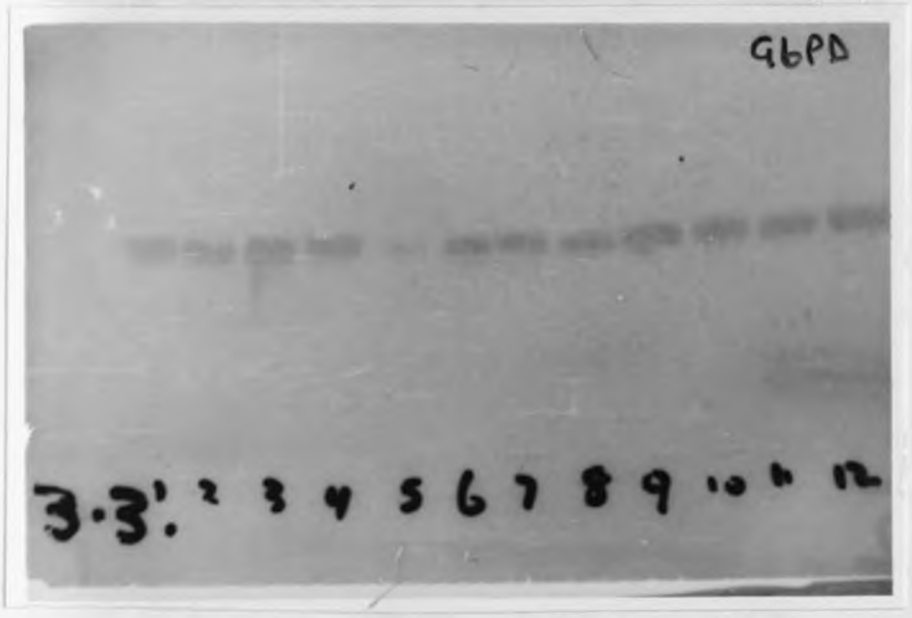
Plates 5a-d. Isoenzyme profiles of *L.donovani* isolates from Baringo and Turkana Districts, Kenya.

Table 10. *L. donovani* isolates number 1-12 in Plates 5a-d and Figs 5a-d.

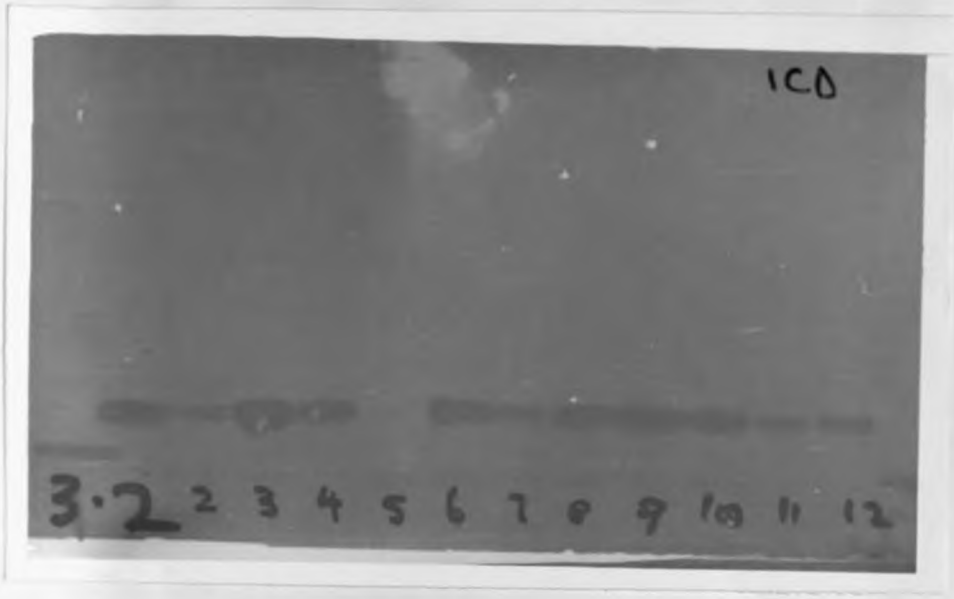
1. NLB-065	5. NLB-652	9. NLB-654
2. NLB-656	6. NLB-658	10.NLB-660
3. NLB-645	7. NLB-653	11.NLB-655
4. NLB-657	8. NLB-659	12.NLB-061



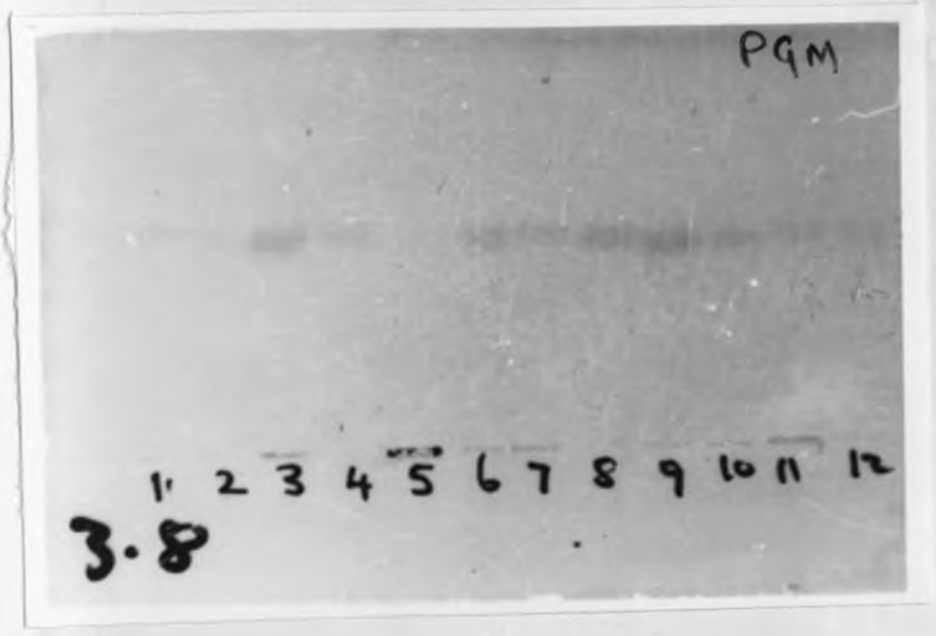
(5a) Zymogram pattern for MDH enzyme.



(5b). Zymogram pattern for G6PD enzyme.

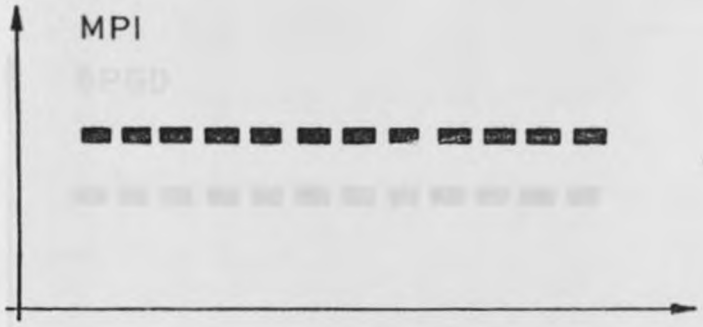


(5c). Zymogram pattern for ICD

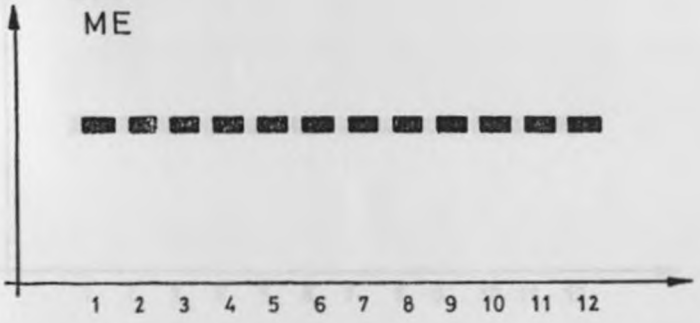


(5d). Zymogram pattern for PGM enzyme.

5(a)



5(b)



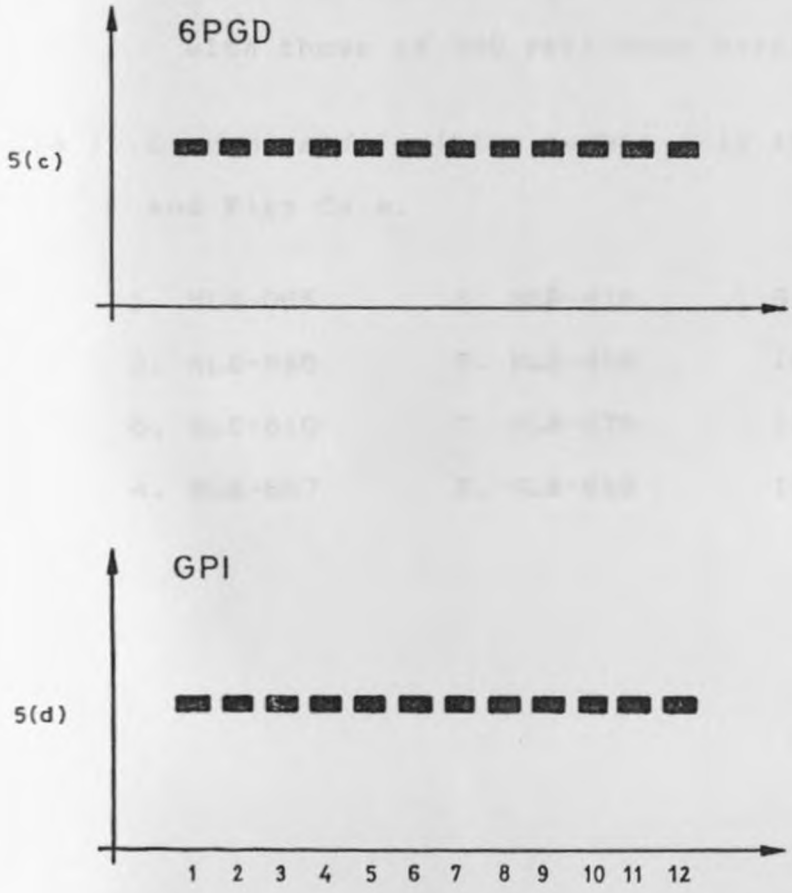


Fig. 5a - d Diagramatic representations of zymogram patterns for four enzymes from 12 L. donovani strains including reference strains

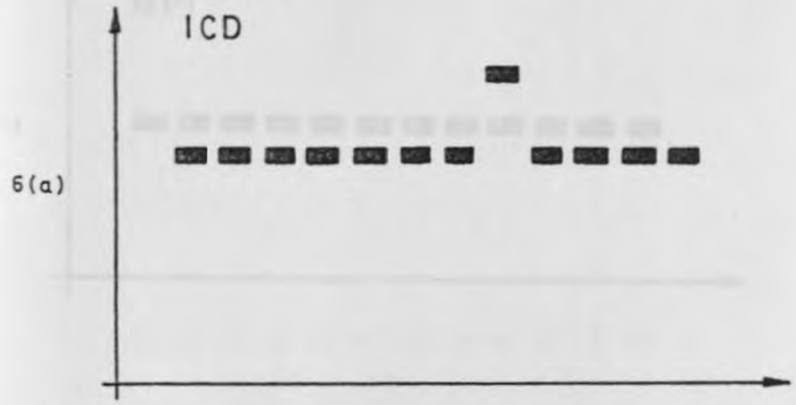
Plates 6a-b. Isoenzyme migration patterns of *L. donovani* isolates from Baringo and Machakos Districts with those of WHO reference strains.

Table 11. *L. donovani* isolates number 1-12 in Plates 6a-b and Figs 6a-e.

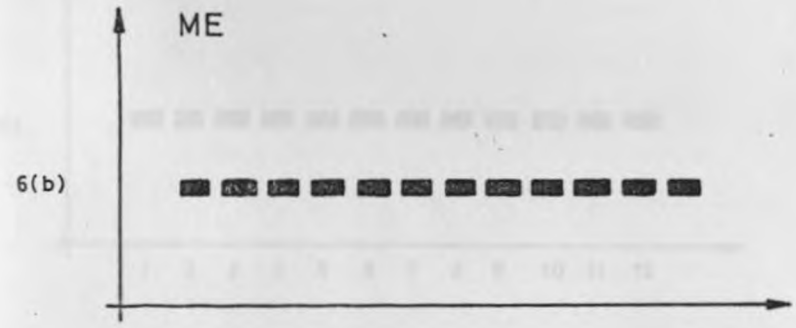
1. NLB-065	5. NLB-616	9. NLB-762
2. NLB-656	6. NLB-658	10. NLB-660
3. NLB-610	7. NLB-578	11. NLB-771
4. NLB-657	8. NLB-659	12. NLB-771

(6a). Zymogram pattern for 6PGD enzyme.

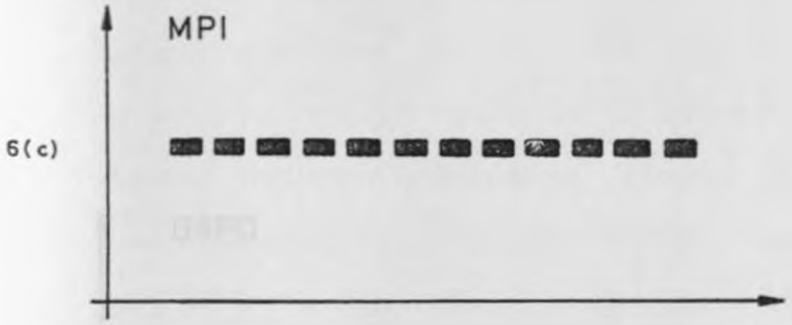
(6b). Zymogram pattern for MDH enzyme.



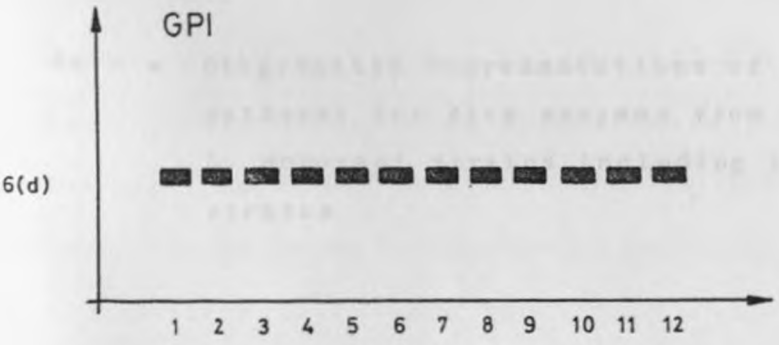
6(a)



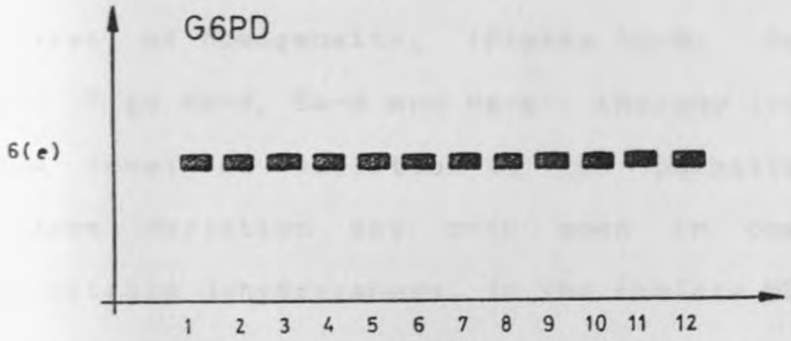
6(b)



6(c)



6(d)



6a - e Diagramatic representations of zymogram patterns for five enzymes from 12 L. donovani strains including reference strains

3.4 D I S C U S S I O N

Enzyme variation among *L. donovani* isolates

Enzyme profiles of all the 15 *L. donovani* isolates from kala-azar patients in Baringo, Machakos and Turkana districts in Kenya showed a striking degree of homogeneity, (Plates 4a-b, 5a-d, 6a-b) and Figs 4a-d, 5a-d and 6a-e), thereby indicating a low level of variation in the parasite,s genome. Enzyme variation was only seen in one enzyme, isocitrate dehydrogenase, in the isolate NLB 659 from Baringo District. This low level of enzyme variation suggests that the *L. donovani* parasites from kala-azar endemic areas in Kenya are not affected by their geographical location. The slight variation seen in the enzyme ICD could be attributed to parasite-host relationship or parasite vector relationship.

Similar results were reported by Le Blancq and Peters (1980) using *L. donovani* isolates from several countries in the Old World.

Characterization using immunological techniques such as monoclonal antibodies and lectin binding carbohydrates have also indicated homogeneity of *L. donovani* sensu lato in the Old World (Schottelius, 1982, Jaffe *et al*, 1984).

Leishmania donovani is thought to be anthroponotic in India and Kenya. Isolates from these regions constitute four closely related

zymodemes, (LON-41, LON-51, LON-56 and LON-44) which differ in one or two enzymes from *L. donovani* sensu stricto (Le Blancq and Peters, 1986). This enzymatic similarly may suggest convergence with adaptive advantage of anthroponotic transmission. The first appearance of epidemic kala-azar in Kenya was reported in the 1950's (Manson Bahr and Southgate, 1964). It is not clear whether this *L. donovani* was introduced from India with *P. martini* as the vector (Le Blancq and Peters, 1986).

The sign and cause of infection of visceral leishmaniasis are similar in the Old World (Manson-Bahr 1971). However, it has been found that in some countries like Kenya, Sudan and India, recovery from visceral leishmaniasis in man, may be followed by the parasite migrating to the skin to give post-kala-azar dermal leishmaniais (PKDL). Serological tests by Bray *et al*, (1973) indicated that the parasite causing the visceral infection causes the subsequent PKDL.

Results from this study suggests that in Kenya, there is only one strain of *L. donovani* which is responsible for kala-azar in all the endemic areas.

CHAPTER FOUR

ADAPTATION OF LEISHMANIA MAJOR IN THE VISCERA OF BALB/c MICE BY REPEATED INTRAPERITONEAL PASSAGE

4.1 Introduction

Balb/c mice are very susceptible to infection with *L. major*. This parasite causes lesions at the sight of infection and then visceralizes (Trotter *et al*, 1980). *Leishmania major* has been found to visceralize in hamsters, Balb/c mice, mole rats and wild rats, culminating in the death of Balb/c mice, mole rats and wild rats (Githure, 1981).

Some enzymes of the cutaneous *Leishmania* are more thermolabile at higher temperatures than the same enzymes in visceral *L. donovani* (Janovy *et al*, 1970). This probably explains why *L. major* do not thrive well in the visceral organs, where the body temperature is higher than in the skin.

In the present study, *Leishmania major* was adapted to high temperatures of the viscera by several intraperitoneal passages in Balb/c mice. Electrophoretic mobility of the enzymes of *L. major* that has been adapted to viscerotropism was investigated to determine any differences due to high temperature adaptation.

4.2 Materials and Methods

4.2.1 Intraperitoneal passaging of *Leishmania major* promastigotes in Balb/c mice.

Leishmania major promastigotes (NLB 144) were allowed to grow to stationary phase. Promastigotes were counted using a haemocytometer and a parasite concentration of 2.2×10^7/ml was obtained. A group of 10 clean Balb/c mice were inoculated intraperitoneally with a parasite concentration of 1×10^6/ml using 26 gauge needle. Two mice from the group were sacrificed after one month and sections of liver and spleen cultured in ambitubes containing NNN medium with an overlay of Schneiders Drosophila medium supplemented with 20% foetal bovine serum. The cultures were incubated at 25^oC and observed daily under a microscope for the presence of promastigotes.

The positive culture was used to inoculate another group of clean Balb/c mice once the parasites reached infective stage.

The animal passage was done six times to adapt the parasite to higher temperatures of the viscera. The effect of passaging on the virulence of the parasite was determined by sacrificing the mice from each passage with pre-patent period, one week less than the previous passage. Some parasites

from each passage were mass cultivated and lysates prepared for electrophoresis as in Chapter 2. Cellulose acetate electrophoresis of the passaged *L. major* was carried out using the same nine enzymes as in Chapters two and three .

4.3 RESULTS

4.3.1 Infectivity of the passaged *Leishmania major* in Balb/c mice.

Intraperitoneal passaging of the cutaneous *Leishmania major* strain resulted in visceralization of the parasite. Pre-patent period for the first passage was much longer (eight weeks) than for a normal cutaneous infection which is usually 3-4 weeks. However, this incubation period became much shorter after each successive passage with mice showing splenomegally 14 days PI. Cultures of liver and spleen were positive for promastigotes.

All the mice in the six groups did not develop a conspicuous cutaneous lesion except two mice which developed a cutaneous lesion at the site of inoculation after three passages. The ulceration was very severe and they both died after six weeks. The pre-patent period for this cutaneous infection was much longer than when the parasites are inoculated subcutaneously. The mice with no cutaneous lesion looked healthy.

In the course of infection, some parasites disseminated to other parts of the body particularly the footpads which became swollen.

4.3.2 **Enzyme profiles of passaged** *Leishmania major*.

Cellulose acetate electrophoresis of *Leishmania major* which adapted to viscerotropism was carried out and compared with *Leishmania donovani* and *Leishmania major* reference strains in nine enzymes used in Chapters two and three.

The viscerotropic *L. major* showed isoenzyme banding patterns similar to those of cutaneous *L. major* for the enzymes, MDH, PGM, MPI, 6PGD, ME. GPI and G6PD enzymes showed banding patterns similar to those of viscerotropic *L.donovani*, (plates 7a-g). Two enzymes, GPI and MDH showed enzyme polymorphism. The enzymes NH andICD did not stain.

Table 12.

Results of intraperitoneal passaging of L. major promastigotes in 10 Balb/c mice.

	Incubation period (days)	Culture results Liver	Spleen	Presence of cutaneous lesion.
Passage no				
1	30	+	+	no
2	25	+	+	no
3	20	+	+	+(2 mice)
4	18	+	+	no
5	15	+	+	no
6	14	+	+	no

Plates 7a-g. Isoenzyme banding patterns of isolates of *L. major* which adapted to viscerotropism compared with those of *L. major* and *L. donovani* reference strains.

Table 13. Isolates number 1-8 in Plates 7a-f.

1. NLB-061 (*L. donovani reference strain*).
2. NLB-144 IP1.
3. NLB-144 IP3.
4. NLB-144 IP2.
5. NLB-144 IP4.
6. NLB-144 IP6.
7. NLB-144 IP5.
8. NLB-144 (*L. major* reference strain).

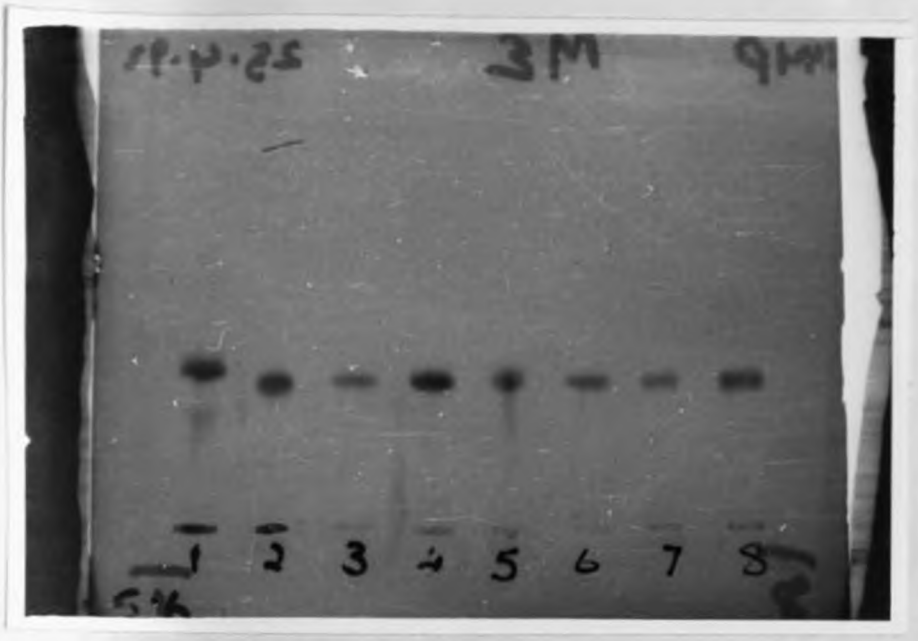
IP-- Intraperitoneal passage

(7a). Zymogram pattern for GPI.

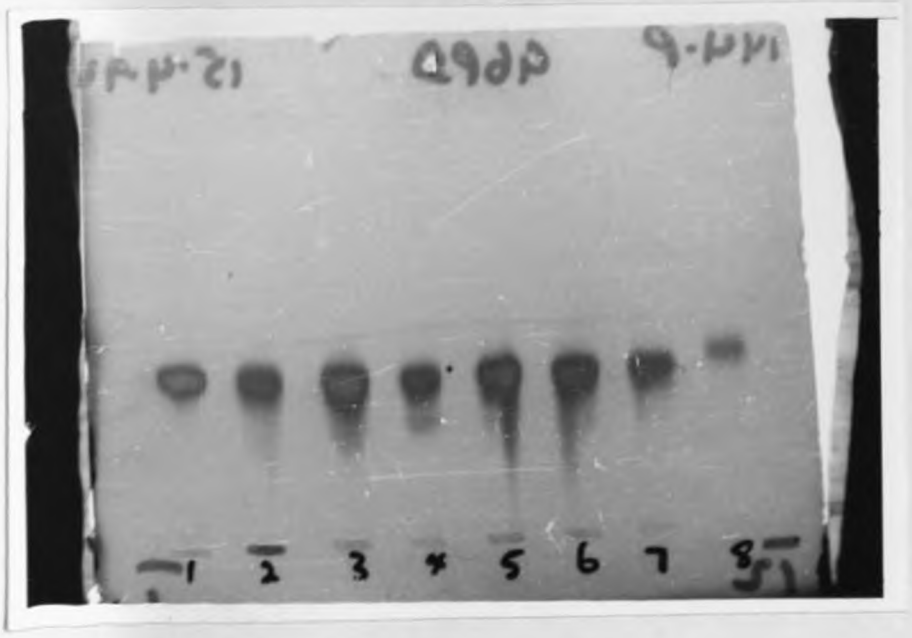
(7b). Zymogram pattern for MDH.

(7c). Zymogram pattern for PGM.

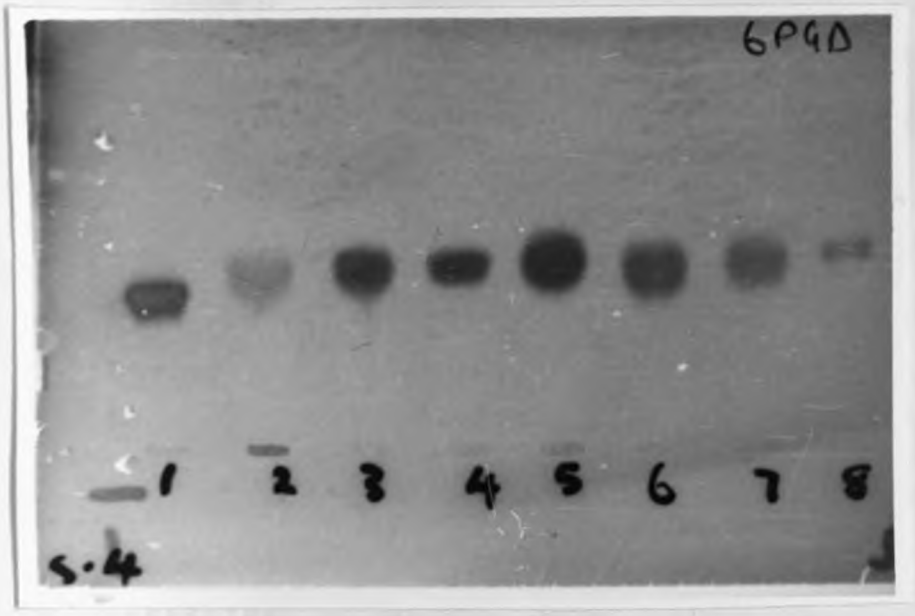
(7d). Zymogram pattern for MPI.



(7e). Zymogram pattern for ME.



(7f). Zymogram pattern for G6PD.



(7g). Zymogram pattern for 6PGD.

4.4 DISCUSSION

Janovy (1970), showed that some enzymes of the cutaneous *Leishmania mexicana* are more thermolabile at higher temperatures than the same enzymes in viscerotropic *Leishmania donovani*. This may therefore explain why *Leishmania major* which is a cutaneous parasite does not thrive in the visceral organs where the body temperature is higher than in the skin. However, it has been reported that in Balb/c mice, *L. major* causes lesions at the site of inoculation and then visceralizes (Trotter *et al*, 1980).

In the present study, *L.major* was gradually adapted to progressively increasing body temperature by several intraperitoneal inoculation of promastigotes in Balb/c mice. This resulted in visceralization of the parasite with no conspicuous cutaneous lesion. These results suggests that, there was selection of mutants which were temperature tolerant and behaved as the viscerotropic *L.donovani*.

Results also showed that several intraperitoneal passaging of promastigotes in Balb/c mice resulted in increased virulence of the parasites which adapted to viscerotropism. Pre-patent period became shorter after each successive passage with mice showing splenomegally 14 days post-inoculation.

The results also support the use of rodents in

maintaining *Leishmania* stocks through passaging of the promastigote stage since the virulence of that stock is maintained (Hendricks *et al*, 1978). This method is superior to *in vitro* culturing technique in maintaining *Leishmania* stocks. The *in vitro* culturing technique when repeated several times has been found to cause loss of virulence and the ability of the parasite to re-establish the infection in a mammalian host (Schneider and Hertigi, 1966).

The viscerotropism of this cutaneous parasite did not cause the death of the mice . The two mice which developed a cutaneous lesion at the site of inoculation died due to the gross ulceration and secondary infection of the lesion. The lesion could have been due to presence of promastigotes at the tip of the needle which were left at the site of inoculation. However, the long pre-patent period suggests that the parasites were very few.

Viscerotropism of cutaneous *Leishmania* parasites had also been reported earlier. Githure (1981), observed that *Leishmania major* can visceralize in hamsters, Balb/c mice, striped mice, mole rats and wild rats culminating in the death of Balb/c mice, mole rats and wild rats. He found that mole rats inoculated with "spleen derived amastigotes" did not develop a conspicous cutaneous lesion but at necropsy, the skin, liver, spleen and kidney were

found to be heavily parasitized. This can be compared with *L.donovani* infection where no cutaneous lesion is evident at the site of inoculation.

Mebrahtu *et al*, (1989) also reported visceralizing *L. tropica* in Kenya. Schnur *et al*, (1981) found that four Israeli strains responsible for visceral leishmaniasis typed as *L. tropica* . These parasites showed a tendency to cause leishmaniasis recividans in certain immunologically defective subjects and visceral leishmaniasis in others. Similar results have been found in Brazil where active cutaneous leishmaniasis is caused by *L. donovani chagasi* (Oliveira *et al*, 1982). In the Mediterranean region, *L. infantum* has been found to cause cutaneous lesions (Rioux *et al*, 1986).

These results demonstrate that some species of *Leishmania* are not restricted exclusively to the skin or viscera and that a given parasite may on occasion cause cutaneous or visceral disease. This is very important in treatment and emphasizes the need to characterize *Leishmania* parasites using intrinsic characters rather than extrinsic characters alone.

Since it has been shown that some enzymes of *Leishmania* are thermolabile (Janovy, 1970), electrophoretic mobility of the enzymes of *L. major* that adapted to viscerotropism was carried out. Results showed that the passaged isolates were

similar to the original stock isoenzymatically. This emphasizes the need to characterize *Leishmania* parasites particularly those isolated from rodents before drawing conclusions of their identity.

CHAPTER FIVE

CONCLUSION

Distinguishing species on the basis of clinical features may prove to be misleading since the primary lesion of kala-azar might be diagnosed as oriental sore (Adler, 1964). Balb/c mice are very susceptible to infection with *Leishmania* parasites. In the present study, experiments using four clones of *L. major* strain and four of *L. donovani* strain has demonstrated the use of these mice in characterizing strains and species of *Leishmania*. Infectivity of these clones in Balb/c mice revealed population heterogeneity which was not evident using isoenzyme electrophoresis.

Leishmania donovani causes kala-azar and post-kala-azar dermal leishmaniasis in Kenya. Earlier reports of biochemical and serological tests have indicated low levels of variation in the genome of *L. donovani* from the Old and New World (Le Blancq and Peters 1986; Schotelius 1982; Bray *et al*, 1973). In the present study, similar results were obtained from isoenzyme electrophoresis of *L. donovani* isolates from kala-azar endemic areas in Kenya. This therefore, suggests the presence of only one strain of *L. donovani* in Kenya. Results also suggest that this parasite is not affected by its geographical

location.

In the present study, several intraperitoneal passaging of *L. major* resulted in visceralization of the parasite. The infection resembled that of viscerotropic *L.donovani* in that no conspicuous cutaneous lesion was formed at the site of inoculation. Dissemination of the parasites to the footpads during the course of infection suggests their inability to adapt fully to high temperatures. The virulence of the parasite increased after each successive passage therefore emphasizing the use of laboratory animals in increasing the virulence of a particular strain of *Leishmania*.

Characterization of the passaged *L. major* using cellulose acetate electrophoresis showed that the parasite did not change the enzymatic pattern as compared with the reference strain. Presence of homogeneity among *L.donovani* clones and *L.donovani* isolates from Kenya isoenzymatically suggests that one type of vaccine can be used in the treatment of visceral leishmaniasis.

The technique of infectivity to mice has been used to differentiate strains of *Leishmania* and strains and clones of *Trypanosoma cruzi* (Khairy and El-hashimi, 1980; Postan *et al*, 1983). In the present study, similar results were obtained using clones of

L.major and *L.donovani*. This suggests the suitability of this technique in conjuction with others when characterizing *Leishmania* parasites.

Results from the three study experiments, suggests that isoenzyme electrophoresis technique cannot be used alone to characterize *Leishmania* parasites. This emphasizes the use of more than one technique to determine the genetic difference of *Leishmania* parasites.

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