THE EPIDEMIOLOGY OF LEISHMANIASIS IN BARINGO AND MACHAKOS DISTRICTS WITH EMPHASIS ON ANIMAL RESERVOIRS

BY

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE UNIVERSITY OF NAIROBI

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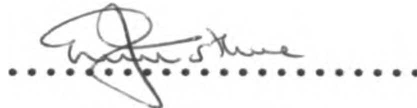

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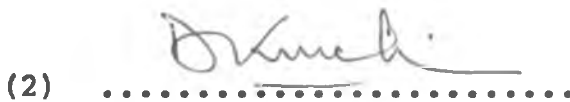

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SUMMARY

Identification of animal reservoirs of leishmaniasis is an absolute pre-requisite to understanding the epidemiology of the disease. A 16-month search for animal reservoirs of leishmaniasis began in Marigat Location of Baringo District in May 1986 and in Masinga Location of Machakos District in December 1987. Epidemics were reported in these areas during the 1950s and 1970s and current endemicity of the disease suggest that animal reservoirs maintain the infection.

Examination for leishmanial parasites was done, using culture and smear techniques. From Marigat, a total of 1,128 rodents belonging to 7 genera and 11 carnivores belonging to 3 genera were examined; and from Masinga, 633 rodents belonging to 10 genera and 95 carnivores belonging to 5 genera were examined. From Marigat, 3 (0.5%) Arvicanthis niloticus, 36 (12.5%) Tatera robusta and 2 (0.8%) Mastomys natalensis were positive for leishmanial parasites. From Masinga, no leishmania infection was detected but 67 (60.4%) Acomys subspinosus, 12 (8.4%) Mastomys natalensis, 2 (4.0%) Lemniscomys striatus, 2 (33.3%) Herpestes sanguineus, 1 (100%) Helogale parvula and 1 (1.2%) Canis familiaris were found positive with trypanosome flagellates.

Forty-two isolates from sandflies, 34 from humans and 13 from lizards all obtained from the study sites were

included for comparison with isolates from wild animals.

All isolates were characterized by isoenzyme analysis using nine enzymes. The enzymes examined were: malate dehydrogenase (MDH), phosphoglucomutase (PGM), glucose phosphate isomerase (GPI), isocitrate dehydrogenase (ICD), nucleoside hydrolase (NH), glucose 6-phosphate dehydrogenase (G6PD), malic enzyme (ME), 6-phosphogluconate dehydrogenase (6PGD) and mannose phosphate isomerase (MPI). The isolates were compared with five Old World Leishmania reference strains and five well characterized rodent trypanosomes of the subgenus Herpetosoma. All the isolates from rodents in Marigat (except one from a Mastomys) were identified as Leishmania major. Of the 34 isolates from humans, 31 were identified as L. donovani and three as L. major. Thirty-four isolates from Sergentomyia spp. and 13 from lizards were all identified as L. adleri. Five P. duboscqi and 3 P. martini isolates were identified as L. major and L. donovani respectively.

All the isolates from animals from Masinga and one from a Mastomys from Marigat differed from the Leishmania reference strains. They were, however, morphologically similar to rodent trypanosomes. The enzyme profiles of the three mongoose isolates were identical to each other but different from rodent and dog isolates.

The results of the studies in Marigat suggest that rodents are the reservoirs of cutaneous leishmaniasis in Baringo District, with Tatera robusta serving as the main reservoir. Leishmania major was not isolated from animals in Masinga.

The prevalence of mammalian trypanosome flagellates was very high in Acomys (60.4%) collected in Masinga but in Marigat, only one Mastomys (0.004%) was found infected with trypanosome flagellates.

Mastomys and Acomys were found to be susceptible to experimental infection with L. donovani while dogs, cats and goats were found to be refractory to the infection. Although rodents were susceptible, none were found naturally infected with L. donovani. The results in this study argue against the notion that wild mammals and/or dogs serve as reservoirs of visceral leishmaniasis and support the suspicion that man is probably the only reservoir of infection in Kenya.

Chapter 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. The genus *Leishmania*

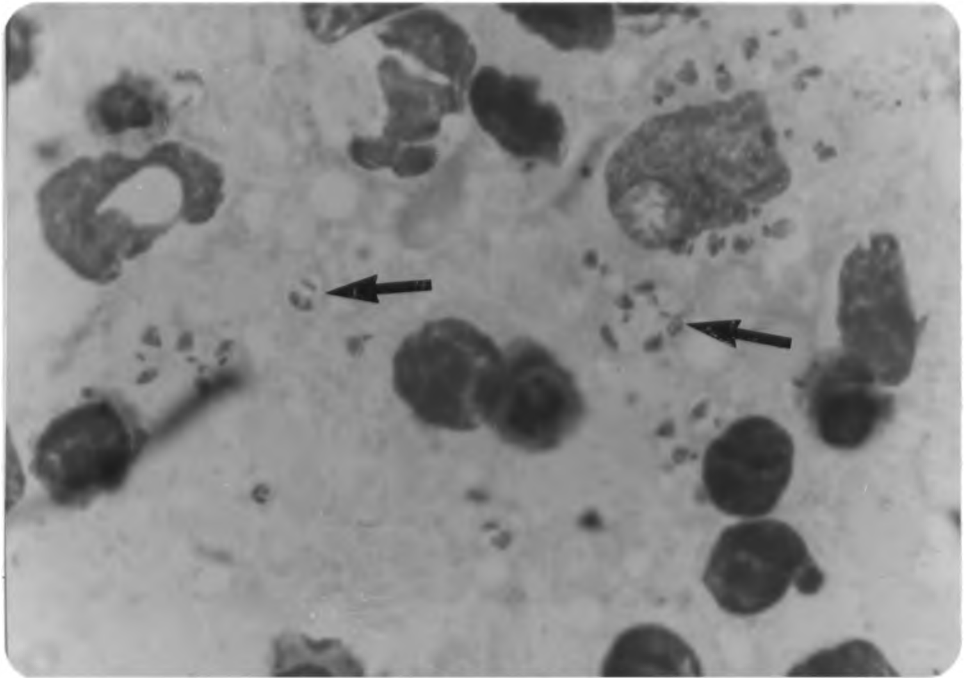
Leishmaniasis is a disease caused by flagellate protozoa belonging to the genus *Leishmania*. The current systematic position of the genus is as follows: Kingdom Protista: Subkingdom Protozoa: Phylum Sarcomastigophora: Class Zoomastigophora: Order Kinetoplastida: Family Trypanosomatidae: Genus *Leishmania* (Molyneux & Ashford, 1983). These parasites are heteroxenous and are transmitted from infected to non-infected hosts by sandflies of the genus *Phlebotomus* in the Old World or *Lutzomyia* in the New World. They are intracellular and exist as round, non-motile amastigotes inside the macrophages of the reticulo-endothelial system of their vertebrate hosts (Plate la). Amastigotes divide by binary fission inside host cells. A sandfly feeding on an infected host picks up amastigotes with the blood meal. These transform into elongated, motile promastigotes in the gut of the fly. This process is mimmicked in culture (Plate lb). The promastigotes also multiply by binary fission and then migrate forward to the proboscis, but do not invade the salivary glands, and are passively deposited in the wound on the skin caused by the piercing proboscis as the fly feeds. The development cycle in the fly takes about 6-10 days depending on the ambient temperature and physiological status of the fly (Hommel, 1978; Lawyer et al., 1984).

Plate 1a

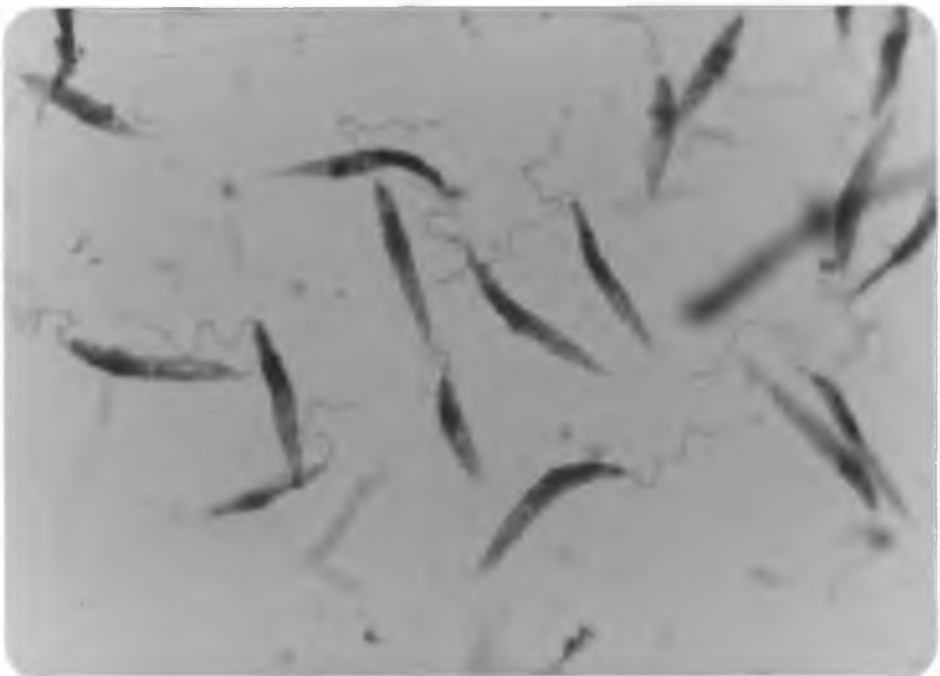
Leishmania donovani amastigotes (arrow) in liver of a hamster

Plate 1b

Leishmania donovani promastigotes in Schneider's culture medium



1a



1b

1.2. Importance of leishmaniasis

Awareness of the importance of leishmaniasis increases as the knowledge of the disease accumulates. A little over 10 years ago, leishmaniasis was included among the six parasitic diseases selected for special emphasis by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. At least 14 different species and subspecies of *Leishmania* are currently known to infect man world-wide and to cause different forms of disesase (WHO, 1984) (Appendix 1). Leishmaniasis is endemic in all continents except Australia (Lainson, 1982) and it has been reported to occur in over 100 countries (WHO, 1984), affecting about 12 million people (WHO, 1988). There are three different forms of the disease, namely, visceral, cutaneous and mucocutaneous leishmaniasis.

Leishmaniases are generally chronic and debilitating rather than rapidly fatal diseases, and therefore large number of cases do not capture the attention of the press or public health authorities as do more dramatic infectious diseases like malaria. The importance of visceral leishmaniasis is often under-estimated and patients tend to seek medical attention late in the course of their illness, often as a result of concurrent infections, or by mistaking the symptoms for malaria. In untreated cases, the mortality rate can be

as high as 90% (Molyneux & Ashford, 1983). Management of hospitalized patients and the cost of medication can be very expensive.

1.3. Leishmaniases in the Old World

There are four well recognized *Leishmania* species that cause human disease in the Old World, namely, *L. donovani*, *L. major*, *L. tropica* and *L. aethiopica*. The sub-species of *L. donovani* are *L. d. donovani* found in India, *L. donovani* s.l. found in China and East Africa, *L. d. infantum* found in the Mediterranean region and *L. d. chagasi* found in South America (WHO, 1984). It is still debatable whether these sub-species should be classified as distinct species. The *L. donovani* found in India and East africa will hereafter be referred to as *L. donovani*.

1.3.1. Visceral leishmaniasis caused by L. donovani and L. d.infantum

Leishmania donovani and *L. d. infantum* are both viscerotropic parasites of wild and domestic canids, and man. *L. donovani* is found mainly in India, China and Eastern Africa. In these countries, the infection is commonly found in children and young adults, 5-20 years of age. *L. d. infantum*, which is commonest in 1 to 4 year age-group, is found in the Mediterranean

region. Often more males than females are infected, the ratio being 4:3. This is probably because of behavioural activities of the males who have a tendency of staying outdoors late in the evenings thereby exposing themselves to sandfly bites. Visceral leishmaniasis results when amastigotes invade the reticulo-endothelial cells of various organ systems of the body, mainly the spleen, liver, bone marrow and lymph nodes. The incubation period ranges from 10 days to over one year and the onset of the disease is gradual. Common symptoms are recurrent fever, anaemia, loss of weight enlargement of the liver and spleen.

Successful treatment with anti-leishmanial drugs often leads to some degree of immunity and resistance to re-infection which may be life-long. In China, India and Kenya cutaneous lesions referred to as post kala-azar dermal leishmaniasis occur in some cases of visceral leishmaniasis long after treatment (Leng Yan-Jia, 1982; Thakur, 1984; Rashid et al., 1986). In Kenyan post kala-azar dermal leishmaniasis, the most common types of lesions are hypopigmented papular and nodular rashes usually on the face but occasionally spread over the whole body (Rashid et al., 1986). Parasites are present in these nodules but visceral involvement is rarely encountered.

Reservoirs and Vectors

Wild and domestic canids have been found naturally infected with *L. d. infantum* in the Mediterranean region (Bettini et al., 1980), Soviet Union (Lysenko, 1971), China (Leng Yan-Jia, 1982), Senegal (Desjeux et al., 1983) and with *L. donovani* in Kenya (Mutinga et al., 1980). Wild cats and rodents were also found infected with *L. donovani* in the Sudan (Hoogstraal & Heyneman, 1969), while *L. d. infantum* was isolated from *Rattus rattus* in Italy (Bettini et al., 1980). There is no known animal reservoir of visceral leishmaniasis in India. The search for animal reservoirs in Kenya has not yielded any conclusive evidence that wild animals are involved in the transmission of this disease.

Several species of sandflies are vectors of visceral leishmaniasis including, *Phlebotomus argentipes* in India (Swaminath et al., 1942), *P. chinensis* in China (Bray, 1974), *P. perniciosus* in the Mediterranean region (Adler & Theodor, 1935), *P. ariasi* in France (Maroli & Bettini, 1977), *P. orientalis* in the Sudan and Ethiopia (Hoogstraal & Heyneman, 1969) and *P. martini* in Kenya (Minter et al., 1962; Perkins et al., 1988). In Kenya, sandflies are believed to breed and rest, among other places, inside termite hills inhabited by *Macrotermes bellicosum*. These termite

hills are prominent features in semi-arid regions of Kenya and are encountered frequently in Masinga and Marigat locations. Heisch (1954) reported that termite hills harboured many sandflies soon after the rainy season. Epidemiological investigations by Southgate & Oriedo (1962) in Kitui District revealed a correlation between proximity to termite mounds and the number of visceral leishmaniasis cases. Wijers (1963) suggested that transmission might occur in early mornings or evenings when people sit near termite hills. However, studies by Ho et al. (1982) in Masinga Location showed that visceral leishmaniasis occurred in homesteads regardless of whether they lived near or far from termite hills.

1.3.2. Cutaneous leishmaniasis caused by L. major

Leishmania major is primarily a zoonotic infection of rodents in the Middle East, Asia and Africa. Infection with this parasite causes simple cutaneous leishmaniasis or oriental sore, which is first manifested as a firm papule at the site of insect bite and grows slowly but progressively. The incubation period is often less than four months. A crust develops centrally on the lesion and becomes detached, exposing a crater-like ulcer. The edge of the wound thickens thus limiting the size of the ulcer.

Self-cure usually occurs in about 2-8 months, leaving a depressed scar and, fortunately, a strong immunity against challenge by both L. major and L. tropica (WHO, 1984).

Reservoirs and Vectors

Different species of rodents have been found naturally infected with L. major in different parts of the Old World. For example, Psammomys obesus and Meriones spp. are the proved reservoirs in Israel, Saudi Arabia and India (Gunders et al., 1968; Mohan & Suri, 1975; Schlein et al., 1984; Peters et al., 1985), and Mastomys erythroleucus and Tatera gambiana are the proved reservoirs in Senegal (Dedet et al., 1979). Rhombomys opimus are the proved reservoirs in the Soviet Union (Lysenko, 1971) and Iran (Nadim et al., 1979). L. major has also been isolated from Arvicanthis niloticus in Senegal and Ethiopia (Ranque et al., 1974; Haile & Lemma, 1977), and Xerus rutilus, Arvicanthis niloticus, Tatera robusta, Taterillus emini, Aethomys kaiseri and Mastomys natalensis in Kenya (Heisch, 1963; Ngoka & Mutinga, 1978b; Githure et al., 1984a; 1986a). L. major has also been isolated from dogs in Egypt (Schnur et al., 1985) and Saudi Arabia (Peters et al., 1985). Some of these hosts cannot be considered as reservoirs because of the low infection rate reported.

The proved vectors of this parasite live in close association with rodents inside rodent burrows and include *Phlebotomus papatasi* in the Middle East and the Soviet Union (Lysenko, 1971; Nadim et al., 1979; Schlein et al., 1982), *P. duboscqi* in West Africa and Kenya (Dedet et al., 1979; Beach et al., 1982; 1984), *P. mongolensis* in Central Asia (Lysenko, 1971) and *P. salehi* in India (Mohan & Suri, 1975).

1.3.3. Cutaneous leishmaniasis caused by *L. aethiopica*

Leishmania aethiopica, also a zoonotic infection, has been reported in the highlands of Ethiopia and Mt. Elgon in Kenya (Kungu et al., 1972; Bray et al., 1973). This parasite causes simple cutaneous leishmaniasis. It occasionally causes a diffuse cutaneous form which presents as non-ulcerating nodules resembling lepromatous leprosy in immunologically deficient individuals (Bryceson, 1970a). Simple cutaneous leishmaniasis caused by *L. aethiopica* generally undergoes spontaneous healing between one to five years. It responds poorly to conventional doses of pentavalent antimony drugs (Bryceson, 1970b). However, it has been shown to respond to local heat therapy (Mutinga and Mngola, 1974) and high-dose pentavalent antimony drugs (Chulay et al., 1983).

Reservoirs and Vectors

This parasite has been isolated from the hyraxes *Procavia capensis* and *Heterohyrax brucei* in Kenya and Ethiopia (Bray et al., 1973; Mutinga, 1975), and from a giant rat, *Cricetomys gambianus* in Kenya (Mutinga, 1975). The hyraxes live in colonies and are active during the day. They do not burrow, but live in deep rock crevises or in hollow trees which are rich in organic matter suitable for the breeding of sandflies (Mutinga, 1975).

The vectors of the disease are found within altitudinal limits of 1600m to 2700m in areas with an annual rainfall above 800mm. The vectors are *P. aculeatus*, *P. pedifer* and *P. longipes* in Kenya and Ethiopia (Mutinga, 1971; Schottelius, 1982).

1.3.4. Cutaneous leishmaniasis caused by L. tropica

Leishmania tropica causes anthroponotic cutaneous leishmaniasis infection in man in North Africa, the Middle East, Soviet Union, India and Southern Europe. This parasite was recently isolated from five patients who had probably acquired the infection in districts around the Nyandarua Range, Kenya (Mebrahtu et al., 1987; 1988). The parasite normally produces chronic lesions which take long to ulcerate and, similar to the cutaneous form caused by *L. aethiopica*, self-cures

after about a year or longer. The incubation period is usually 2-8 months. Occasionally, the infection gives rise to a chronic tuberculoid lesion known as leishmaniasis recidivans which may last for several years and respond little to treatment. In self-cured individuals, specific immunity to challenge with *L. tropica* is normally expressed.

Reservoirs and Vectors

Leishmania tropica normally has a human-fly-human transmission cycle with no known animal reservoir. However, this parasite was reported from a naturally infected *Rattus rattus* from Iraq, probably an accidental host (Bray & Dabbagh, 1968). The possibility of dogs acting as reservoirs has been investigated and, although dogs are found infected, these infections run concurrently with those in humans. Therefore, the dog can be termed as an alternative host rather than a reservoir host for *L. tropica* (Sherman et al., 1973). Moreover, this parasite exists in areas where there are no dogs (Nadim et al., 1979). Man is therefore considered to be the only important reservoir of infection.

P. sergenti, a sandfly found in urban areas, is considered to be the main vector of this disease. Its anthropophilic feeding habits enable it to maintain *L.*

tropica infection in humans only without the need for alternative reservoirs.

1.4. Experimental infection of animals with Leishmania

A variety of experimental animals have been used in the study of leishmaniasis. Caution should be exercised when extrapolating from experimental to natural conditions. In experimental infections, alternative routes of infection may be used other than biting through the skin that is used by sandflies. In addition, the number of parasites delivered by a sandfly through its bite has not been established and an even larger inoculum may be delivered in experimental infections. Despite these drawbacks, experimental infection in laboratory animals has shed light on the genetic basis of host susceptibility and resistance to leishmaniasis. Mice and hamsters have been used to compare infectivity of different strains of Leishmania spp. (Githure, 1981; Al-Taqi & Mohammed, 1981; Githure & Gardener, 1981) and also to study the genetics of the immune response (Bradley & Kirkley, 1977), and response to chemotherapy (Trotter et al., 1980). Hamsters have been used as animal models of visceral leishmaniasis (Stauber, 1966; Schnur et al., 1973).

The phylogenetic closeness of nonhuman primates to man has been increasingly exploited and these animals have been used as models for various diseases afflicting man. The susceptibility of East African nonhuman primates to infection with L. donovani and L. major showed that vervet monkeys (Cercopithecus aethiops), sykes monkeys (Cercopithecus mitis) and baboons (Papio anubis) can be used as models for vaccine or drug trials against these infections. Monkeys infected with L. major self-cure without treatment in about three months post-infection and are immune to challenge with a homologous strain of the parasite (Githure et al., 1987). Infection with L. donovani caused disease varying from fatal in some animals to self-cure in others after about eight months post-infection (Githure et al., 1986b; Shatry et al., 1987a). The susceptibility of these animals to the parasites suggested that nonhuman primates are probably naturally infected with leishmanial parasites in Kenya. This possibility was investigated by Binhazim et al. (1987) who isolated L. major from one of 35 vervet monkeys examined for leishmanial parasites in Thika, Kenya. Studies are currently in progress in Kenya to test candidate vaccines against L. major infection in the vervet model.

1.5. Characterization of Leishmania species

Identification of morphologically similar parasites responsible for different diseases in man is important to clinicians, research scientists, and epidemiologists. In Kenya, leishmanial parasites isolated from humans, wild animals or sandflies and examined microscopically, could be either L. donovani, L. major, L. tropica or L. aethiopica. In addition, sandflies may be infected with either L. adleri, Crithidia spp., Leptomonas spp. or Herpetosoma spp. Crithidia-like organisms were recently isolated from man, sandflies and a dog in Kenya. These were found to be enzymatically similar to flagellates isolated from dogs and rodents in Egypt (Morsey et al., 1985; Githure et al., 1986c).

Parasites of the genus Leishmania are morphologically identical and it is routinely difficult to distinguish one from another, especially in the promastigote form (Garnham, 1971). However, Gardener et al., (1977) have shown that morphometric approaches can be used to differentiate L. major amastigotes from L. tropica amastigotes by size. L. major amastigotes are larger than L. tropica amastigotes (Gardener et al., 1977). This technique is tedious and is not applicable to many Leishmania species. Classification of Leishmania was formerly on geographical distribution

and clinical manifestations in man. In the Old World, visceral leishmaniasis was associated with L. donovani whereas cutaneous leishmaniasis was associated with L. major, L. aethiopica, and L. tropica. This classification was later found to be inadequate when it was realized that clinical manifestations of infection are the expression of both the genetic variability of the host (Turk & Bryceson, 1971) and of the parasite (Lainson & Shaw, 1972). It is now clear that a single species has the ability to cause more than one disease syndrome in man owing to the multiple tissue tropism of the parasite. L. donovani, a parasite that causes visceral leishmaniasis can also cause simple cutaneous lesions or post kala-azar dermal leishmaniasis in man (Thakur, 1984; Belazzoug et al., 1985; Rashid et al., 1986). This syndrome can be mistaken for diffuse cutaneous leishmaniasis caused by L. aethiopica. In addition, diffuse cutaneous leishmaniasis occurs in immunologically deficient individuals, whereas the same parasite causes simple cutaneous lesions in normal individuals (Bryceson, 1970a). In some cases, diffuse cutaneous leishmaniasis can easily be mistaken for lepromatous leprosy which is caused by a bacterium, Mycobacterium leprae. Cutaneous lesions caused by Leishmania can also be mistaken for fungal infections.

The predilection of the parasites for organ system

of the host is not strictly specific since *L. tropica*, a parasite that causes cutaneous leishmaniasis, has been reported to viscerilize in man (Schiliro et al., 1978; Aljeboori & Evans, 1980; Schnur et al., 1981; Mebrahtu et al., 1988b) and *L. major*, another parasite that causes cutaneous leishmaniasis, has been isolated from the viscera of rodents (Dedet et al., 1979; Githure et al., 1984a). To distinguish between various species of *Leishmania*, a wide variety of techniques have been developed. They include serological techniques, such as agglutination test and excreted factor serotyping and biochemical techniques, such as isoenzyme electrophoresis and DNA hybridization (Chance & Walton, 1982).

1.5.1. Serologial techniques

An agglutination test known as the Noguchi-Adler test (Adler, 1964) was used for many years to differentiate species of *Leishmania*. This technique makes use of the ability of *Leishmania* to aggregate when cultured in a homologous antiserum. The test differentiates *L. major*, *L. tropica*, *L. donovani*, *L. d. infantum*, *L. braziliensis* and *L. mexicana* (Adler, 1964).

Excreted factor serotyping, modified from the Noguchi-Adler test, is a simple immunodiffusion

precipitation reaction between factors excreted by promastigotes in culture and hyperimmune rabbit antiserum (Schnur et al., 1972). Because different leishmanias produce different types of excreted factors, these exoantigens can be used as taxonomic markers. Using the technique, several stocks of leishmanias from Ethiopia, Kenya and the Sudan were examined (Schnur & Zuckerman, 1977; Githure et al., 1986a). All the Middle East L. major strains typed as subserotype A while the Sudanese and Kenyan L. major and L. donovani strains typed as subserotype B_2. The Ethiopian and Kenyan L. aethiopica typed as B_1. Although the Kenyan and Israeli L. major strains showed different serotypes, they were found to have the same enzyme profiles for 12 enzymes (Githure et al., 1986a). Conversely, L. major and L. donovani were found to have the same excreted factor serotypes, thus limiting the use of this technique for differentiating between these two species of Leishmania in Kenya (Githure et al., 1986a).

The immunological differentiation of Leishmania has gained new dimensions with the production of monoclonal antibodies that can distinguish between various species and subspecies. McMahon-Pratt & David (1981) developed monoclonal antibodies that distinguish between L. mexicana and L. braziliensis, parasites that cause

simple cutaneous and muco-cutaneous leishmaniases, respectively. This technique has clearly differentiated L. major, L. tropica, L. donovani, L. enriettii and L. mexicana (McMahon-Pratt & David, 1981).

1.5.2. Biochemical techniques

Leishmania can be identified by comparing the banding patterns of restriction endonuclease-generated kDNA fragments separated by gel electrophoresis. Hybridization of labelled kDNA probes from known species with kDNA from unknown isolates immobilized on nitrocellulose filter paper can also be used to identify isolates of Leishmania. This technique has been shown to be useful in distinguishing between the Old World species of Leishmania (Jackson et al., 1984; Lawrie et al., 1985).

Isoenzyme characterization of leishmanias is the most widely used technique in solving problems related to the taxonomy and epidemiology of leishmaniases. Parasites possessing different enzyme variants of a particular enzyme are genetically different. Isoenzyme electrophoresis is a technique by which isoenzymes are subjected to an electric field in order to separate them according to their charge. Support matrices, such as starch gel, polyacrylamide gel and cellulose

acetate, provide a molecular sieving property which enhances separation. The separated isoenzymes show as bands when stained with appropriate reagents. Each band is a distinct area of enzyme activity on the gel and its position can be defined relative to a reference band.

A practical improvement in the determination of enzyme variations within *Leishmania* spp. has been the introduction of the simple and quick cellulose acetate electrophoresis (Kreutzer & Christensen, 1980; Lanham et al., 1981) rather than starch or polyacrylamide gel techniques which are time-consuming and expensive to run.

With isoenzyme techniques, a number of Old World *Leishmania* stocks, including stocks from Africa and the Middle East, have been extensively studied (Al-Taqi & Evans, 1978; Chance et al., 1978; Rassam et al., 1979; Schnur et al., 1981). In the present study, isoenzyme technique was employed in the characterization of *Leishmania* isolates because it is inexpensive, simple, and can differentiate all the *Leishmania* parasites found in Kenya.

1.6. Animal reservoirs of leishmaniasis in Kenya

Visceral leishmaniasis was first reported in Kenya in 1941 but investigations on the disease did not start

until the early 1950s (Heisch, 1954). The disease occurs predominantly in semi-arid, sparsely populated areas which lie at an altitude of between 900 and 1200m. The disease is found in parts of the Kitui, Machakos, Meru, West Pokot, Baringo and Turkana districts. It is believed to have been introduced into Meru, Machakos and Kitui districts by troops returning from Ethiopia after the Second World War (Cole et al., 1942) and into the Turkana, West Pokot and Baringo districts by cattle traders from the Sudan and Ethiopia (McKinnon & Fendall, 1955). It has been suggested that the epidemics reported in some of these areas in 1950s and 1970s arose as a result of man interfering with the presumed zoonotic cycle of the disease (Ngoka & Mutinga, 1978b).

1.6.1. Outbreaks of visceral leishmaniasis

Visceral leishmaniasis became a notifiable disease by the Ministry of Health, Kenya in 1957 based on the demonstration of the parasites. There are several factors associated with visceral leishmaniasis in Kenya which have contributed to its spread, such as proximity of homesteads to termite hills which are believed to be breeding and resting sites for sandflies (Wijers, 1963), sharing of huts with goats and calves which may attract sandflies inside houses (Southgate & Oriedo,

1962), proximity of cattle corrals to homesteads whereby the cattle dung may provide suitable habitat for sandfly breeding (personal observation), hot climate which encourages people to stay outdoors late in the evenings thereby exposing themselves to sandfly bites (personal observation), and proximity of rodent burrows to human dwellings which provide resting and breeding sites for sandflies (Beach et al., 1984).

There appears to be a lower prevalence of visceral leishmaniasis in Kenya than in countries like India. During the reported outbreak of visceral leishmaniasis in Tseikuru in Kitui District in 1980, only 120 (0.62%) cases of active visceral leishmaniasis were confirmed by splenic puncture of 3,669 patients with splenomegaly, in a population of 19,225 (Mbugua & Siongok, 1981). In Marigat, Baringo District, a survey of 5,459 inhabitants yielded 1,145 individuals with splenomegaly of whom 25 (0.46%) were positive for visceral leishmaniasis (Leeuwenburg et al., 1983). An apparent epidemic of visceral leishmaniasis was described by Heisch (1954) in Kitui District in about 1,500 people who were said to have suffered from the disease based on splenomegaly. Another epidemic, reported in about 2,000 people in Machakos and Kitui districts in 1977-80 (Ngoka & Mutinga, 1978a) was also based on splenomegaly. The question of how many of

these people had spleen enlargement due to visceral leishmaniasis and how many due to malaria should be considered when interpreting the epidemics reported in Kenya.

1.6.2. Criteria for incriminating an animal reservoir

The occurrence of epidemics suggest the existence of an animal reservoir. The search for reservoirs in Kenya has been going on since the early 1950s. The demonstration of leishmanial parasites either in stained smears or culture made from wild or domestic animals is not by itself sufficient to incriminate an animal as a reservoir host of leishmaniasis. The parasite may be one that is never found in man, for example, *L. enriettii*, *L. hertigi*, or *L. gerbilli* of rodents. On the other hand, the parasite might be a human pathogen in an accidental host. It is therefore very important that parasites isolated from animals and sandflies are characterized and compared with isolates from man. Even if isolates from animals are identical to those from man, further investigations are necessary before an animal can be incriminated as a reservoir. Unfortunately, this is not always done and some investigators have incriminated animals as reservoirs based on a single isolation of a parasites that resemble those found in man. The fact that these

animals live in close contact with man, or that they are reservoirs in other foci, is not enough evidence to incriminate them beyond doubt. For example, the importance of dogs as reservoirs of visceral leishmaniasis in Kenya cannot be judged without further information on the prevalence of infection in dogs, the contact between dogs and vectors and the course of infection in the naturally infected dogs.

A WHO Expert Committee (WHO, 1984) drew up some criteria that should be considered before incriminating an animal as a reservoir host of leishmaniasis. These are:

(a) A reservoir host should be sufficiently abundant, long-lived, and be a major food source for the vector.

(b) Parasites isolated from the skin and viscera of animals should be identical to isolates from man.

(c) The course of infection in a reservoir host should be long and relatively nonpathogenic, and the parasites should be readily available to the vector through the skin or blood of the animal so that a high proportion of sandflies feeding on it become infected.

(d) The reservoir's resting and breeding places should provide suitable microhabitats for the vector.

(e) The proportion of the animals becoming infected during their life should usually be considerable, in certain situations exceeding 20%.

Generally, repeated isolation of the same parasite that causes disease in man is enough evidence to incriminate a reservoir. Rare infections are probably accidental and represent dead ends for the parasite.

1.6.3. <u>Isolation of Leishmania from animals in Kenya</u>

The search for animal reservoirs in Kenya began in the early 1950s and is still in progress. A summary of the reports of all isolates from mammals in Kenya is given in Table 1. McKinnon & Fendal (1955) reported that no cases of canine leishmaniasis were recorded by the Veterinary Department in Baringo District prior to commencement of their studies on animal reservoirs. Heisch (1954) examined over 100 dogs, 330 gerbils, 30 mongooses and 30 jackals for leishmaniasis in Kitui and Machakos districts with negative results except for infections observed in hamsters 10 months after they were inoculated with the pooled spleens of three mongooses. He however, doubted the validity of these infections and cautioned that the hamsters could have been mixed up with others previously infected with <u>L. donovani</u> which were kept in the same room. He however isolated trypanosomes from the spleen of a mongoose,

Table 1

Summary of leishmanial parasites isolated from mammals in Kenya

Leishmania	Animal sp.	No. +ve	Locality	References
L. major	Squirrel	1/161*	Baringo	Heisch, 1957
L. major	Gerbil	3/345	W. Pokot	Heisch et al. 1959
L. major	Gerbil	1/27	Baringo	Ngoka & Mutinga,1978b
L. major	Gerbil	7/166	Baringo	Githure et al. 1984a
L. major	Grass rat	5/466	Baringo	Githure et al. 1984a
L. major	Malmmate rat	2/51	Baringo	Githure et al. 1984a
L. major	Emini gerbil	2/17	Baringo	Githure et al. 1984a
L. major	Bush rat	1/2	Baringo	Githure et al. 1984a
L. major	Monkey	1/30	Kiambu	Binhazim et al. 1987
L. aethiopica	Hyrax	3/36	Bungoma	Mutinga, 1975
L. aethiopica	Giant rat	1/83	Bungoma	Mutinga, 1975
L. donovani	Dog	1/288	Machakos	Mutinga et al. 1980
Flagellates+	Dog	1/80	W. Pokot	Ngoka & Mutinga,1978b
Flagellates+	Bat	3/104	Bungoma	Mutinga, 1975
Flagellates+	Mongoose	4/12	Kitui	Mutinga et al. 1982
Flagellates+	Genet cat	1/12	Kitui	Mutinga et al. 1982
Crithidia sp.	Dog	1/63	Turkana	Githure et al. 1986c

* No. of animals positive/No. of animals examined.

+ Uncharacterized isolates.

Helogale parvula which were typed as *Trypanosoma helogalae* (Grewal, 1960).

In these earlier studies, only the smears of organs were examined and no cultures were attempted because of prevailing adverse conditions at the time. As a result, occult infections in these animals could have been overlooked. Nevertheless, isolations were made. Heisch (1963) reviewed the investigations done on animal reservoirs of visceral leishmaniasis since 1952 and compiled a list of 31 animal species of which a total of 1,780 specimens had been examined. These comprised of 1,419 rodents, 103 dogs, 68 bats, 56 jackals, 56 bovids, 46 mongooses, 10 genet cats and 10 hyraxes. All were found to be negative for *Leishmania* on smears, cultures and hamster inoculation except for 3 of 345 *Tatera robusta* (Heisch et al., 1959) and one of 161 *Xerus rutilus* (Heisch, 1957), splenic samples of which were inoculated into hamsters. These isolates were at that time believed to be *L. donovani* and immunization studies against visceral leishmaniasis were conducted using these strains. The parasites caused nodules on human volunteers but did not viscerilize (Manson-Bahr, 1959). They were later identified as *L. major* (Chance et al., 1978). These were the first isolates of *L. major* from rodents in Kenya.

Ngoka & Mutinga (1978b) working on reservoirs in Baringo and West Pokot districts examined 700 animals including 510 rodents, 80 dogs, 54 mongooses, 26 genet cats, 12 monkeys, 9 jackal and 9 bovids. They isolated *L. major* from one out of 27 *Tatera robusta* examined, and flagellates from one out of 80 dogs examined. The isolate from the dog was not characterized and although promastigotes and amastigotes were observed in cultures and smears respectively, these could have been either *Leishmania* or *Trypanosoma*. It would be interesting to re-examine the dog isolate, if it was cryopreserved, to determine its identity. This is because it has been reported that some strains of trypanosomes of the subgenus *Herpetosoma* divide as amastigote forms in the visceral organs and transform into epimastigotes in cultures (Ormerod & Killick-Kendrick, 1956; Molyneux, 1969).

Githure et al. (1984a) examined 789 rodents belonging to ten different genera in Baringo District and isolated *L. major* from 7 of 168 *Tatera robusta*, 5 of 466 *Arvicanthis niloticus*, 2 of 51 *Mastomys natalensis*, 2 of 17 *Taterillus emini* and one of 2 *Aethomys kaiseri*. Isolates from these rodents, *P. duboscqi*, and man, all from Baringo District were characterized as *L. major* by isoenzyme analysis of 12 enzymes, thereby establishing cutaneous leishmaniasis caused by *L. major* as a zoonosis

(Beach et al., 1984; Githure et al., 1986a; Muigai et al., 1987).

Southgate & Oriedo (1962) showed that visceral leishmaniasis in Kitui and Machakos districts occurred more frequently in people sleeping in the company of dogs and goats than in the general population. They speculated that dogs and goats sleeping near or inside huts might act as bait for the sandflies which are thus brought into closer contact with man whom they preferentially bite. This hypothesis has not been verified. Surveys for animals reservoirs in Machakos and Kitui districts were all negative except for the isolation of *L. adleri* from lizards (Heisch, 1958) which was found to be nonpathogenic to man (Manson-Bahr, 1959).

Later, Mutinga (1971) incriminated *P. pedifer* as the vector of *L. aethiopica* in the Mt. Elgon area and Kungu et al. (1972) isolated *L. aethiopica* from man in the same area. In a search for the reservoir of cutaneous leishmaniasis, the former author examined a total of 893 animals including 679 rodents, 104 bats, 70 dogs, 36 hyraxes, 2 mongooses and 2 lizards (Mutinga, 1975). *Leishmania aethiopica* was isolated from 3 of 36 hyraxes (*Procavia* sp.) and 1 of 83 giant rats (*Cricetomys* sp.). The isolates from the hyraxes, *P. pedifer* and man were biochemically indistinguishable (Chance et al., 1978).

In subsequent studies, investigations of animal reservoirs concentrated on dogs and small mammals living close to human habitations because rodents were found naturally infected with L. donovani in the neighbouring Sudan (Hoogstraal & Heyneman, 1969) and dogs were proved to be reservoirs of visceral leishmaniasis in the Mediterranean region (Mansour et al., 1970).

Recently, several isolates were made from wild and domestic animals in Kenya (Table 1) but to date, there has been only one confirmed report of a dog naturally infected with L. donovani in Machakos District. Mutinga et al. (1980) examined 288 emaciated dogs in the Machakos District and found flagellates in the spleens of two dogs. One of the isolates was characterized as L. donovani whereas the other one was thought to be a trypanosome (Schnur, personal communication). This L. donovani isolate was identical to isolates from man in the same area and from P. martini from Kitui (Minter et al., 1962; Chance et al., 1978). Since this was an isolated instance, it does not qualify the dog as a reservoir for L. donovani in Kenya, and further investigations are required.

Mutinga et al. (1982) examined 100 carnivores in Kitui District and found four mongooses of 12 examined and one of 12 genet cats examined to be positive for flagellates. Seventy-six dogs examined were all

negative for flagellates. Unfortunately, no reports are available on the characterization of these isolates for further identification, hence it is not known whether they were actually Leishmania, Trypanosoma or any other flagellates. In 1982, Lightner & Githure (unpublished data) examined 63 dogs, 4 jackals, 4 hyenas and 6 bovids from Turkana District and isolated flagellates from one dog. These were found to be different from five WHO Leishmania reference strains based on 12 isoenzymes but similar in morphology to Crithidia spp. and also similar to isolates from rats and dogs in Egypt (Morsey et al., 1985; Githure et al., 1986c).

In summary, several animal species in Kenya have been examined for Leishmania over the last four decades. So far, only rodents and hyraxes can be incriminated as reservoirs of cutaneous leishmaniasis caused by L. major and L. aethiopica respectively. No animal has to date been incriminated as a reservoir of visceral leishmaniasis in Kenya.

1.7. Aims and Objectives of the study

An observation that can be drawn from leishmaniasis studies in Kenya is that it is very important to cryopreserve all isolates from humans, sandflies and animals for future reference and to facilitate a better understanding of the epidemiology of the disease. Very

few isolates made during early investigations were cryopreserved or well documented making it virtually impossible to verify some of the reported findings.

The general purpose of this study was to identify animal reservoir(s) of leishmaniasis in Kenya. The specific objectives were to:

(a) Isolate leishmanial parasites from wild and domestic animals in search of animal reservoirs.

(b) Investigate the susceptibility of potential animal reservoirs to experimental infection with *L. donovani*.

(c) Identify isolates from animals, sandflies and man using isoenzyme technique.

(d) Consolidate all the information on animal reservoirs in order to re-evaluate the epidemiology of leishmaniasis in Kenya.

To achieve these objectives, two geographically separate endemic foci of leishmaniasis were selected for study. These areas are: Marigat in Baringo District, Rift Valley Province and Masinga in Machakos District, Eastern Province, Kenya. Animals were trapped and examined for leishmanial infection by culturing in NNN medium, the spleen, liver, bone marrow, and a piece of skin from nose and ear and by making impression smears of these organs. Resulting isolates were cryopreserved in liquid nitrogen and later characterized by isoenzyme analysis.

This thesis is therefore arranged so as to answer the points raised above. Chapter 2 deals with isolation of leishmanial parasites from wild and domestic animals while Chapter 3 is involved in investigation of susceptibility of wild and domestic animals to infection with *L. donovani*. Identification of isolates using isoenzyme analysis is dealt with in Chapter 4 and the evaluation of reservoir studies of visceral leishmaniasis is consolidated in Chapter 5.

Chapter 2

ISOLATION OF *LEISHMANIA* FROM ANIMALS IN BARINGO AND MACHAKOS DISTRICTS, KENYA

2.1. INTRODUCTION

In some parts of the Old World, several animal species including dogs, foxes and rodents are found naturally infected with *Leishmania* (Hommel, 1978) and many of them serve as natural reservoirs for the human disease. In most cases man is generally a poor source of leishmanial parasites for the vectors and becomes infected when he interferes with the reservoir/sandfly association. In the Mediterranean region, China and South America, wild and domestic dogs are naturally infected with *L. d. infantum*, *L. donovani* and *L. d. chagasi* respectively (Levine, 1973). The rate of infection in dogs may reach 20% in some countries and infection rate as high as 40% have been reported in the Mediterranean region where the human infection rate is only 1-2% (Levine, 1973). It has been reported that dogs with visceral leishmaniasis in this region have demonstrable parasites in the skin, blood and all visceral organs (Mansour et al., 1970). Dog control in China led to a drastic reduction in human cases indicating that the dog is an important reservoir of human infection in that part of the World (Leng Yan-Jia, 1982).

In Kenya, the search for animal reservoirs of leishmaniasis has resulted in the identification of rock hyraxes as the reservoirs of *L. aethiopica* (Mutinga,

1975) and rodents as reservoirs of *L. major* (Heisch, 1963; Ngoka & Mutinga, 1978b; Githure et al., 1986a). In addition, a vervet monkey was found naturally infected with *L. major* in Thika (Binhazim et al., 1987), and a dog was found naturally infected with *L. donovani* in Machakos District (Mutinga et al., 1980). In the neighbouring Sudan, *Rattus rattus*, *Acomys* sp. and *Arvicanthis niloticus* were found naturally infected with *L. donovani* (Hoogstraal & Heyneman, 1969; Chance et al., 1978) which is probably different from *L. donovani* in Kenya. *Rattus rattus* were also found naturally infected with *L. d. infantum* in Italy (Bettini et al., 1980). The importance of isolations of *L. donovani* from these areas is difficult to interpret in epidemiological terms because the relative numbers of positive animals examined was too small to conclude that they are in fact reservoirs of the disease. Furthermore, the feeding behaviour of the sandfly vector on these animals is poorly known.

In Kenya, experimental infection of wild rodents, *Mastomys natalensis*, *Rattus rattus* and *Heterocephalus glaber* has shown these rodents to be highly susceptible to *L. donovani* infection (Githure, 1981; Shatry et al., 1987a; Githure et al., 1988).

The hypothesis advanced for this study was that, since rodents have been found to be susceptible to

experimental infection with Kenyan L. donovani strains and since some rodents and dogs have been found naturally infected with this parasite in neighbouring Sudan and elsewhere, it is likely that they could also be found naturally infected in Kenya as well.

The objectives of this study were, to isolate and cryopreserve leishmanial parasites from wild and domestic animals in Baringo and Machakos districts of Kenya, for future reference and to search for animal reservoir(s) of visceral leishmaniasis in these regions.

2.2 MATERIALS AND METHODS

2.2.1. Study Areas

Small mammals were trapped in two leishmaniasis endemic areas: Marigat in Baringo District, Rift Valley Province and Masinga in Machakos District, Eastern Province of Kenya. These areas were selected because (a), investigations on sandfly vectors and case-finding surveys for human infections had been carried out by KEMRI scientists in these areas since 1980 but thorough studies on animal reservoirs were lacking due to inadequate isolation methods, (b), both study areas had experienced a recent population influx due to new job opportunities created by local developments; in Marigat, the Perkerra Irrigation Scheme, and in Masinga, the construction of Masinga Dam on the Tana River, (c), it

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had previously been reported that epidemics of visceral leishmaniasis in these areas was as a result of man interfering with the fly-animal-fly contact of *L. donovani*, and (d), both areas are accessible by good roads all year round.

Marigat Location

Marigat lies approximately 0°28'N and 35°58'E, about 260km north-west of Nairobi (Fig. 1). The town of Marigat lies inside the Rift Valley at an altitude of 1,067m. The study site was the Perkerra Irrigation Scheme and its environs, situated 3km east of Marigat town. This area is relatively flat and lies between the Kamasia Hills to the west and the Laikipia Escarpment to the east both of which rise to about 2,500m above sea level. Two permanent water courses, the Perkerra and Molo rivers traverse the area and empty into Lake Baringo, about 20km to the north of Marigat town. The annual mean minimum and maximum temperatures are 17.3 and 33.3°C; total rainfall received during the study was 1,052.6mm (Appendix 2a).

The area has sandy loam and alluvial-type friable soil which is ideal for burrowing animals. To the south, towards Lake Bogoria, the soil is volcanic with many rocky hills. The land is characterized by cultivated fields bordered by tall open *Acacia*-*Balanite*

Plate 2a

Part of Marigat study area during the dry season.

Plate 2b

Part of Perkerra Irrigation Scheme in Marigat showing banana and maize plantations.

2a

2b

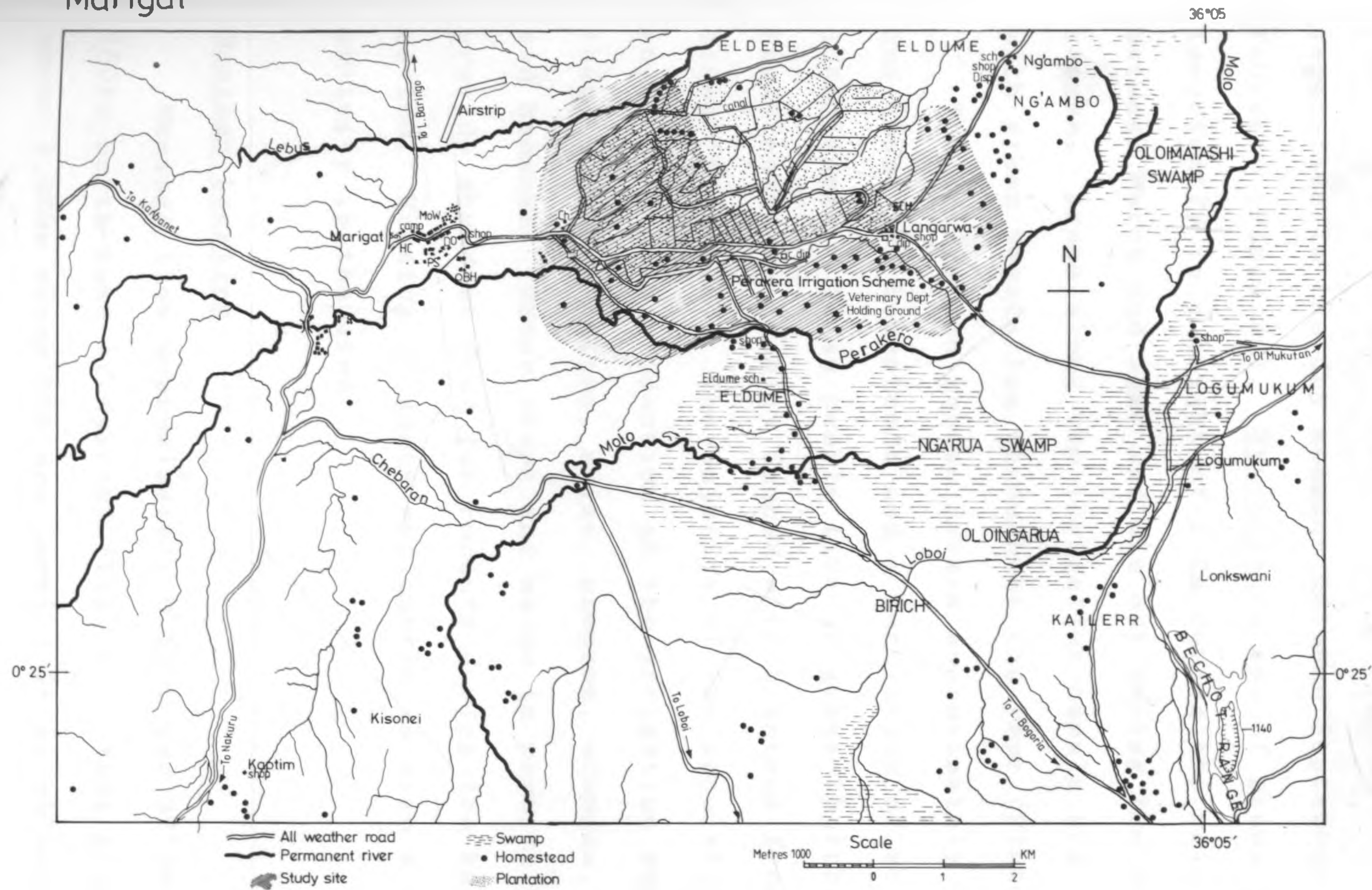


Fig. 1

woodland and thorny bushes, with little ground vegetation in the dry seasons between September and February (Plate 2a). *Hyperrhania* sp. of grass grows luxuriantly to a height of 1-2m during the rainy season between March and June and die out during the dry season. Areas along the irrigation canals are covered with green vegetation throughout the year (Plate 2b).

The irrigation scheme covers approximately $6km^2$ and has about 180 homesteads and a population of about 1,100 inhabitants. These people live in grass-thatched huts. Homesteads are usually fenced with branches from *Acacia* trees (Plate 2a). The majority of the population are engaged in crop production at the irrigation scheme; these includes onions, maize, pawpaws, oranges, cabbages and bananas. These crops and maize in particular, provide abundant food for rodents during the harvest period. Herding of goats and cattle is also a common activity in the area.

Masinga Location

Masinga lies approximately 0°53'S and 37°36'E, about 150km north-east of Nairobi (Fig. 2). This area lies about 1,088m above the sea level. It is situated south of the Tana River and is adjacent to the Masinga Dam which was constructed in 1977-1981. The mean annual minimum and maximum temperatures are 17.6 and 29.3°C.

Masinga

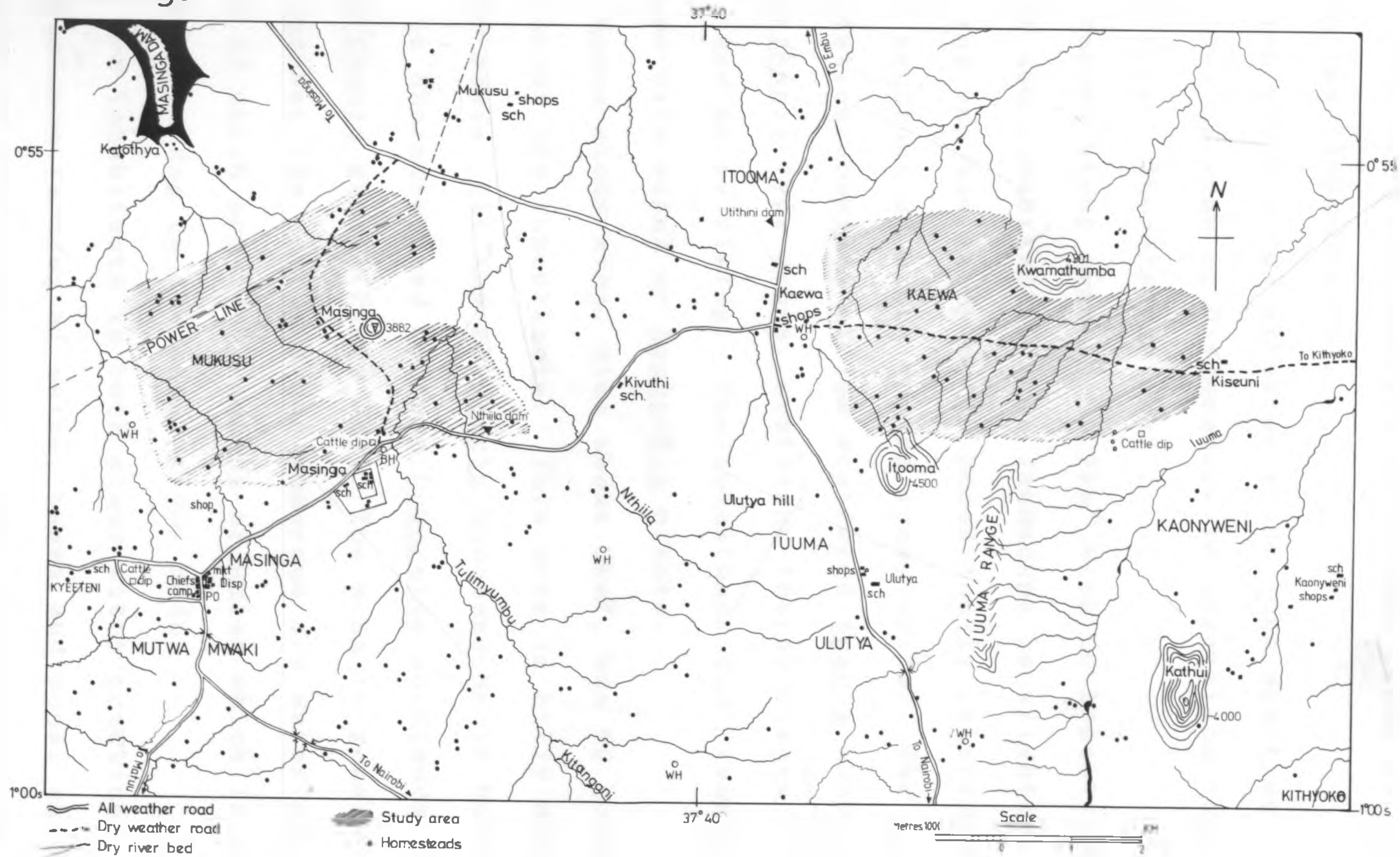


Fig. 2

The total recorded rainfall during the study period was 1,268.3mm (Appendix 2b).

Two study sites situated about 10km apart were selected because of differences in vegetation cover and soil type. These areas are:

(a) Mukusu study area about 3km^2 with 60 homesteads and about 450 inhabitants. The landscape is slightly sloping and has sandy soils occasionally interspersed with sections of black cotton loamy soils. The vegetation consists of low scattered Acacia woodland, Hyperrhania type grass, scattered thorny bushes (Plate 3a), and maize fields. The homesteads and "shambas" are fenced with sisal or Euphorbia plants.

(b) Kaewa study area, also about 3km^2, has 50 homesteads and about 350 inhabitants. This area is hilly with loamy sands. It has no Acacia woodland as in Mukusu, but is characterized by open Combretum woodlands and Hyperrhania grass (Plate 3b). The area is generally dry throughout the year and has numerous dry water courses, some of which provide a source of water which is scooped from holes dug in the sandy river beds.

The inhabitants in both study sites practice subsistence farming of maize, beans and peas, and also herding of goats and cattle. Most people live in grass-thatched huts (Plate 3b) while a few live in houses with brick walls and roofed with corrugated iron sheets (Plate 3a).

Plate 3a

Mukusu study area in Masinga showing *Acacia* trees, sisal hedge and different types of soils.

Plate 3b

Kaewa study area in Masinga showing *Combretum* woodland and sandy soil.

3a

3b

2.2.2. Preparation of culture media

Schneider's medium was obtained commercially from GIBCO (Grand Island Biological Company, USA) lyopholized in 1 litre sachets. This was reconstituted with 1000ml distilled water just prior to use, and supplemented with $NaHCO_3$=0.4gm, cystine=0.1gm, tyrosine=0.5gm and yeastolate=2.0gm. The medium was then filter-sterilized by passing through 0.2µm Nalgene millipore filters (Nalge, California, USA) under vaccum pressure. It was further supplemented with 20% heat-inactivated fetal bovine serum (Flow Laboratories, UK), antibiotics (250U/ml penicillin, 250µg/ml streptomycin, 250µg/ml gentamycin), and antifungal (500µg/ml 5-fluorocytosine).

NNN diphasic medium was prepared by adding 4gm blood-agar base (DIFCO Laboratories, Detroit, Michigan, USA) into 85ml distilled water. This was autoclaved at 121°C for 20 minutes and allowed to cool to about 45°C. Blood obtained aseptically from a rabbit by cardiac puncture was defibrinated by shaking vigorously in a sterile bottle containing a few glass beads for about 10 minutes. The defibrinated blood was transfered to a sterile flask and haemolysed by freeze-thawing three times. Fifteen ml of this blood was added to 85ml of the warm agar and mixed by swirling gently. Aliquots of 1.5ml of this mixture were then dispensed into tissue culture ambitubes (Miles Scientific Inc, IL, USA) and

allowed to solidify as slants. A 1ml overlay of Schneider's medium, prepared as described above, was added to the slants just prior to use.

2.2.3. Trapping animals

Two types of traps were used to trap animals: Wire cage traps measuring 15.2 x 17.7 x 28cm (Kenya Food Association, Nairobi) (Plate 4a) and Havahart traps measuring 17.7 x 17.7 x 76cm (Lititz, PA, USA) (Plates 4b). Between twenty and thirty-five traps (maximum of 30 wire cage traps and 5 Havahart traps) were set for 4-5 nights every month for a period of 16 months in each study site from May 1986 to November 1987 in Marigat and from December 1986 to May 1988 in Masinga. Traps were set out in the evenings beside rodent runs and burrow openings, inside and outside homesteads, along fences, around termite mounds, in and around cattle 'bomas' and under shrubs, and checked in the mornings for nocturnal animals. They were re-set in the mornings and re-checked in the evenings for diurnal animals. Maize-flour paste (Ugali), found to be attractive to rodents, was used as bait. The trapping frequency in each month was calculated as the number of animals trapped divided by the number of traps used multiplied by the trap-nights. The trapped animals were put in plastic holding cages with wire lids and fed and watered

Plate 4a

Wire cage trap (arrow) set near a termite mound in Masinga study area.

Plate 4b

Havahart trap set near a rodent burrow in Marigat study area.

4a

4b

ad libitum until necropsy, either in a field laboratory or in the KEMRI laboratory in Nairobi.

2.2.4. Isolating flagellates from small mammals

Trapped animals were sprayed with Permethrin (Cooper, Wellcome, UK) and combed to remove ectoparasites. They were then killed with chloroform, identified, sexed, and examined for any signs of cutaneous lesions, which when seen were aspirated according to the method of Hendricks & Wright (1979). The animals were then soaked in a tray half-filled with a mixture of 70% ethanol and 5% centrimide to kill bacteria on the skin, and necropsied. Individual cultures of the spleen, and liver were made by macerating the organs with a pair of scissors and inoculating into NNN medium. Pieces of skin were removed from the nose and ear, cut into tiny pieces with a pair of scissors and inoculated into NNN culture medium. Bone-marrow aspirates were made by severing the femur with scissors and withdrawing the marrow with a 3ml syringe fitted with a 23 gauge needle and inoculating into NNN medium. Smears of the organs and aspirates were also made and stained with Giemsa. The smears were examined for amastigotes under oil immersion at x100 magnification. The cultures were incubated at 25°C in a Precision Low Incubator (Chicago, USA) and

examined daily for 14 days using an inverted microscope (Leitz, W. Germany) to detect promastigotes. Cultures were considered negative if no promastigotes were seen within 14 days of incubation. Resulting isolates were assigned a Nairobi Leishmania Bank number and cryopreserved in liquid nitrogen until required.

2.2.5. Isolating flagellates from dogs

Homesteads with dogs were visited and the owners requested to restrain their dogs. Dogs were anaesthetized using 2-(2,6-xylidino)-5,6-dihydron-4H-1,3-thiazine hydrochloride (Rompun® solution 2%) (Bayer, Leverkusen, Pakistan) at a dosage of 1.0ml/10kg body weight injected intramuscularly in the thigh muscle. For the purpose of follow-up in case a dog was found positive, the name of the owner, sex and name of the dog and the exact area of residence were recorded. Splenic and liver aspirates were performed with a 10ml syringe fitted with a 21 gauge needle. These aspirates were cultured individually in NNN medium. Smears of the aspirates were also made and stained with Giemsa. Examination of cultures and smears was done as described in the above section.

2.2.6. Isolating Leishmania from humans

The Clinical Research Centre (CRC) of KEMRI conducts

case-finding surveys for leishmaniasis patients in Marigat and Masinga. In addition, patients from these and other endemic foci are routinely referred to CRC in Nairobi by the local health officials for treatment. Thirty-four human isolates, 19 from Marigat, and 15 from Masinga were included in the study for comparison with animal isolates. The isolation of parasites from the spleen of patients was done by the medical officers at CRC according to the method prescribed by WHO (1984). Percutaneous spleen aspiration was performed with a 21 gauge needle fitted with a 10ml syringe and a portion of the aspirate was inoculated into NNN medium overlayed with Schneider's medium and the rest used to make smears. Isolation of parasites from cutaneous lesions on patients was done according to the method of Hendricks & Wright (1979). In patients with lesions, 0.1ml of sterile saline was injected intradermally into a lesion with a 26 gauge needle fitted with a 2ml syringe. The injected saline was withdrawn from the lesion and used to inoculate into NNN medium and to make smears. The cultures and smears were examined as described above.

2.2.7. Isolating flagellates from lizards

Trapped lizards were killed with chloroform and then bled intracardially using a 2ml syringe fitted with a 23

gauge needle. A drop of blood was cultured in NNN medium and the rest used to make thin and thick blood smears. Cultures and smears were examined as described above.

2.2.8. Isolating flagellates from sandflies

Isolates from sandflies collected in Marigat were included in the study for comparison with animal isolates. No isolates from sandflies from Masinga were available during the study. Isolation and examination of flagellates from sandflies was done according to the method of Perkins et al. (1988). The flies were dissected and if flagellates were seen upon examination of the digestive tract, the flagellates were aspirated with a 1ml syringe and injected into a 5ml vaccine vial containing 2ml Schneider's medium. The vials were transported to Nairobi within 2 days of parasite isolation where the cultures were subcultured into four separate tubes, two of which contained NNN medium and two with Schneider's medium. The cultures were examined as described above.

2.2.9. Cryopreserving Leishmania and other flagellates

Isolates from people, wild animals and sandflies were grown to a concentration of about 10^8 promastigotes

Plate 5

Liquid nitrogen refrigeration unit showing (a) Cryo-med programmable freezer (b) storage cylinders (c) reservoir tanks.

5

per ml in Schneider's medium. The parasite suspension was mixed with an equal volume of Schneider's medium containing 15% Dimethyl Sulfoxide (DMSO) (Sigma Chemicals CO. USA) to give a final concentration of 7.5% DMSO. The mixture was allowed to stabilize at room temperature for about 30 min after which 1ml was dispensed into 2ml cryotubes (Nunc Int-Med, Denmark). The tubes were labelled with date and Nairobi Leishmania Bank number and then cryopreserved using a programmable Cryo-Med freezer (MVE, New Prague, MN, USA) which was connected to a liquid nitrogen supply tank and programmed to freeze the parasites at the rate of 1°C per minute down to -80°C (Githure et al., 1984b; Plate 5). They were then removed from the freezer and stored in liquid nitrogen at a temperature of -196°C. The isolates were labelled with a 4-element coding system recommended by the WHO (Chance & Walton, 1982). A detailed history of each isolate and location in the cryobank was recorded on standard WHO stabilate forms and the information stored in an IBM Personal Computer for easy access and retrieval.

2.3. RESULTS

2.3.1. Animals trapped in Marigat

Trapping of animals in Marigat was conducted for a period of 16 months, from May 1986 to Nov 1987. During

and after the wet season from March through June 87, the study site was covered with fast growing vegetation consisting of *Hyperrania* sp. of grass and shrubs. This vegetation thrived for about three months after the long rains and dried out during the dry season from October through February. The trapping frequencies of the most commonly trapped rodents, namely, *Arvicanthis*, *Tatera*, *Mastomys* and *Acomys* are shown in Table 2. A peak trapping frequency for *Arvicanthis*, *Tatera*, and *Mastomys* was observed in September indicating that breeding was intense soon after the long rains in April-June. A number of pregnant females and young *Tatera*, *Mastomys* and *Arvicanthis* were trapped in July to September soon after the rains. All the animals trapped were mainly nocturnal except the *Arvicanthis*, *Elephantulus*, *Xerus* and lizards.

A total of 1,128 rodents of 7 genera, 11 canids of 3 genera and 28 lizards of 2 genera were examined. The number of animals per species trapped in this area is shown in Table 3. The majority of the mammals trapped were *Arvicanthis*, with a total of 552, followed by *Tatera* (289), *Mastomys* (256) and *Acomys* (27). *Elephantulus*, *Xerus* and *Crocidura* were rarely trapped. One genet cat and one mongoose were also trapped.

Of the 10 mammalian genera examined, only three were found infected with *Leishmania*. The positive animals

Table 2

Trapping frequency (%) of some rodents trapped in Marigat study area.

Month	Tatera	Arvicanthis	Mastomys	Acomys
May '86	3.8	16.3	2.5	0.0
Jun '86	14.0	18.0	2.0	2.0
Jul '86	14.0	33.0	6.0	4.0
Aug '86	22.0	48.0	0.0	0.0
Sep '86	12.8	47.8	21.7	5.6
Oct '86	6.7	28.0	10.7	4.7
Nov '86	30.7	13.3	0.7	2.0
Dec '86	5.3	35.3	0.0	0.0
Jan '87	8.7	19.3	4.0	0.3
Feb '87	9.3	19.3	21.0	0.3
Mar '87	5.0	25.0	14.2	3.3
Apr '87	8.3	6.7	2.5	0.8
May '87	13.0	8.0	8.0	0.0
Jul '87	28.3	11.7	1.7	1.7
Sep '87	38.3	66.7	55.0	3.3
Nov '87	17.3	35.3	30.7	0.0

were 36 (12.5%) Tatera robusta (Plate 6a), 3 (0.5%) Arvicanthis niloticus (Plate 6b), and 2 (0.8%) Mastomys natalensis (Plate 6c). Twelve (42.9%) lizards were also found positive (Plate 6d,6e). The infection rate in Tatera is shown in Fig. 3. Positive Tatera were trapped in the dry and wet season during the study period. The highest infection rates for Tatera were observed in the months of June, February and November. Very few Arvicanthis and Mastomys were found positive and these too did not show seasonality in infection rate. Only three Arvicanthis were found positive for Leishmania, of which two were trapped in May 1986 and one in May 1987. The two positive Mastomys were trapped in March 1987 and September 1987.

All the slide smears examined were negative for amastigotes.

2.3.2. Animals trapped in Masinga

Trapping of animals in Masinga was also conducted for a period of 16 months from December 1986 to May 1988. In the Mukusu and Kaewa study areas, green vegetation, mainly Hyperrania sp. of grass and shrubs covered the ground shortly after the wet season. This soon dried and died out, leaving only the open short Acacia woodland in Mukusu village and open Combretum woodland in Kaewa study site. The trapping frequencies

Table 3

Animals examined for Leishmania in Marigat, Baringo District.

Animal sp.	Common name	Females	Males	Total	% +ve
Arvicanthis niloticus	Grass rat	1/287*	2/265*	3/552*	0.5
Tatera robusta	Gerbil	22/178	14/111	36/289	12.5
Mastomys natalensis	Multimammate rat	0/130	2/126	2/256	0.8
Acomys subspinosus	Spiny mouse	0/12	0/15	0/27	0.0
Xerus rutilus	Ground squirrel	0/0	0/1	0/1	0.0
Crocidura sp.	Shrew	0/0	0/1	0/1	0.0
Elephantulus rufescens	Elephant shrew	0/0	0/2	0/2	0.0
Genetta genetta	Genet cat	0/1	0/0	0/1	0.0
Canis familiaris	Domestic dog	0/3	0/6	0/9	0.0
Ichneumia albicauda	White-tailed mong	0/0	0/1	0/1	0.0
Latastia longicaudata	Lacertid lizard	-	-	10/25	40.0
Varanus sp.	Monitor lizard	-	-	2/3	66.7

* No. of animals positive for Leishmania/No of animals examined.

of the most commonly trapped rodents, Tatera, Acomys, Mastomys and Lemniscomys, are shown in Table 4. The trapping frequencies indicate that the highest percentage of Mastomys, Tatera and Acomys were trapped from January to May. This high population was observed soon after the short rains in November-January (Appendix 2b). Arvicanthis, Lemniscomys, Xerus, Elephantulus, Helogale and Herpestes were mainly diurnal and the rest of the animals were nocturnal.

A total of 633 rodents belonging to 10 genera and 95 canids belonging to 5 genera were trapped. The most commonly trapped rodent was Tatera (228), followed by Mastomys (143), Acomys (111), Lemniscomys (50), Arvicanthis (46), Xerus (25) and Aethomys (24) (Table 5). Elephant shrews, hedgehogs, mongooses and genet cats were rarely trapped. Arvicanthis was very limited in its distribution, all 46 being trapped in one homestead in Mukusu village.

Six of the 15 mammalian genera examined were found positive for flagellates (Table 5). These includes 67 (60.4%) Acomys (Plate 7a), 12 (8.4%) Mastomys (Plate 6c), 2 (33.3%) Herpestes (Plate 7b), 1 (100%) Helogale (Plate 7c), 2 (4.0%) Lemniscomys (Plate 7d) and 1 (1.2%) Canis (Plate 7e). Also found positive was 1 (20.0%) of the Latastia (Plate 6d) trapped.

The infection rate of Acomys is shown in Fig. 4a.

Plate 6

Rodents and lizards found positive for *Leishmania* in Marigat study area.

(a) *Tatera robusta* (gerbil)

(b) *Arvicanthis niloticus* (grass rat)

(c) *Mastomys natalensis* (multimammate rat)

(d) *Latastia longicaudata* (lizard)

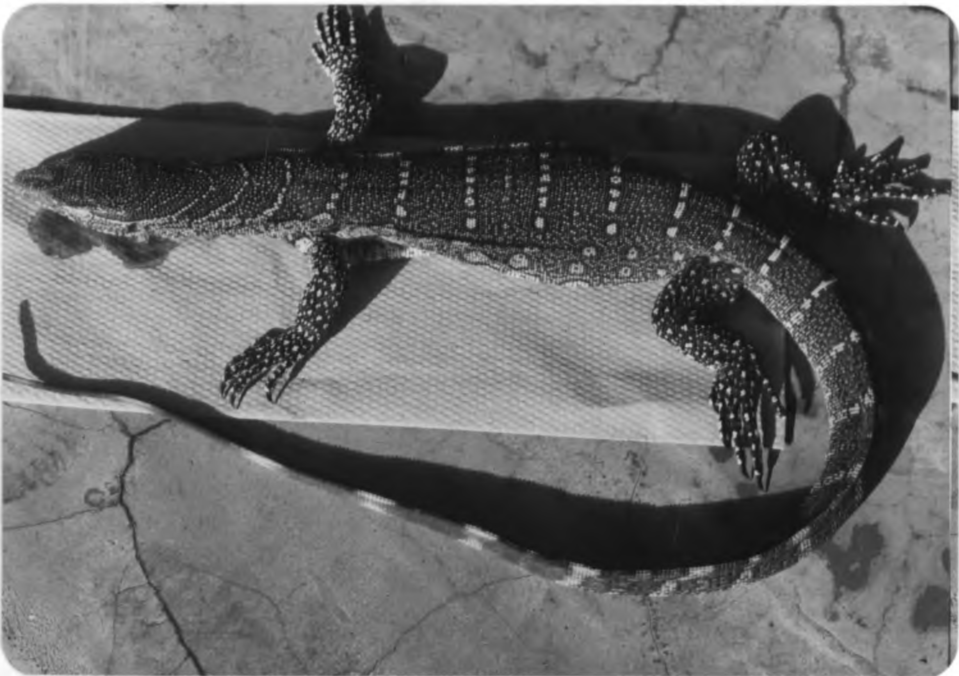
(e) *Varanus* sp. (monitor lizard)

6a

6b

6c

6d



6e

Fig. 3a. *Leishmania* infection rate in *Tatera* spp. trapped in Marigat between May 1986 and November 1987

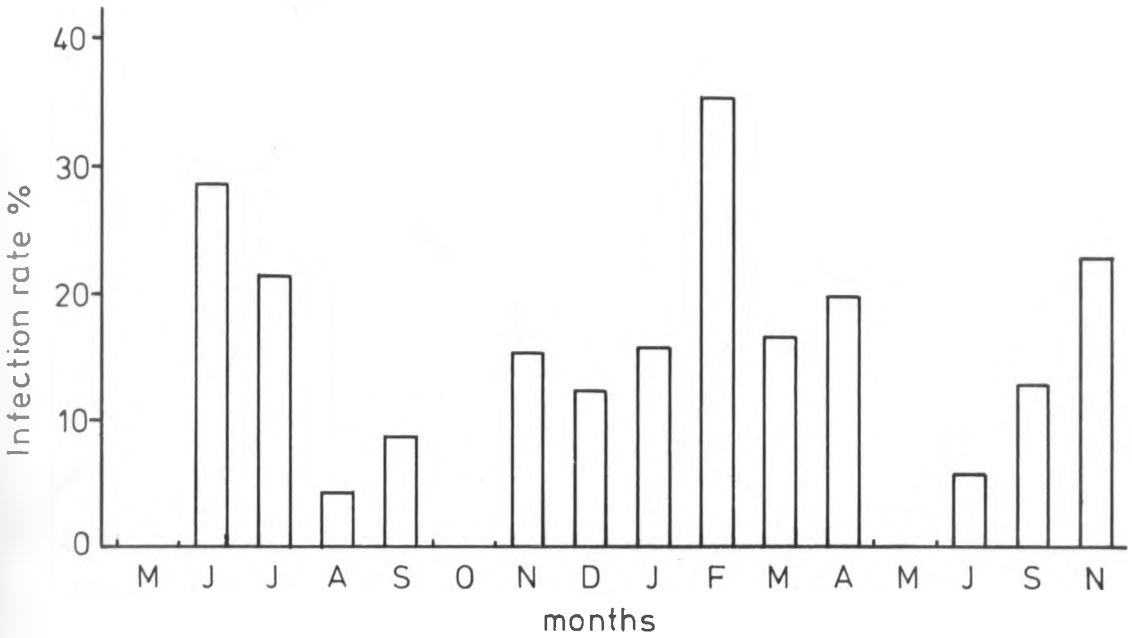


Fig. 3b. Rainfall in Marigat between January 1986 and December 1987

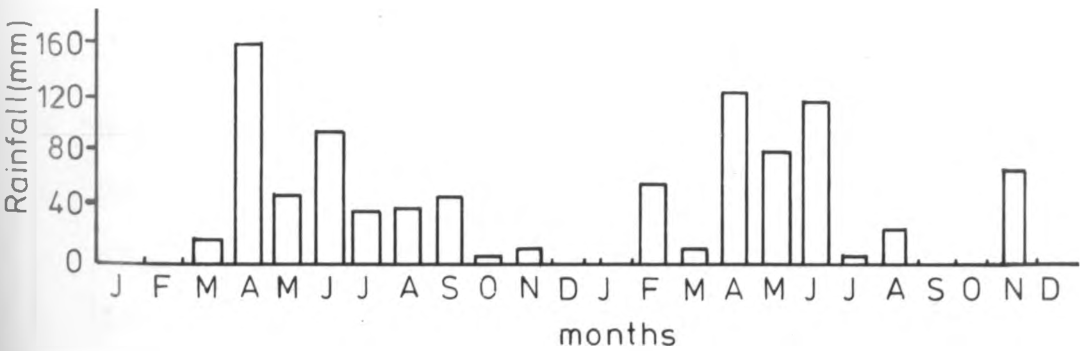


Table 4

Trapping frequency (%) of some rodents trapped in Masinga study area

Month	Acomys	Mastomys	Tatera	Lemniscomys
Dec '86	8.3	6.7	6.7	1.7
Jan '87	1.0	22.0	2.0	0.0
Feb '87	0.0	29.0	12.0	0.0
Mar '87	11.7	5.0	22.5	3.3
Apr '87	19.3	10.7	18.7	9.3
May '87	4.0	19.3	16.7	2.0
Jun '87	8.7	8.0	16.0	3.3
Jul '87	0.8	8.3	6.7	0.0
Aug '87	8.7	6.0	14.0	2.7
Sep '87	5.8	3.3	12.5	4.2
Oct '87	2.5	0.8	17.5	3.3
Nov '87	8.9	1.1	13.3	7.8
Dec '87	3.3	0.0	10.8	0.8
Jan '88	2.5	0.0	17.5	2.5
Mar '88	10.0	1.3	15.0	1.3
May '88	4.0	0.8	16.0	0.8

Table 5

Animals examined for *Leishmania* in Masinga, Machakos District.

Animal sp.	Common name	Females	Males	Total
Tatera robusta	Gerbil	0/119*	0/109*	0/228*
Mastomys natalensis	Multimammate rat	5/78	7/65	12/143
Acomys subspinosus	Spiny mouse	42/67	25/44	67/111
Lemniscomys striatus	Striped mouse	2/29	0/21	2/50
Arvicanthis niloticus	Grass rat	0/34	0/12	0/46
Xerus rutilus	Ground squirrel	0/8	0/5	0/13
Xerus erythropus	Striped G. squirrel	0/5	0/7	0/12
Aethomys kaiseri	Bush rat	0/13	0/11	0/24
Elephantulus rufescens	Elephant shrew	0/4	0/0	0/4
Erinaceus albiventris	Hedgehog	0/0	0/2	0/2
Herpestes sanguineus	Slender mongoose	1/4	1/2	2/6
Ichneumia albicauda	White-tailed mongoose	0/0	0/1	0/1
Helogale parvula	Dwarf mongoose	1/1	0/0	1/1
Canis familiaris	Domestic dog	1/28	0/56	1/84
Genetta genetta	Genet cat	0/1	0/2	0/3
Latastia longicaudata	Lacertid lizard	-	-	1/5

No. of animals positive for flagellates/No. of animals examined.

Plate 7

Rodents and canids found positive for flagellates in Masinga study area.

(a) *Acomys subspinosus* (spiny mouse)

(b) *Herpestes sanguineus* (slender mongoose)

(c) *Helogale parvula* (dwarf mongoose)

(d) *Lemnisomys striatus* (striped mouse)

(e) *Canis familiaris* (domestic dog). Spleen aspirate being taken from a dog in Masinga.

7a

7b

7c

FORWARDAIR
UNITED KINGDOM HEADQUART
TELEX: 933973 CABLES: FORWARDAIR
AIRLINE • AWB No.
DESTINAT
KGS

7d

7e

Fig. 4a Flagellate infection rate in Acomys spp. trapped in Masinga between December 1986 and May 1987

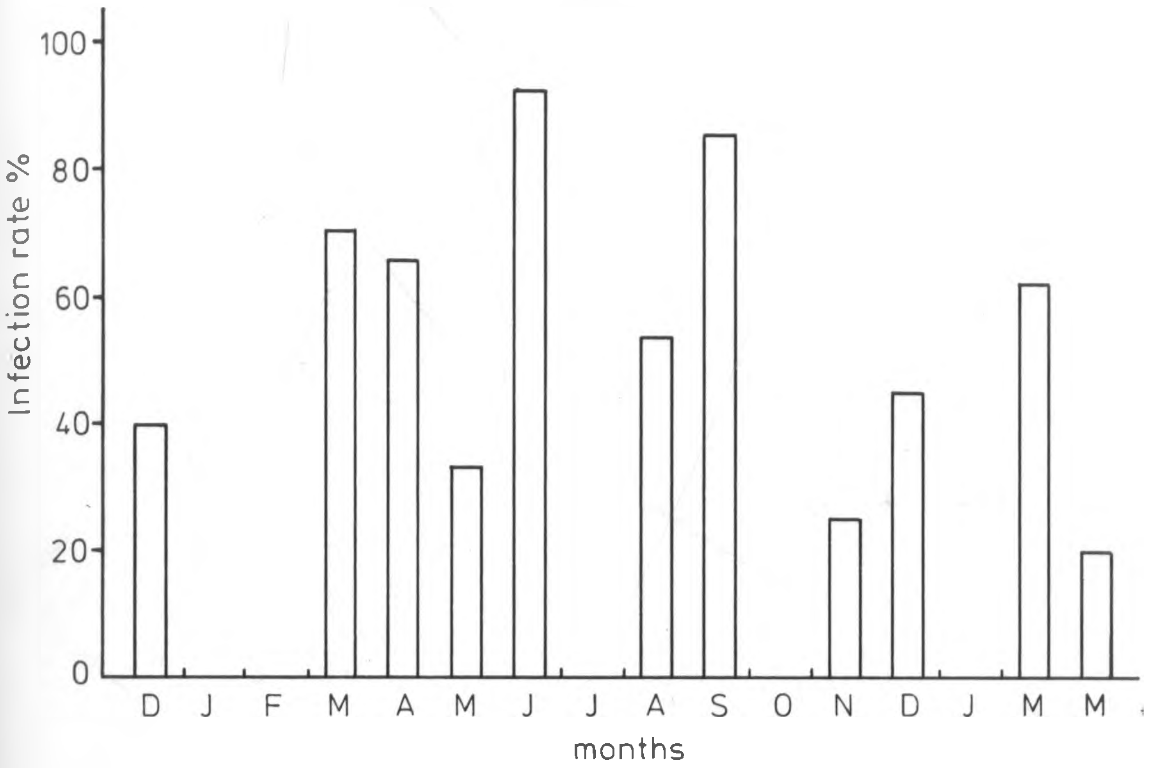
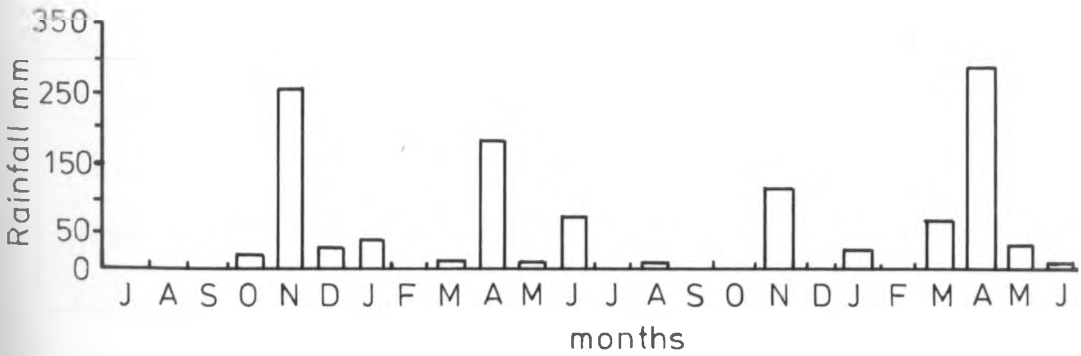


Fig. 4b Rainfall in Masinga between July 1986 and June 1988



High infection rates in Acomys were recorded during and soon after the rainy season in March-September. The infection rate in Mastomys did not show seasonality. The majority of the positive Mastomys (12%) were trapped in May 87. The two positive Lemniscomys were trapped in May 87 and November 87 while the three positive mongooses were trapped in September 87, December 87 and January 88. Of the 84 dogs examined, only one was found positive for flagellates in May 1988 . All slide smears were negative for amastigotes.

2.3.3. A summary of cryopreserved isolates

A total of 126 rodents, 3 mongooses, 1 dog and 13 lizards from Marigat and Masinga were found positive for flagellates. From some animals, more than one isolate was obtained from different organs of the same animal. As a result, 210 isolates were made from the spleen, liver, bone marrow and skin tissues (Table 6). Of the 41 mammals found positive in Marigat, none were positive in all four organs, (i.e. spleen, liver, bone marrow, skin), 4 were positive in three of the organs, 6 were positive in two of the organs while 31 were positive in only one organ (Appendix 3a). By comparison, of the 85 mammals found positive in Masinga, 3 were positive in all four organs by culture, 6 were positive in three organs, 36 were positive in two organs, and 40 were

Table 6

Total number of isolates obtained from different organs in animals.

Area	Animal sp.	No.+ve	Spleen	Liver	B.marrow	Skin	Total
Masinga	*Acomys*	67	48	40	19	7	114
"	*Mastomys*	12	3	10	6	1	20
"	*Lemniscomys*	2	1	1	2	0	4
"	Mongooses	3	3	0	0	0	3
"	Dog	1	1	0	0	0	1
"	*Latastia*	1	(blood)				1
Marigat	*Tatera*	36	22	3	7	17	49
"	*Arvicanthis*	3	0	2	1	0	3
"	*Mastomys*	2	2	0	0	1	3
"	*Latastia*	10	(blood)				10
"	*Varanus*	2	(blood)				2
Total No. of isolates							210

positive in only one organ (Appendix 3b). Of the 33 lizards examined in Marigat and Masinga, 13 were found positive in cardiac blood cultures (Appendix 3c). More isolates were made from the spleens of animals trapped in Masinga (69.3%) than from the skin (3.8%). Isolates from the bone marrow and liver were respectively 35.7 and 38.7% of the total. In rodents from Marigat, more isolates were made from the spleen (53.7%) and skin (32.4%) than from the bone marrow (17.6%) and liver (21.7%). The isolates made from the skin were from apparently normal skin with no conspicuous lesions.

Of the 210 isolates cultured in NNN medium, 24 (11.4%) were lost to bacterial and fungal contamination and the remaining 186 (88.6%) isolates were successfully cryopreserved in liquid nitrogen for future reference.

From February 1981 to August 1988, 752 individuals suspected of suffering from leishmaniasis were examined at the Clinical Research Centre of KEMRI. Of these, 385 isolates were found positive for leishmanial parasites and 353 isolates were successfully cryopreserved in the Nairobi Leishmania Bank. Of these, 158 were isolated from patients in Baringo District and 102 from patients in Machakos District. The remaining 93 were from patients in Kitui, Nakuru, Turkana, West Pokot, Bungoma, Meru and Samburu districts. In addition, 340 isolates from sandflies, 184 from rodents, 3 from mongooses, 2

Table 7

Total number of isolates deposited in the Nairobi Leishmania Bank, between February 1981 to August 1988.

Host	1981	1982	1983	1984	1985	1986	1987	1988	Total
Man	29	7	33	38	61	82	59	44	353*
Rodents	0	14	-	-	-	34	124	12	184
Sandflies	-	-	26	-	49	100	125	40	340
Mongooses	-	-	-	-	-	-	2	1	3
Dogs	1	-	-	-	-	-	-	1	2
Lizards	-	-	-	-	-	7	6	-	13

* No of isolates excluding repeat isolates from the same patient.

from dogs and 13 from lizards were also cryopreserved (Table 7) for future reference.

2.4. DISCUSSION

In this study, animals from Marigat and Masinga were examined for leishmanial infection by necropsy and by culture of various organ tissues. The resulting isolates were cryopreserved in liquid nitrogen for identification and future reference.

Success in trapping animals in a given area is subject to great variations depending on the rainfall, vegetation, habitat, type of the trap and population density of rodents in the area. In general, the two study sites, Masinga and Marigat are ecologically and climatically similar, but the species diversity and the type of flagellates in animals differed. Rodents were more abundant in Marigat but in Masinga there was greater species diversity. The predominant rodent species in Marigat was the diurnal *Arvicanthis*, while in Masinga the nocturnal *Tatera* predominated. *Arvicanthis* was rarely trapped in Masinga and was found in only one homestead in Mukusu village. *Lemniscomys*, *Herpestes* and *Xerus* were frequently seen wandering about during the day in Masinga, but were rarely seen in Marigat where only one *Xerus* was trapped.

Lacertid lizards, trapped commonly near termite

hills, were prevalent in Marigat and Masinga, while monitor lizards were trapped only in Marigat along the irrigation canals.

The results of this study show for the first time a very high infection rate of animals with flagellates. The low prevalence of leishmanial infections reported in wild animals by previous investigators may have been due to insensitive sampling techniques available at that time. Use of a sensitive culture medium to isolate leishmanial parasites from vectors and mammalian hosts is very important in obtaining a true picture of the infection rate. A less sensitive medium will often give false negative results. In addition, fungal and bacterial contamination normally inhibit the growth of Leishmania in culture and may also give false negative results (Kimber et al., 1981). Sensitivity of various culture media was evaluated by inoculating cultures with known numbers of amastigotes and determining the minimum number of amastigotes that will yield a positive culture. NNN medium was found to be more sensitive than either RPMI, Grace's or Schneider's medium (Githure, 1982). Schneider's medium was found to be more suitable for large scale cultivation of the parasites (Hendricks et al., 1978; Lightner & Githure, 1983). NNN medium overlayed with Schneider's medium was found to be twice as sensitive as either of the media used alone (Githure

et al., 1984c). The sensitivity of this medium combination coupled with the sterile technique used resulted in the high infection rates obtained in the animals.

Three of ten mammalian genera collected were found infected in Marigat, compared to six of fifteen genera in Masinga. The infected animals were, Arvicanthis, Mastomys and Tatera in Marigat and Acomys, Mastomys, Lemniscomys, Herpestes, Helogale and Canis in Masinga. In Marigat, Tatera had the highest infection rate (12.5%), while in Masinga, Acomys had the highest infection rate (60.4%). None of the Tatera or Arvicanthis trapped in Masinga and Acomys trapped in Marigat were found infected. Mastomys and lacertid lizards were the only animals species found infected at both study sites. In both sites, the infection rate did not correlate with the wet or dry seasons and transmission is believed to take place throughout the year. In Senegal, where the infection rate in rodents was 0.02% in Arvicanthis niloticus, 0.07% in Mastomys erythroleucus and 0.06% in Tatera gambiana, Dedet et al. (1979) observed that transmission of L. major occurred throughout the year, independent of the seasons.

Four organs (spleen, liver, bone marrow and skin) were sampled from each rodent with the aim of identifying the most commonly infected tissues. It is

hoped by culturing the target organ, time, effort and money can be saved in future searches for animal reservoirs. In this study, animals from Masinga were most commonly infected in the spleen (mean 69.3%). Likewise, those from Marigat were infected mainly in the spleen (53.7%) and skin (32.4%). Rodents with cutaneous infections demonstrated by culture examination did not have conspicuous lesions on the skin. In Ethiopia and West Africa, L. major occurs in rodents as visceral infection (Haile & Lemma, 1977; Dedet et al., 1979) while in Israel, it occurs predominantly on the ears of rodents with no visceral involvement (Schlein et al., 1984).

The results of this study show that the use of a sensitive culture medium in isolating parasites from animals, sandflies and man yields a high number of isolates which can be successfully cryopreserved in liquid nitrogen. This is the first report showing a very high infection rate in animals examined for leishmaniasis in Kenya. Since these flagellates are almost all morphologically identical, it was important to identify them biologically and biochemically; this is the subject of Chapters 3 and 4.

Chapter 3

EXPERIMENTAL INFECTION OF ANIMALS WITH *LEISHMANIA* AND OTHER FLAGELLATES

3.1. INTRODUCTION

One of the criteria for incriminating an animal reservoir of leishmaniasis is that the animal should be susceptible to infection with the parasite from man. In view of this, many attempts have been made to infect wild caught rodents with L. donovani in order to evaluate their susceptibility and potential usefulness as models, and reservoirs of visceral leishmaniasis (Heyneman & Mansour, 1963; Stauber et al., 1966; Pozio et al., 1981; Nolan & Farrel, 1987). Some potential animal reservoirs of visceral leishmaniasis in Kenya namely, Mastomys natalensis, Rattus rattus, and Heterocephalus glaber have been found to be susceptible to infection with L. donovani (Githure, 1981; Shatry et al., 1987b; Githure et al., 1988).

In Iraq, Al-Taqi & Mohammed (1981) investigated the susceptibility of wild rodents and laboratory animals to infection with L. tropica. All the animals used in their study were refractory to infection and they concluded that there was no animal reservoir of L. tropica in Iraq. To investigate the role played by dogs in the transmission of visceral leishmaniasis in the Mediterranean and East Africa, Mansour et al. (1970) injected three groups of 4 puppies and 4 adult dogs with 1-2 x 10^8 amastigotes/kg body weight of L. donovani strains from the Mediterranean, the Sudan and Kenya.

All the three strains of Leishmania were found to infect dogs but showed considerable variation in infectivity. There was no significant difference in the infection rate between puppies and adult dogs. In dogs infected with the Mediterranean strain, parasites were demonstrated in the blood, spleen, liver, bone marrow, skin, lymph nodes, kidneys and lungs at necropsy 512 days post-infection. The Mediterranean strain showed a chronic heavy infection for over 500 days while the Kenyan and the Sudanese strains caused declining infections which disappeared completely at 128 and 256 days respectively. They concluded that the short duration of infection produced by the Sudanese and the Kenyan strains and the spontaneous cure in the latter suggest that the dog may be excluded as a potential reservoir of visceral leishmaniasis in these two countries. In contrast, recovery of parasites from the peripheral blood in the dogs infected with the Mediterranean strain and the persistence of infection for over 500 days indicated a well-adapted host-parasite relationship and suggested that dogs could be the reservoirs of visceral leishmaniasis in the Mediterranean region.

Some laboratory animals, for example Balb/c mice and golden hamsters, have been found useful in differentiating between L. major and L. donovani on the

basis of the disease they cause. The former parasite causes ulcerating lesions on the skin of these animals whereas the latter causes only visceral leishmaniasis, even when inoculated intradermally (Schnur et al., 1973). Balb/c mice have been found to be very susceptible to *L. major* and *L. donovani* infections (Bradley & Kirkley, 1977; Trotter et al., 1980).

This study was designed to evaluate the susceptibility of wild and domestic animals suspected as animal reservoirs of visceral leishmaniasis and, to determine the infectivity of flagellates from sandflies and wild animals in Balb/c mice. Any isolates causing lesions on the nose of a mouse was an *L. major* suspect and any that caused splenomegaly and no lesion on the nose was an *L. donovani* suspect. The identity of these isolates was later confirmed by isoenzyme analysis.

3.2. MATERIALS AND METHODS

3.2.1. Susceptibility of wild and domestic animals to *L. donovani*

To determine if some of the animals found at the study sites can act as reservoirs of visceral leishmaniasis, dogs, cats, goats, multimammate rats, and spiny mice were inoculated with a Kenyan strain of *L. donovani*. Two dogs and two goats were purchased in Masinga and quarantined in KEMRI Animal House for 40

days prior to experimental infection. During this period, they were dewormed and checked for leishmanial infection by culturing aspirates from the spleen, liver, and skin of the nose and ears in NNN medium and by examining impression smears of these organs. All were found by these procedures to be negative for *Leishmania*. Three stray cats, one male and two females caught in the KEMRI compound were put in the animal house and allowed to breed. Both females gave birth four times to a total of 15 kittens, 12 of which were used in this study.

Mastomys, *Acomys*, *Arvicanthis* and *Tatera*, trapped in Masinga study site, were put in separate cages in groups of 2 females and one male, for the purpose of breeding. *Mastomys* produced several litters totalling over 100 rats in one and a half years in captivity, while *Acomys* gave birth to only five litters totalling 16 mice during this period. *Arvicanthis* and *Tatera* failed to breed in captivity and experiments could not be done with these two rodent species.

Infection and sampling of animals

Two dogs weighing 5.1kg and 9.0kg, and two goats weighing 14kg each were inoculated intradermally with an 8-day old stationary phase culture of *L. donovani* (3 x 10^7 promastigotes in 0.4ml of Schneider's medium) on the

right ear and nose. The parasite culture was derived from the spleen of a hamster previously infected with MHOM/KE/83/NLB-065 isolated from the spleen of a visceral leishmaniasis patient from Machakos District. Twenty-six female Mastomys with an average weight of 65gm (range 55.1 to 72.0gm) and 16 Acomys with an average weight of 16.4gm (range 13.6 to 18.9gm) were inoculated subcutaneously on the nose with the same strain (1 x 10^7 promastigotes in 0.2ml).

To test the virulence and infectivity of the inoculum used for dogs, goats, Mastomys and Acomys, 20 Balb/c mice with an average weight of 21.1gm (range 16.8 to 24.8gm) were inoculated subcutaneously on the nose with 1 x 10^7promastigotes of the same strain of L. donovani.

The mice were sampled at 3 weeks post-inoculation to check for infectivity of the inoculum. The cultures of the spleen and skin at site of inoculation were found to be positive and therefore, all the animals were sampled at 6, 9, 12, 15, 18, 21 and 24 weeks post-inoculation. Two Mastomys, two mice and two Acomys were sacrificed at each sampling interval and the spleen, liver, femur bone marrow and skin of the nose cultured into NNN medium. Impression smears of these tissues were also made. At each sampling interval, the dogs were anaesthetized with Rompun and percutaneous liver and spleen aspirations

done using a 21 gauge needle fitted with a 10ml syringe while the skin aspirate at the sites of parasite inoculation was taken with a 26 gauge needle fitted with a 1ml tuberculin syringe. The aspirates from the skin of the nose and right ear, spleen and liver were inoculated into NNN medium and also used to make smears. The goats, restrained by two assistants, were sampled in the same manner as the dogs.

In a separate experiment, 12 cats weighing an average of 1500gm (range 800 to 1600.8gm) were divided into 2 groups. One group of six cats received subcutaneous inoculation on the right ear with 1×10^7 promastigotes in 0.1ml containing L. donovani strain, NLB-065; the other group of six cats was inoculated intravenously with the same strain on the lower fore-leg with 3×10^7 amastigotes/kg body weight. Two cats were sacrificed at each sampling interval of 4, 8, 12, 16, 20 and 24 weeks PI and samples of the spleen, liver, femur bone-marrow, and skin of the right ear inoculated into NNN medium and also used to make smears. All cultures were incubated at 25°C and examined daily for a period of 14 days for the presence of promastigotes. Smears were stained with Giemsa and examined for amastigotes.

3.2.2 Susceptibility of mice to sandfly and animal isolates

Groups of Balb/c mice were inoculated with isolates from sandflies and animals trapped in Marigat and Masinga to determine the infectivity of these parasites and the disease they cause in mice. The isolates from Masinga comprised 62 from *Acomys*, 7 from *Mastomys*, one from *Lemniscomys*, 3 from mongooses, and one from a dog. Those from Marigat comprised 32 from *Tatera*, 3 from *Arvicanthis*, 2 from *Mastomys* and 42 from sandflies (Table 8).

Each isolate was inoculated intraperitonealy and subcutaneously into groups of mice. The inoculum into each route contained 1×10^7 promastigotes in 0.1ml of medium. Mice were killed at intervals of 4 and 12 weeks post-inoculation to examine them for signs of skin lesions and to culture the nose skin, liver, spleen, and femur bone-marrow to detect promastigotes. Smears of these organs were also made and examined for amastigotes.

3.3 RESULTS

3.3.1. Susceptibility of mice to sandfly and animal isolates

Thirty-seven rodent isolates from Marigat were used to inoculate groups of Balb/c mice (Table 8). It was

observed that all isolates, with the exception of NLB-926 from *Mastomys*, produced ulcerating granulomatous lesions on the noses of mice 4 weeks post-inoculation (Plate 8) and viscerilized in some mice, leading to death. The lesions were typical of those caused by *L. major* reference strain, MHOM/IL/67/LRC-L137=NLB-326. The isolate from *Mastomys* (NLB-926) was found to be infective to mice, but no lesion was observed at the site of inoculation. The spleen and skin cultures made from mice injected with this isolate were positive at 4 weeks post-inoculation but negative for flagellates at 12 weeks post-inoculation.

Seventy-four isolates from animals in Masinga were used to inoculate groups of Balb/c mice. Of these, only 2 isolates, (NLB-750 and NLB-924) caused infection in mice. The infection was demonstrated by examining the spleen and skin cultures 4 weeks post-inoculation but not on smears, and all the organs were negative by 12 weeks post-inoculation. In a separate experiment, the course of infection caused by NLB-750, NLB-924 and NLB-926 in mice inoculated intraperitoneally in groups of mice was monitored by examining parasitaemia in wet preparations of tail blood. It was observed that parasitaemia increased by 3 weeks post-inoculation, after which it was cleared and all mice were negative for flagellates 5 weeks post-inoculation.

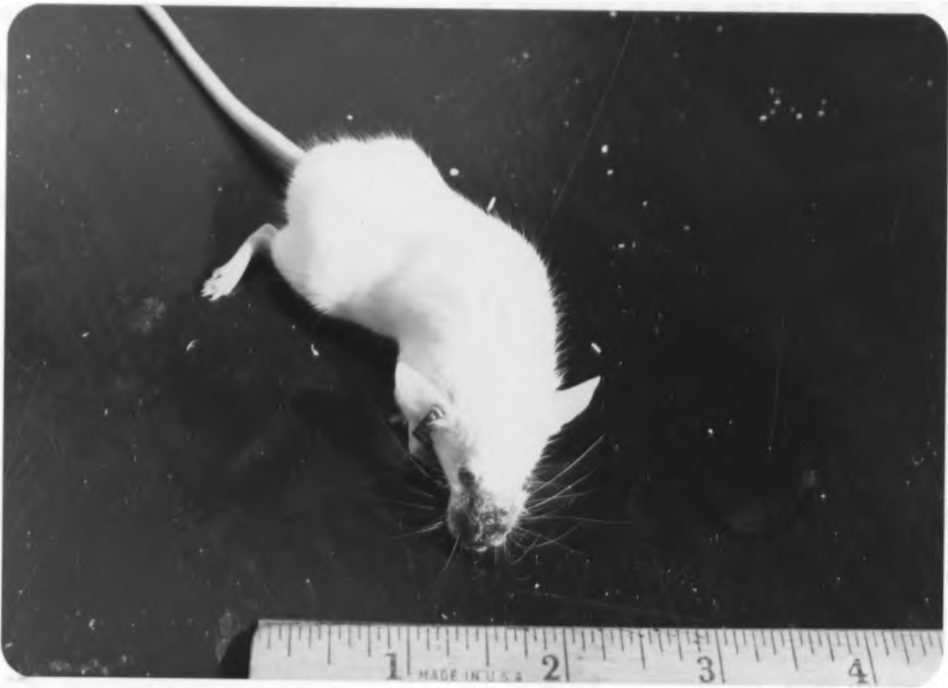
Table 8

Susceptibility of Balb/c mice to animal and sandfly isolates 4 and 12 weeks post-inoculation

Donor	No. of Isolates	Study site	No. of mice inoc.	No.of infective isolates
Acomys	62	Masinga	252	2
Mastomys	7	Masinga	28	0
Lemniscomys	1	Masinga	4	0
Mongooses	3	Masinga	22	0
Dog	1	Masinga	8	0
Tatera	32	Marigat	128	32
Arvicanthis	3	Marigat	12	3
Mastomys	2	Marigat	8	1
S.schwetzi	6	Marigat	24	0
S.antenatus	18	Marigat	72	0
S.africanus	1	Marigat	4	0
S.clydei	9	Marigat	36	0
P.duboscqi	5	Marigat	20	5
P.martini	3	Marigat	18	3

Plate 8

Balb/c mouse showing an ulcerated lesion on the nose 4 weeks after subcutaneous inoculation with *L. major* from a *Tatera*.



8

Forty-two sandfly isolates from Marigat were inoculated into mice. Of these, only eight caused infection in mice. These were, 5 isolates from P. duboscqi, which caused ulcerating lesions on the noses of mice 4 weeks post-inoculation, and 3 isolates from P. martini which caused infection in the spleen and liver of mice 12 weeks post-inoculation. Parasites isolated from the skin and viscera of these mice were grown and cryopreserved in liquid nitrogen for characterization.

3.3.2. Susceptibility of wild and domestic animals to L. donovani

Positive cultures made from the skin, spleen and liver of control mice at 3 weeks post-inoculation indicated that the inoculum used was viable and infective to animals. The skin and spleen of Mastomys infected with L. donovani were positive in culture 6 weeks post-inoculation and the spleen was subsequently positive throughout the sampling intervals (Table 9). L. donovani infection in Acomys and mice was variable and mice were persistently negative in the liver. In contrast, the sampled tissues from two dogs and two goats injected with the same inoculum of L. donovani were found negative in cultures and smears throughout the 24 weeks of the experiment (Table 9).

Table 9

Culture results of animals inoculated subcutaneously with 1×10^7 promastigotes of L. donovani, NLB-065.

Animal	No. of Animals	Organ*	Weeks Post-inoculation 6	9	12	15	18	21	24
		Spleen	+/-	+/-	+/-	+/-	+/+	+/+	+/-
Mastomys	26	Liver	-/-	-/-	-/-	+/-	+/+	+/-	+/+
		Skin	+/-	+/-	+/-	-/-	+/-	-/-	+/-
		Spleen	-/-	-/-	+/-	-/-	+/-	-/-	+/-
Acomys	12	Liver	-/-	-/-	-/-	-/-	+/+	-/-	-/-
		Skin	-/-	-/-	+/-	-/-	-/-	-/-	-/-
		Spleen	-/-	-/-	+/-	+/-	+/-	+/+	-/-
Mice	20	Liver	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		Skin	+/-	-/-	+/-	-/-	-/-	-/-	-/-
		Spleen	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Goats	2	Liver	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		Skin	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		Spleen	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Dogs	2	Liver	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		Skin	-/-	-/-	-/-	-/-	-/-	-/-	-/-

* Results are for two animals at each sampling interval.

Similarly, the two groups of cats inoculated on separate occasions with amastigotes and promastigotes of *L. donovani* did not become infected as demonstrated in cultures and smears made from organs of these animals throughout the 24 weeks of the experiment.

3.4. DISCUSSION

In early studies on vector and animal reservoirs, the course of infection of leishmanial parasites in a susceptible animal was used to identify the parasites. In this study, isolates from rodents and *P. duboscqi* from Marigat were partially identified as *L. major* on the basis of the lesion they caused on the noses of mice. Isolates from *P. martini* from Marigat were partially identified as *L. donovani* on the basis that they were viscerotropic in mice. None of the isolates from *Sergentomyia* spp. caused infection in mice. Similarly, none of the isolates from Masinga caused infection apart from 3 rodent isolates which were detected only on examination of wet blood smears.

One criterion of a good *Leishmania* reservoir is that it should be susceptible to the parasite infecting man. In view of this, wild rodents, domestic dogs, cats and goats were inoculated with *L. donovani* to evaluate their potential as animal reservoirs of visceral leishmaniasis in Kenya. It was observed that *Mastomys* were highly

susceptible to the infection, more so than the mice control. They developed a heavy parasite load in the spleen throughout the course of the experiment, with no apparent ill-health. This confirms the work of Githure (1981), where *Mastomys* infected with *L. donovani* showed a tremendous enlargement of a parasitized spleen with a weight of 7gm as compared to a normal spleen weight of 0.3gm at 34 weeks post-inoculation. Nolan & Farrel (1987) have also shown that this rat is highly susceptible to *L. donovani* infection.

In contrast, the cats, dogs and goats were quite refractory to *L. donovani* infection. Although the numbers of these animals used were small, the fact that the inoculum which caused infection in rodents failed to produce infection in any of these animals, indicates that they have some degree of resistance to *L. donovani* infection. Mansour et al. (1970) inoculated four dogs with a Kenyan *L. donovani* strain and demonstrated a transient declining infection which resolved by 128 days post-inoculation. They injected dogs intravenously with a large number of amastigotes which are capabable of surviving in visceral organs for some time without multiplying. Dogs infected with the Mediterranean strain of *L. donovani* developed a chronic, heavy infection, with parasites observed in the visceral organs, the skin and peripheral blood 512 days

post-inoculation (Mansour et al., 1970).

Although the numbers of domestic animals (cats, dogs and goats) used in this study was small, the results suggest that these animals were not susceptible to *L. donovani* infection and are probably not involved in natural transmission of visceral leishmaniasis in Kenya. Thorough studies on experimental infection of these domestic animals need to be done.

Mastomys and *Acomys* were found to be susceptible to infection with *L. donovani*, but surprisingly these have not been found with natural infection of this parasite in previous or the present studies. This implies that there is no interaction of the vector with these rodents in their natural habitats. The results of this study suggest that rodents do not play a significant role in the transmission of *L. donovani* in Kenya. The implication of rodents as reservoirs of leishmaniasis is further discussed in Chapters 4 and 5.

Chapter 4

CHARACTERIZATION OF *LEISHMANIA* BY CELLULOSE ACETATE ELECTROPHORESIS

4.1. INTRODUCTION

Identification of Leishmania by morphology is unreliable because differences between species are not clear cut, and promastigotes of the same species can differ in morphology depending on culture conditions and stage of growth. More reliable identification methods include isoenzyme analysis, the use of monoclonal antibodies, analysis of kinetoplast DNA fragments resulting from treatment with restriction endonuclease, analysis of membrane lipids and hybridization of total kDNA. Isoenzyme analysis has been widely used to identify protozoan parasites, namely, Leishmania (Kreutzer & Christensen, 1980), Trypanosoma (Godfrey & Kilgour 1976), Eimeria (Shirley & Rollinson, 1979), Plasmodium (Carter & Voller, 1975), and Amoeba (Sargeaunt et al., 1978).

There have been several mistaken identities of flagellates isolated from humans, sandflies and wild animals in Kenya. For example, an isolate made from the spleen of a ground squirrel in Baringo District (Heisch, 1957) and thought to be L. donovani was later identified as L. major using isoenzyme analysis (Chance et al., 1978). Another isolate made from a dog in Turkana District was thought to be L. donovani (Lightner & Githure, unpublished data) but was later found to be different from the Old World Leishmania reference

strains and similar to *Crithidia* sp. (Githure et al., 1986c). Isolates from the spleens of antimony-resistant cases of visceral leishmaniasis were assumed to be *L. donovani* based on clinical manifestations, but were later identified as *L. tropica* (Mebrahtu et al., 1989). Attempts to incriminate dogs as reservoirs of visceral leishmaniasis in Kenya have been founded on the assumption that any flagellate from the viscera of dogs is *Leishmania*. However, it has been established that dogs can also harbour flagellates other than *Leishmania* (Githure et al., 1986c; Schnur, personal communication). Similarly, flagellates from sandflies can be either *Leishmania*, *Trypanosoma* or *Crithidia*. Isolates from the incriminated vector of visceral leishmaniasis have been assumed to be *L. donovani*, but a recent study has shown that *P. martini* can also harbour flagellates other than *Leishmania* (Perkins et al., 1988).

As a result of these and other assumptions made in the past, it has become necessary to characterize all isolates from humans, sandflies and wild animals in order to understand the epidemiology of leishmaniasis in Kenya. Correct identities of parasites also allows accurate experimentation on drugs or vaccines. Prior to this study, only about 35 isolates of *Leishmania* from Kenya had been characterized using biochemical,

serological and immunological techniques (Peters et al., 1977; Chance et al., 1978; Le Blancq & Peters, 1986; Githure et al., 1986a). All these isolates were characterized in overseas institutions for lack of facilities locally. This study was designed to characterize by cellulose acetate electrophoresis of nine enzymes, leishmanial isolates from man, sandflies and wild animals from two endemic foci in Kenya.

4.2. MATERIALS AND METHODS

4.2.1. Preparation of parasite lysates

Isolates from patients, sandflies and wild animals were stored as stabilates at -196°C in liquid nitrogen with 7.5% DMSO as cryoprotectant. They were removed from liquid nitrogen and thawed at room temperature. Parasites were grown in 5ml Schneider's medium in $25cm^2$ tissue culture flasks (Corning Glass Works, Corning, N.Y.) and, when the growth was good, were transferred into $75cm^2$ culture flasks containing 20ml of Schneider's medium. The growth of the parasites was monitored daily by examining the culture flasks under an inverted microscope. Promastigotes were harvested during the log phase of growth by centrifugation at 1,500g for 15min at 4°C. The supernatant was discarded and the pellet of promastigotes washed three times in cold phosphate buffered saline, pH 7.2. The washed pellet was

resuspended in an equal volume of distilled water to lyse the promastigotes before freezing at -80°C. The frozen samples were then removed from the freezer and allowed to thaw at room temperature. The samples were lysed further by snap-freezing and thawing about three times in liquid nitrogen. Soluble extracts of the lysed promastigotes were prepared by centrifugation at 4,000g for 30 minutes at 4°C. The lysate supernatant was dropped with a pasteur pipete into a 100ml plastic beaker half-filled with liquid nitrogen. The resulting 15µl beads were counted and put in cryotubes labelled with the lysate number and date, and then stored in liquid nitrogen until required for electrophoresis.

4.2.2. Enzymes examined

Isolates from humans, other mammals, reptiles and sandflies were examined by cellulose acetate electrophoresis according to the methods of Kreutzer & Christensen (1980) and Lanham et al. (1981). The preparation of buffers and staining reagents and the running conditions were similar to those of Lanham et al. (1981), with slight modifications. Of the 12 enzymes attempted, only 9 which gave clear band resolutions were routinely studied. These were, malate dehydrogenase (MDH, E.C. 1.1.1.37); malic enzyme (ME, E.C. 1.1.1.40); isocitrate dehydrogenase (ICD, E.C.

1.1.1.42); 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44); glucose 6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49); phosphoglucomutase (PGM, E.C. 2.7.5.1); nucleoside hydrolase (NH, E.C. 3.2.2.2); mannose phosphate isomerase (MPI, E.C. 5.3.1.8), and glucose phosphate isomerase (GPI, E.C. 5.3.1.9). Enzyme numbers and abbreviations are listed according to the Enzyme Commission Nomenclature (1973).

4.2.3. Preparation of tank and staining buffers

500ml of tank buffer for each enzyme or a group of enzymes was prepared as shown in Table 10. Ten per cent sucrose was added to the tank buffer to reduce the current and to improve the resolution of the bands. Fifty ml of this buffer was put in each of the two tank compartments and 400ml used to soak the cellulose acetate plates (Helena Laboratories, Beaumont, Texas, USA). Labelled plates were pre-soaked in the appropriate buffer for at least 30 minutes before loading them with eight or 12 samples.

Paper wicks were dipped in tank buffer on each side of the compartment and coolant sponges put in the centre compartment to help prevent overheating or drying of the plates during electrophoresis.

250ml of staining buffer for each or a group of enzymes was prepared as in Table 10. These buffers were

Table 10.

Running and staining conditions for cellulose acetate electrophoresis of Leishmania isolates

Enzyme	Tank buffer	Time min	Voltage	Staining buffer	dH_2O	Staining reagents: Ions	Coenzymes	Enzymes	Substrates	Visualizing
MDH	1	40	200	A, 7ml	8ml	-	NAD 10mg	-	1M L-malate 2ml	MTT PMS Agar 5mg 2mg 1.2%
ME	7	30	200	B, 13ml	2ml	MgCl 0.1M	NADP 10mg	-	1M L-Malate 2ml	MTT PMS Agar 5mg 2mg 1.2%
ICD	5	30	200	B, 13ml	-	MnCl 0.1M	NADP 10mg	-	Isocitric acid 10mg	MTT PMS Agar 5mg 2mg 1.2%
6PGD	2	30	200	B, 13ml	2ml	MgCl 0.1M	NADP 10mg	-	6-Phosphogluconic acid 10mg	MTT PMS Agar 5mg 2mg 1.2%
G6PD	7	30	200	B, 13ml	2ml	MgCl 0.1M	NADP 10mg	-	Glucose-6-Phosphate 10mg, ATP-10mg	MTT PMS Agar 5mg 2mg 1.2%
PGM	4	30	200	A, 13ml	1ml	MgCl 0.1M	NADP 10mg	G6PD 100µl	Glucose-1-Phosphate 20mg	MTT PMS Agar 5mg 2mg 1.2%
NH	5	25	200	D, 13ml	4ml	-	-	Xanthine oxidase 50µl	Inosine 20mg	MTT PMS Agar 5mg 2mg 1.2%
MPI	6	30	200	B, 13ml	-	MgCl 0.1M	NADP 10mg	GPI 100µl	Mannose-6-Phos 10mg	MTT PMS Agar 5mg 2mg 1.2%
GPI	4	30	200	A, 13ml	1ml	MgCl 0.1M	NADP 10mg	G6PD 100µl	Fructose-6-Phos 10mg	MTT PMS Agar 5mg 2mg 1.2%

Footnotes for Table 10

Tank Buffers

1. Tris barbital/Sodium barbital* pH 9.0 I=0.075/1mM Mg acetate
2. 0.1M Tris/0.0176M Maleic acid/0.01M EDTA/0.01M Mg acetate pH 7.4
3. 0.25M Tris/0.05M EDTA/0.055M Boric acid/0.075M Mg acetate pH 9.0
4. 0.1M Tris/0.1M Maleic acid/0.01M EDTA/0.01M Mg Acetate pH 7.4 adjusted with NaOH
5. 0.08M Na_2HPO_4/0.192M Na_2HPO_4 pH 8.0
6. 0.2M K_2HPO_4 pH 7.0 adjusted with KOH
7. Tris barbital/Sodium barbital* pH 9.0 I=0.02/1mM Mg acetate

Staining Buffers

A. 0.3M Tris/HCl pH 8.0

B. 0.3M Tris/HCl pH 7.4

C. 0.004M Na_2HPO_4/0.096M Na_2HPO_4 pH 8.0

D. 0.3M Tris/HCl pH 7.0

* Prepared from pre-packed sachets Cat. # 5805 (Helena Laboratories, Beaumont, Texas).

stored at 4°C until required but discarded after two weeks.

4.2.4. Preparation of staining reagents

The staining reagents shown in Table 10 were obtained from Sigma Chemical Co. (St. Louis, MO 63178, USA). They were dissolved in appropriate volumes of staining buffer. 1.2% molten agar (60°C) was added to the reagent mixture in a beaker, mixed briefly, and then poured into labelled petri dishes. This was allowed to solidify in the dark because the tetrazolium salts used for visualization are sensitive to light. The staining plates were used soon after preparation.

4.2.5. Electrophoresis

Lysate beads were removed from liquid nitrogen and allowed to thaw individually in the wells of a microtitre plate. Ten µl samples were then pipetted into an 8 or 12 well application system (Helena Laboratories, Beaumont, Texas, USA). The unknown isolates were run together with WHO *Leishmania* reference strains and well-characterized Kenyan *Leishmania* strains (Table 11a). Well characterized rodent trypanosome of the subgenus *Herpetosoma* (Table 11b) were also included in the runs. The cellulose acetate plates were removed from the soaking buffer and blotted with Helena blotting

pads. Each plate was transferred to a base-aligning plate and the applicator lowered into the sample wells for 5 seconds and pressed onto the cellulose acetate plates. The loaded plate was then placed face down across the two paper wicks and held in place by weighted microslides. Tanks for different enzyme runs were connected in parallel to a power supply (LKB, Biochrom, U.K) (Plate 9), and voltage and time set according to the enzyme schedule.

4.2.6. Staining of plates

At the end of the running time, the current was switched off and the plates removed from the tank and placed individually onto the agar gel containing specific staining reagents for each enzyme. They were incubated in the dark at room temperature until band development was of optimum resolution. The enzyme reaction was stopped by removing the plate from the agar and fixing in 5% acetic acid for 2 minutes. The plates were then washed with tap water and allowed to dry at room temperature. The banding patterns were compared with the reference strains included in the runs and then recorded by photographing the plates using white-light background illumination and or by drawing the zymograms. Afterwards the cellulose acetate plates were wrapped with aluminium foil and stored for reference.

Table 11a

WHO Leishmania reference strains used

Strain Code	Leishmania sp.
MHOM/ET/62/HU3 = NLB-325	*L. donovani*
MHOM/KE/83/NLB-065	*L. donovani**
MHOM/IL/67/LRC-L137 = NLB-326	*L. major*
MHOM/KE/83/NLB-144	*L. major**
MHOM/SU/60/Strain-OD = NLB-305	*L. tropica*
MHOM/ET/72/L100 = NLB-310	*L. aethiopica*
RLAT/KE/54/Heisch-146 = NLB-493	*L. adleri**

* Well characterized Kenyan strains.

Table 11b

Rodent trypanosomes reference strains used*

Strain Code	Trypanosoma sp.
MRAT/00/00 = NLB-506	*T. lewisi*
MMUS/00/00 = NLB-507	*T. musculi*
MMIC/00/00 = NLB-508	*T. microti*
MEVO/00/00 = NLB-509	*T. evotomys*
MAPO/00/00 = NLB-510	*T. grosi*

* Donated by Professor D.H. Molyneux, University of Salford, UK.

Plate 9

Cellulose acetate electrophoresis apparatus showing (a) power pack (b) electrophoresis tanks (c) cellulose acetate plates, and (d) sample applicator kit.

9

4.3. RESULTS

4.3.1. Isolates from Marigat

A total of 50 isolates from rodents trapped in Marigat were examined by isoenzyme analysis of nine enzymes. These included 44 isolates from *Tatera*, 3 from *Arvicanthis*, and 3 from *Mastomys* (Appendix 3a). Isolates obtained from different organs of the same animal were compared with each other and found to be indistinguishable by all the enzymes. Therefore, in subsequent comparative studies, only one isolate from each animal was selected and compared with other isolates from the same animal species. The 32 isolates from *Tatera* were found to be indistinguishable by the nine enzymes examined (Table 12). Similarly, the 3 *Arvicanthis* isolates were found to be indistinguishable from each other. The two isolates from *Mastomys* differed from each other for all the nine enzymes. One of them (NLB-926) was not a leishmania on the basis of the morphology and isoenzyme profiles. The other isolate (NLB-706) was identical to the *Tatera* and *Arvicanthis* isolates. Comparison of *Mastomys*, *Tatera* and *Arvicanthis* isolates with the *Leishmania* reference strains showed identical enzyme profiles and were all indistinguishable from *L. major* reference strain, NLB-326 except for NLB-708, and NLB-544 which showed variations in G6PD, MDH and PGM (Plates 10a, 10c and

10d). They were all found to be different from L. donovani and L. tropica reference strains in the nine enzymes examined.

Nineteen isolates from patients from Marigat were examined, 14 of which were obtained from the spleen, 3 from the skin, and 2 from nasal swabs (Appendix 4). The 14 isolates from the spleens and the two from nasal swabs were found to have identical enzyme profiles, and were indistinguishable from the L. donovani reference strain. Two MDH variants, (NLB-462 and NLB-491) were identical to each other but differed from the reference strain (Plate 10f).

A list of the sandfly isolates examined by cellulose acetate electrophoresis is shown in Appendix 5. Six Sergentomyia schwetzi, 18 S. antennatus, 9 S. clydei and one S. africanus were found to have identical enzyme profiles and were indistinguishable from the L. adleri reference strain.

Twelve isolates from lizards were also found to have the same enzyme profiles as the L. adleri reference strain by all the enzymes examined.

Five isolates from P. duboscqi had enzyme profiles identical to the L. major reference strain while 3 isolates from P. martini were found to be identical to L. donovani reference strain in all the enzymes examined.

4.3.2. Isolates from Masinga

A total of 123 isolates obtained from Masinga were examined by cellulose acetate electrophoresis. These included 103 isolates from *Acomys*, 13 from *Mastomys*, 3 from *Lemniscomys*, 2 from *Herpestes*, 1 from *Helogale*, and 1 from *Canis* (Appendix 3b). Isolates obtained from different organs of the same animal were found to have identical enzyme profiles for all the nine enzymes examined. Therefore, only one isolate from each animal was selected for comparison with isolates within and among animal species, and also with the reference strains. Three different enzyme profiles were observed in isolates from *Acomys*. Enzyme variation was observed in MDH, ICD and NH (Plate 12).

The 3 isolates from mongooses were compared with each other and found to be indistinguishable in 8 out of the 9 enzymes examined. In the MDH variant, one isolate, NLB-1002 had two bands, only one of which was held in common with the other two mongoose isolates. The morphology of mongoose isolate was also different from other animal isolates in that the flagellum was about three times the length of the body (Plate 11d).

The dog isolate was found to be different from the *Acomys*, *Mastomys*, *Lemniscomys* and mongoose isolates in all the enzymes (Fig. 5). In addition, the dog isolate appeared crithidial-like in its morphology and showed

close resemblance to a Crithidia-like flagellates isolated from a dog in Turkana District (Plate 11e, 11f). However, the two isolates differed from each other in all the enzymes examined (Fig. 5).

Isolates from animals in Masinga were found to be enzymatically different from the Leishmania reference strains (Fig. 5) and also different from the rodent trypanosome reference strains although an isolate from Mastomys, NLB-810, had some enzymes profiles (MDH, PGM, and GPI) held in common with T. lewisi (NLB-506) reference strain. Of the 7 Mastomys isolates examined, 6 enzymes produced the same banding pattern for all isolates examined and 3 enzymes, (MDH, NH & GPI) showed variation for one isolate, NLB-810 from the rest of the Mastomys isolates. The morphology of Acomys and Mastomys is shown in Plates 11a, 11b which shows a characteristic pointed posterior end as in T. musculi (Plate 11c).

The lizard isolate (NLB-728) from Masinga was found to be identical to the 12 isolates from lizards trapped in Marigat and indistinguishable from L. adleri reference strain.

Fifteen isolates from patients in Masinga were examined by cellulose acetate electrophoresis, of which 14 were from the spleen and one from nasal swabs (Appendix 4). These were found to have identical enzyme

Plate 10

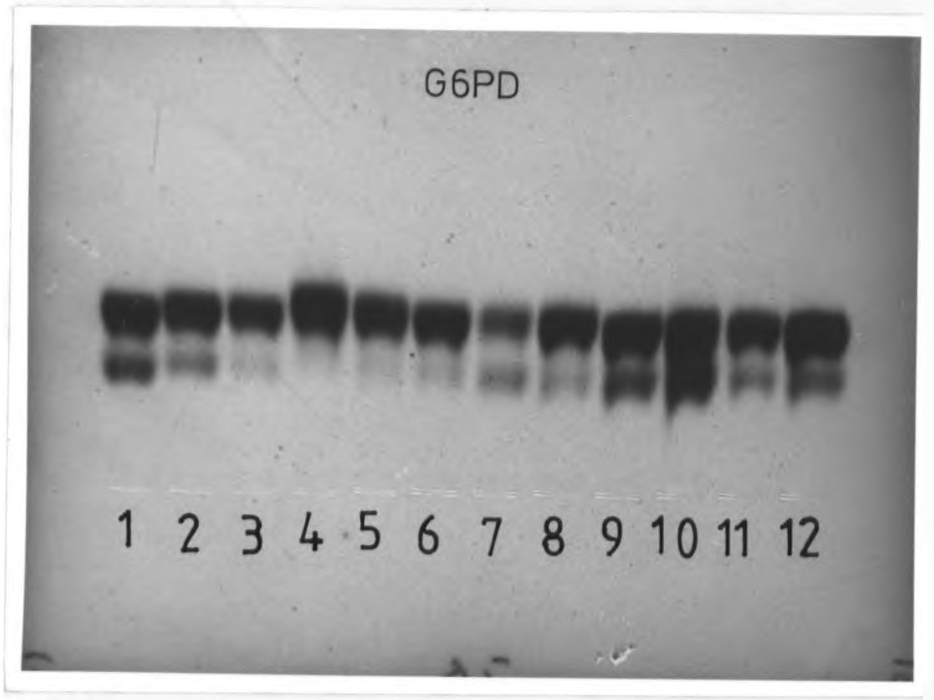
Leishmanial parasites from rodents in Marigat.

(10a)

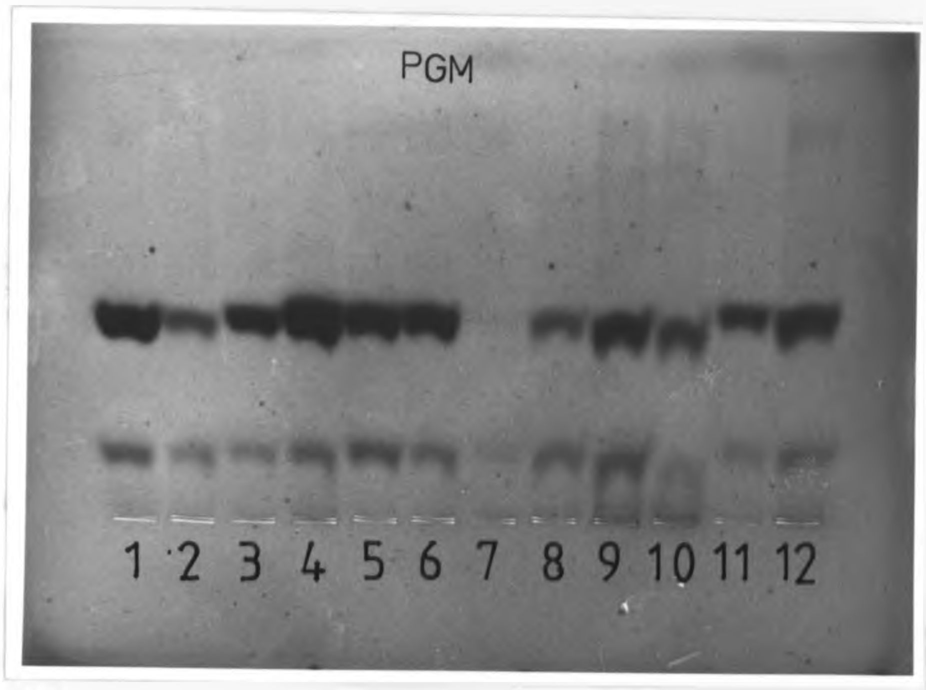
1. NLB-515, _Tatera_ skin
2. NLB-497, _Arvicanthis_ liver
3. NLB-780, _Arvicanthis_ B.marrow
4. NLB-544, _Tatera_ spleen
5. NLB-540, _Tatera_ skin
6. NLB-541, _Tatera_ skin
7. NLB-496, _Arvicanthis_ liver
8. NLB-706, _Mastomys_ skin
9. NLB-630, _Tatera_ B.marrow
10. NLB-631, _Tatera_ liver
11. NLB-629, _Tatera_ spleen
12. NLB-326, _L. major_ reference strain.

(10b)

Arrangement of samples same as shown above.



10a



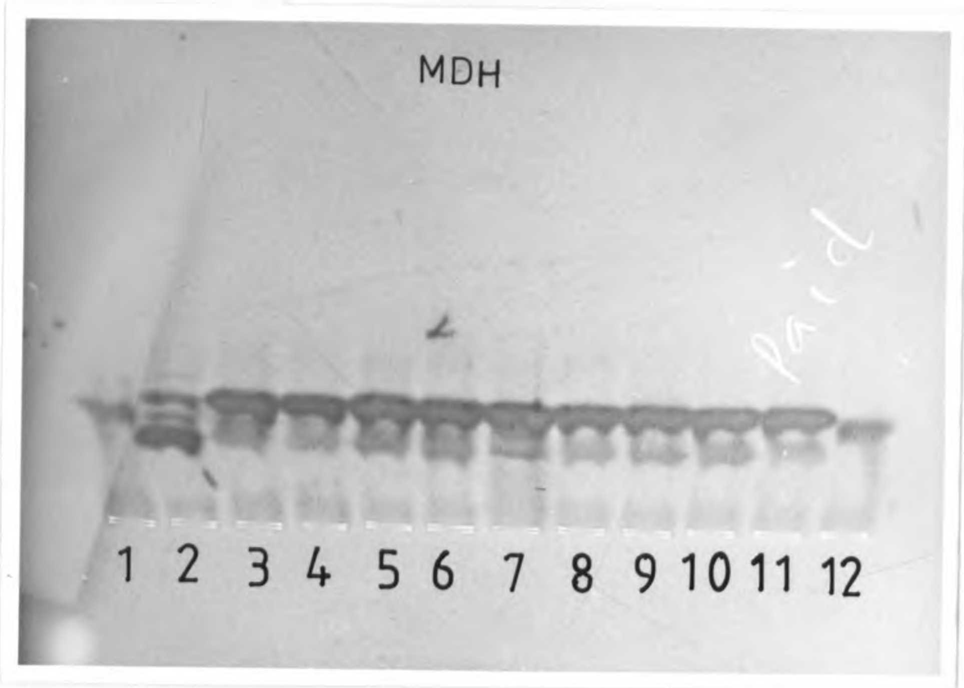
10b

(10c)

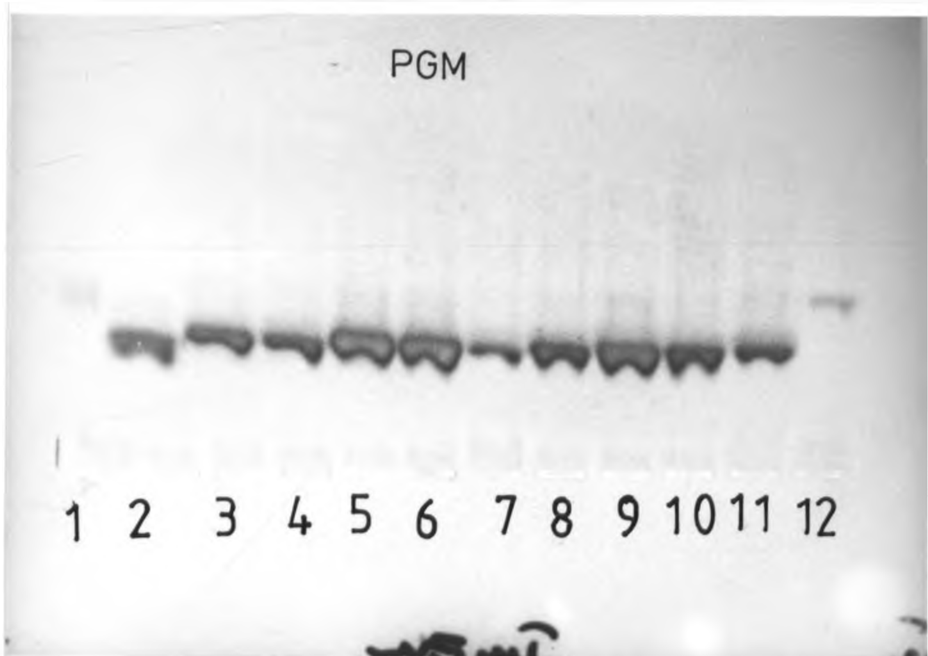
1. NLB-325, *L. donovani* reference strain
2. NLB-708, *Tatera* skin
3. NLB-922, *Tatera* B.marrow
4. NLB-938, *Tatera* skin
5. NLB-965, *Tatera* skin
6. NLB-326, *L. major* reference strain
7. NLB-927, *Tatera* spleen
8. NLB-634, *Tatera* liver
9. NLB-630, *Tatera* B. marrow
10. NLB-560, *Tatera* spleen
11. NLB-707, *Mastomys* spleen
12. NLB-305, *L. tropica* reference strain

(10d)

Arrangement of samples same as shown above



10c



10d

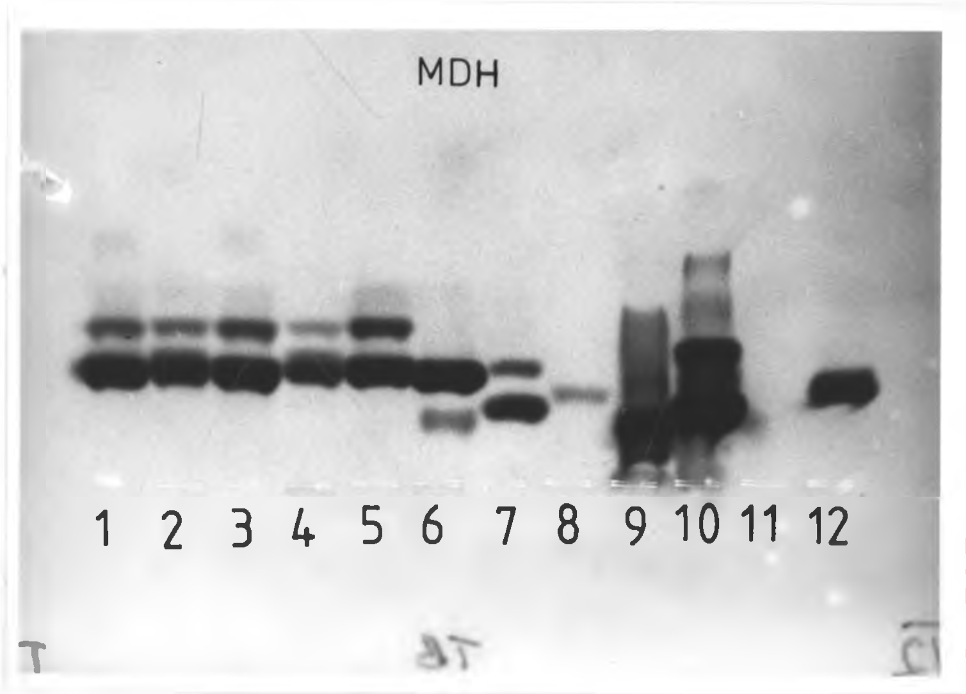
(10e)

1. NLB-326, *L. major* reference strain
2. NLB-455, Man skin
3. NLB-958, *Tatera* spleen
4. NLB-496, *Arvicanthis* liver
5. NLB-765, *P. duboscqi*
6. NLB-1029, *Acomys* spleen
7. NLB-804, *Mastomys* B.marrow
8. NLB-813, *Lemniscomys* liver
9. NLB-979, *Helogale* spleen
10. NLB-1054, *Canis* spleen
11. NLB-926, *Mastomys* spleen
12. NLB-507, *T. musculi* reference strain

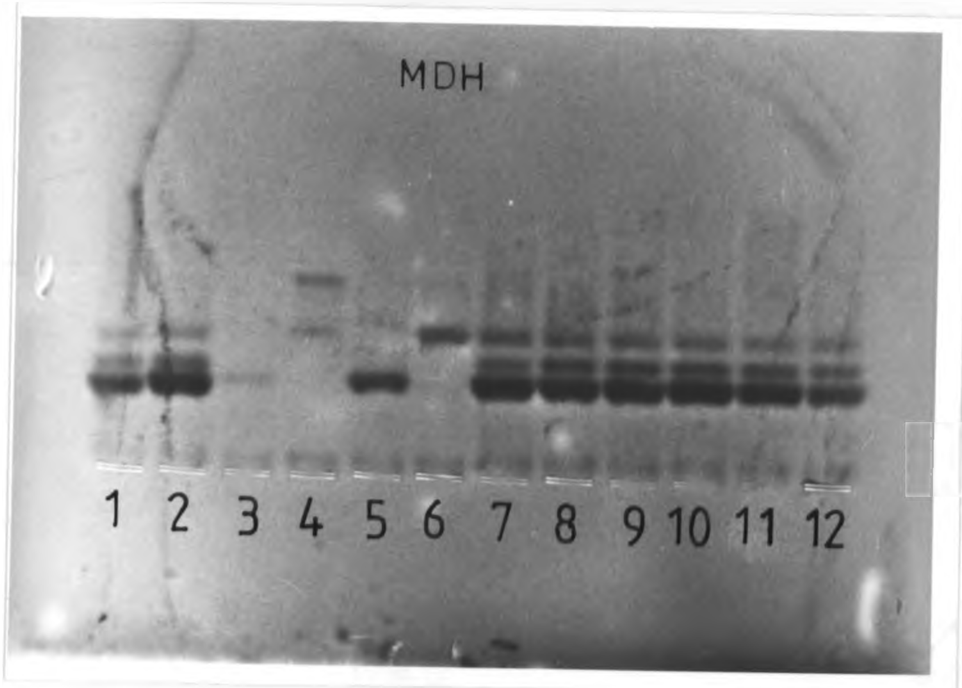
(10f)

Human isolates showing NLB-462 and NLB-491 variants

1. NLB-443, Nasal swab
2. NLB-446, Spleen
3. NLB-453, Spleen
4. NLB-462, Spleen
5. NLB-470, Spleen
6. NLB-491, Spleen
7. NLB-567, Spleen
8. NLB-578, Spleen
9. NLB-573, Spleen
10. NLB-596, Spleen
11. NLB-625, Spleen
12. NLB-325, *L. donovani* reference strain



10e



10f

Plate 11

Morphology of flagellates in a 7-day old culture isolated from rodents and canids from Masinga study area.

(a) Acomys, NLB-842

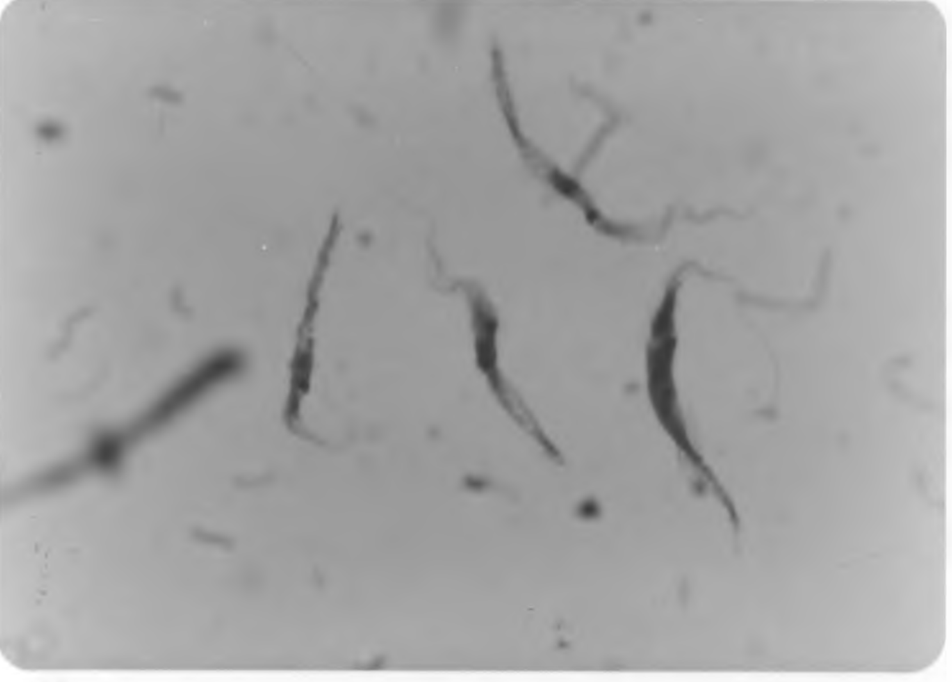
(b) Mastomys NLB-637

(c) Trypanosoma musculi, NLB-507

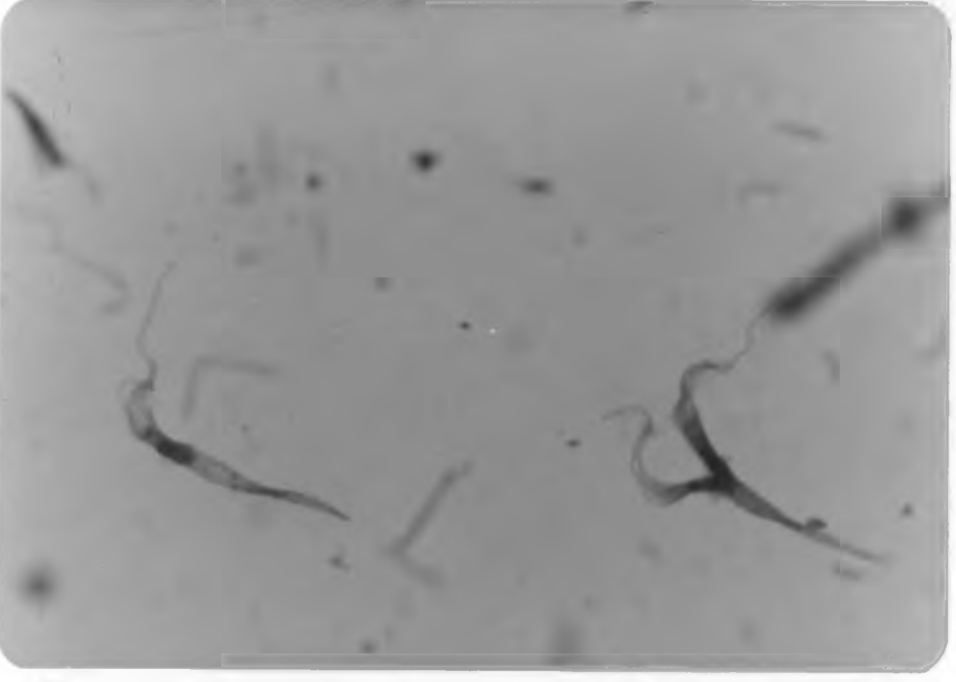
(d) Helogale, NLB-979

(e) Canis, NLB-1054

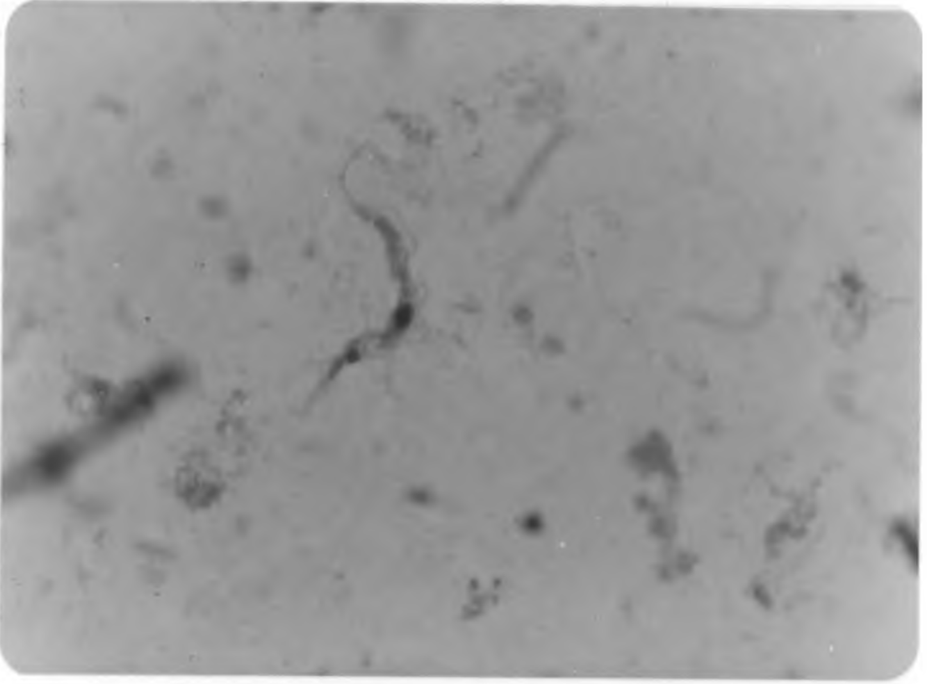
(f) Canis, NLB-039



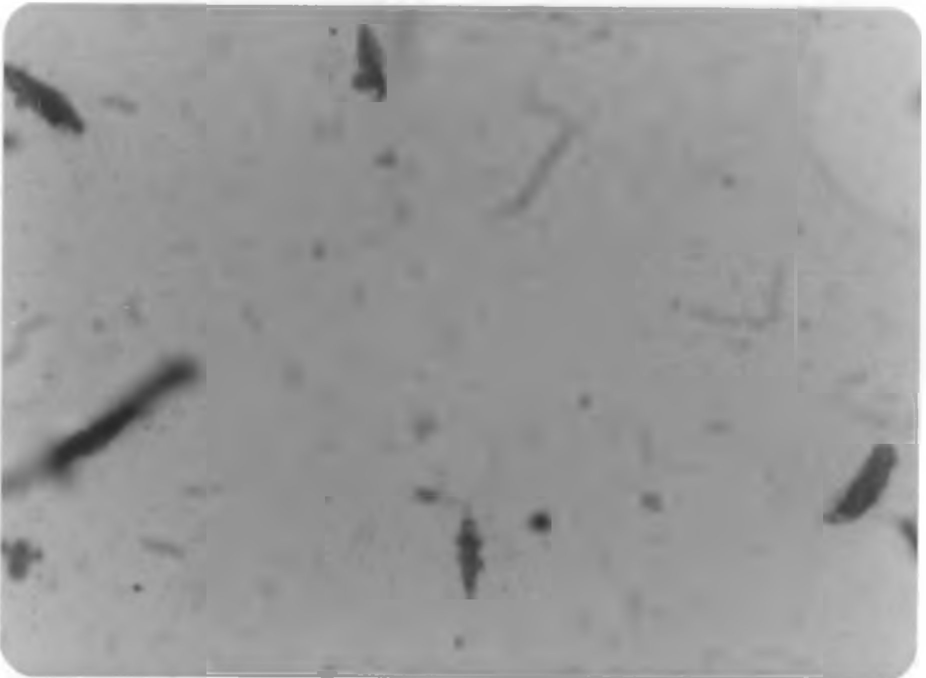
11a



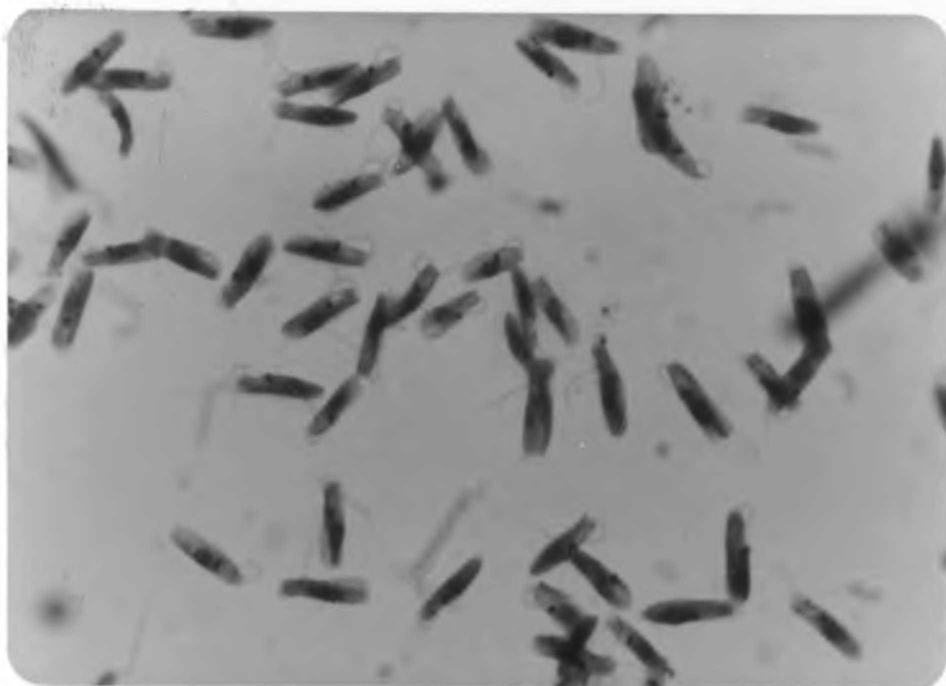
11b



11c



11d



11e

11f

Plate 12

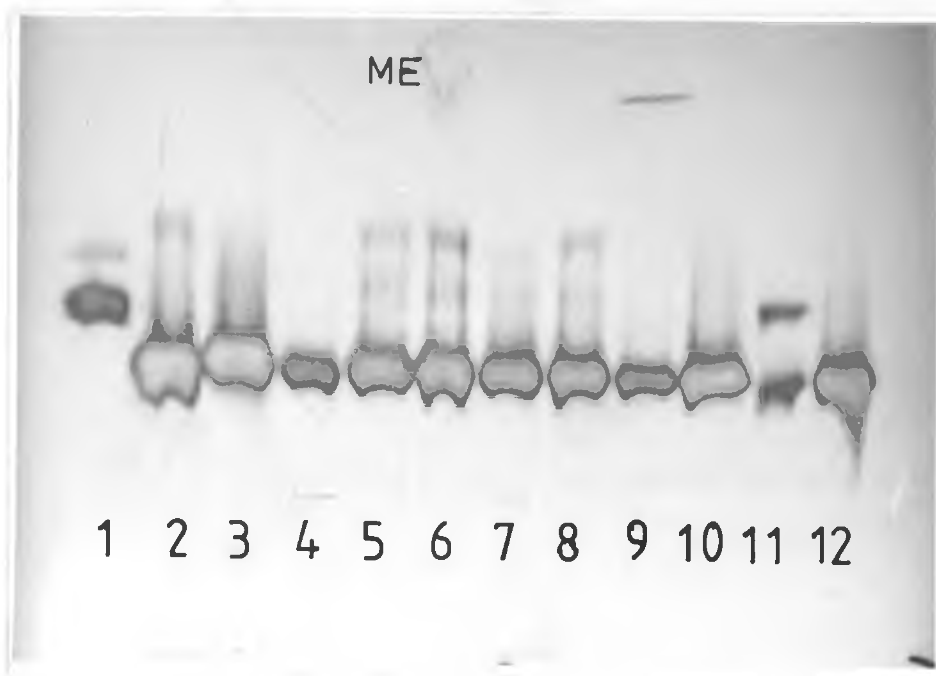
Isolates from rodents from Masinga

(12a)

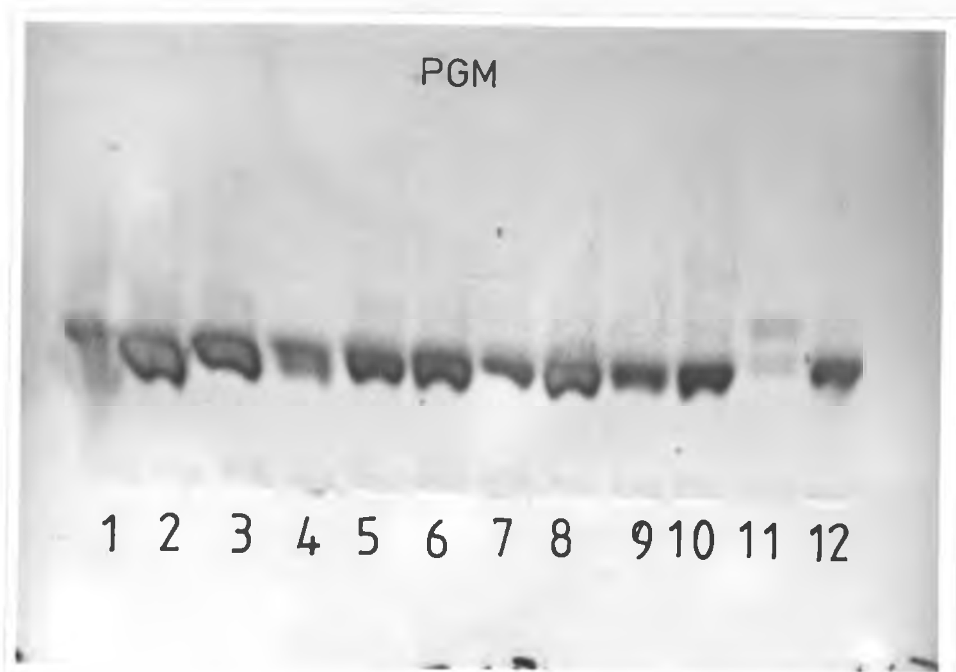
1. NLB-305, _L_. _tropica_ reference strain
2. NLB-636, _Acomys_ B.marrow
3. NLB-641, _Acomys_ B. marrow
4. NLB-712, _Acomys_ spleen
5. NLB-714, _Acomys_ spleen
6. NLB-715, _Acomys_ spleen
7. NLB-716, _Acomys_ spleen
8. NLB-723, _Acomys_ spleen
9. NLB-725, _Acomys_ spleen
10. NLB-774, _Acomys_ liver
11. NLB-325, _L_. _donovani_ reference strain
12. NLB-746, _Acomys_ spleen

(12b)

Arrangement of samples same as shown above



12a



12b

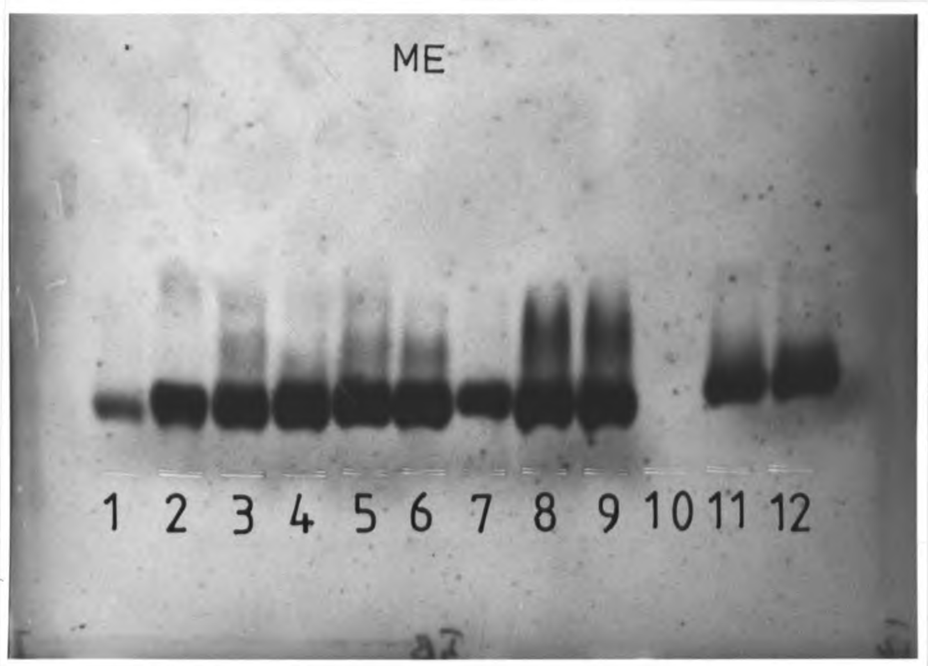
<u>Acomys</u> and <u>Mastomys</u> isolates

(12c)

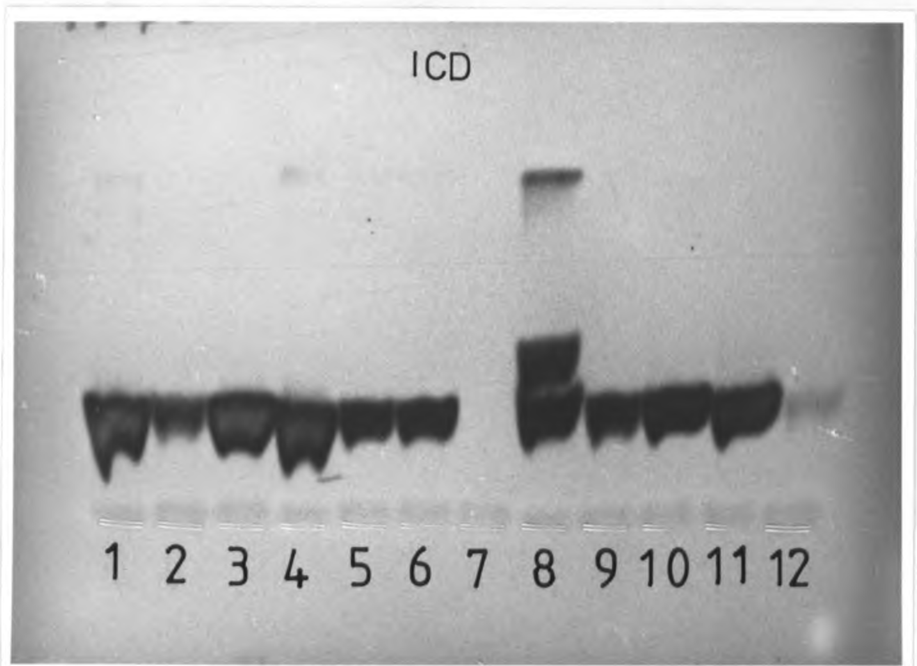
1. NLB-901, <u>Acomys</u> B.marrow
2. NLB-1033, <u>Acomys</u> liver
3. NLB-1029, <u>Acomys</u> spleen
4. NLB-1028, <u>Acomys</u> liver
5. NLB-1026, Acomys liver
6. NLB-1027, <u>Acomys</u> spleen
7. NLB-1055, <u>Acomys</u> spleen
8. NLB-904, <u>Acomys</u> liver
9. NLB-944, <u>Acomys</u> spleen
10. NLB-687, <u>Mastomys</u> liver
11. NLB-749, <u>Mastomys</u> spleen
12. NLB-804, <u>Mastomys</u> B.marrow

(12d)

1. NLB-643, <u>Acomys</u> spleen
2. NLB-768, <u>Acomys</u> skin
3. NLB-809, <u>Acomys</u> liver
4. NLB-924, <u>Acomys</u> liver
5. NLB-990, <u>Acomys</u> B.marrow
6. NLB-980, <u>Acomys</u> spleen
7. NLB-687, <u>Mastomys</u> liver
8. NLB-1033, <u>Acomys</u> liver
9. NLB-1025, <u>Acomys</u> liver
10. NLB-1029, <u>Acomys</u> spleen
11. NLB-1028, <u>Acomys</u> liver
12. NLB-641, <u>Acomys</u> B.marrow



12c



12d

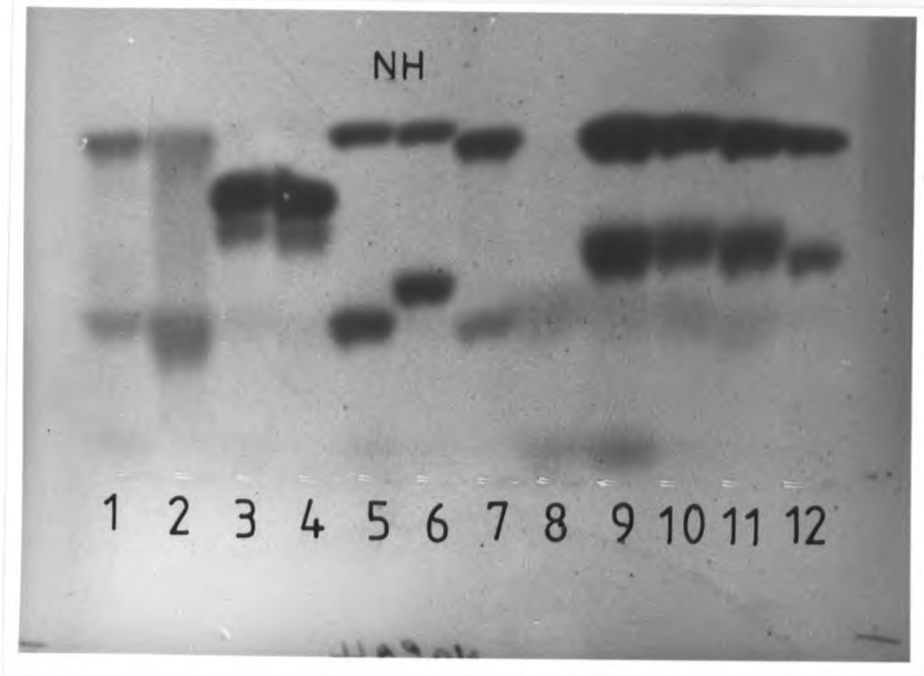
(12e)

Enzyme variants for *Acomys* isolate NLB-1033 and NLB-1055 and a *Mastomys* isolate NLB-810.

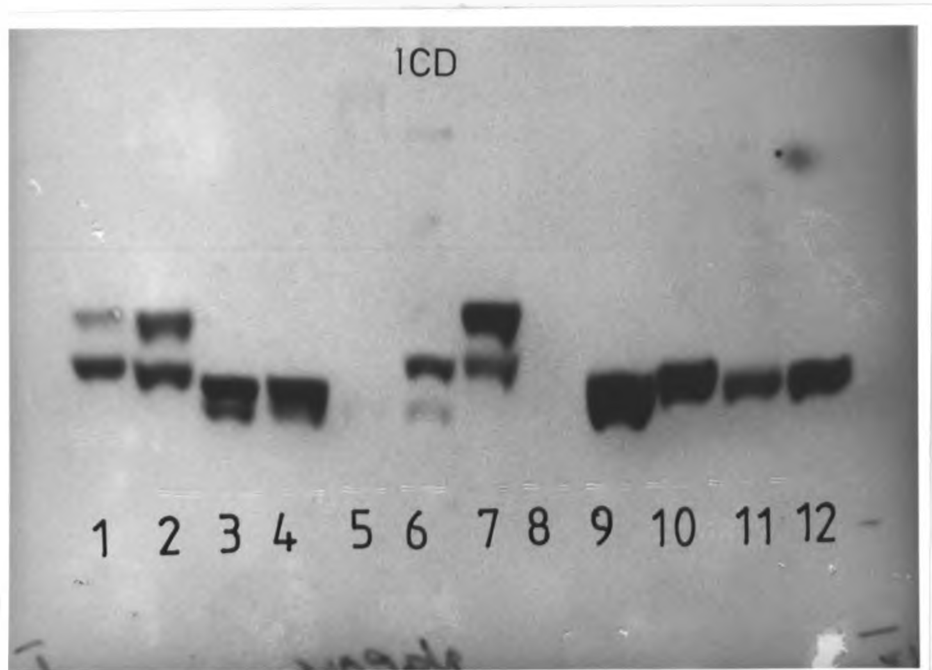
1. NLB-1055, *Acomys* spleen
2. NLB-1033, *Acomys* liver
3. NLB-804, *Mastomys* B.marrow
4. NLB-749, *Mastomys* spleen
5. NLB-810, *Mastomys*, B.marrow
6. NLB-506, *T. lewisi* reference strain
7. NLB-1002, *Herpestes* spleen
8. NLB-743, *Acomy* spleen
9. NLB-754, *Acomys* B.marrow
10. NLB-756, *Acomys* spleen
11. NLB-781, *Acomys* spleen
12. NLB-790, *Acomys* spleen

(12f)

Arrangement of samples same as shown above and showing variants for isolates NLB-1033 and NLB-1055.



12e



12f

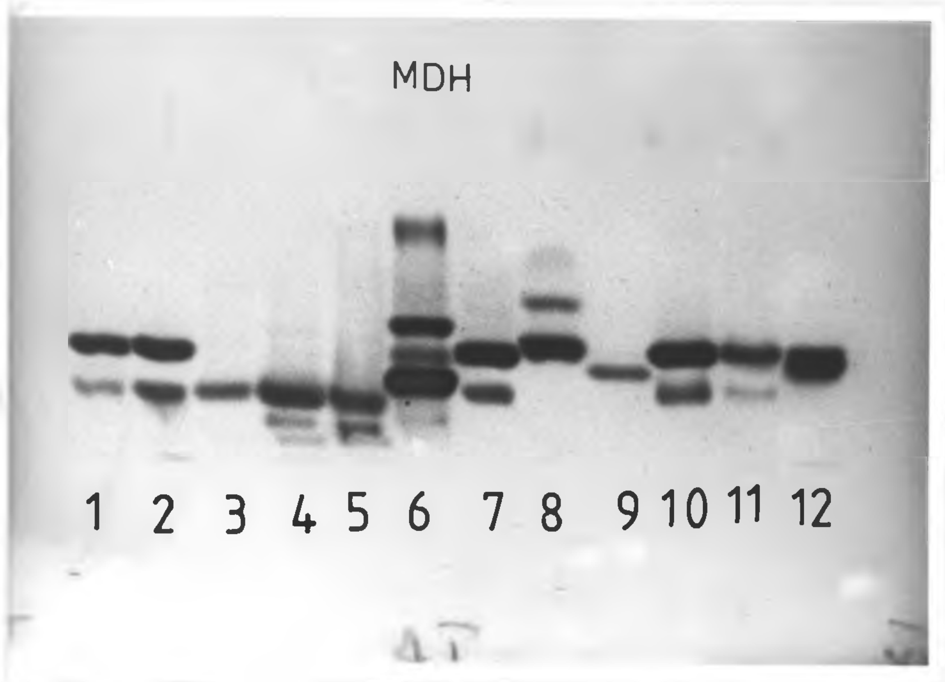
(12g)

Acomys isolate NLB-1033, NLB-1055 and NLB-750 showing enzyme variants.

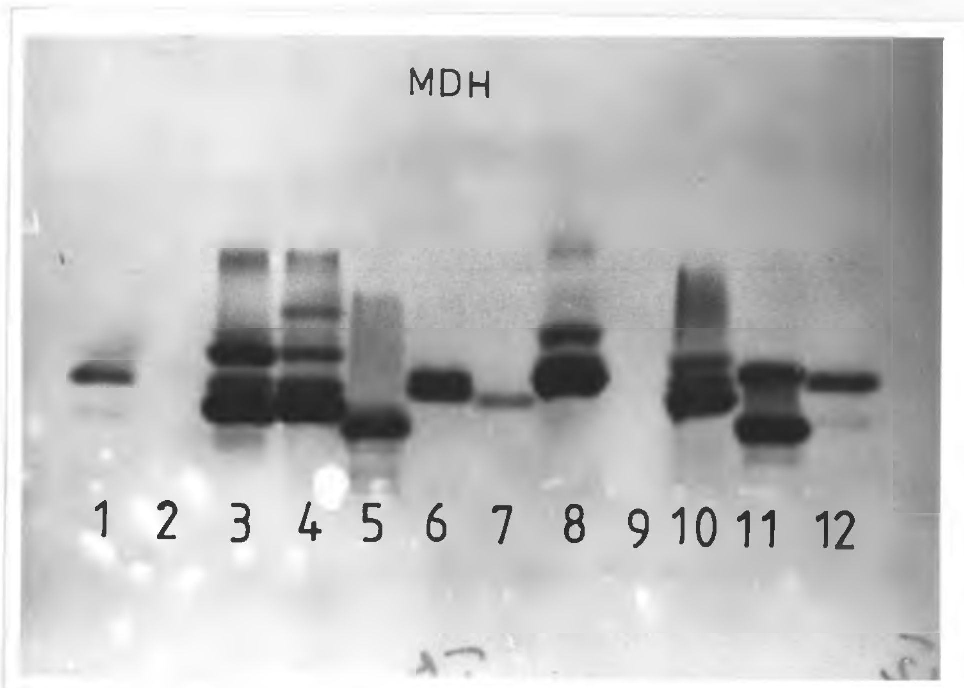
1. NLB-993, Lab infected *Acomys*
2. NLB-924, *Acomys* liver
3. NLB-1002, *Herpestes* spleen
4. NLB-1055, *Acomys* spleen
5. NLB-1033, *Acomys* liver
6. NLB-1054, *Canis* spleen
7. NLB-1034, *Acomys* liver
8. NLB-750, *Acomys* B.marrow
9. NLB-814, *Lemniscomys* spleen
10. NLB-748, *Acomys* spleen
11. NLB-726, *Acomys* skin
12. NLB-507, *T. musculi* reference strain

(12h)

1. NLB-756, *Acomys* spleen
2. NLB-610, Man spleen
3. NLB-1054, *Canis* spleen
4. NLB-039, *Canis* spleen
5. NLB-1002, *Herpestes* spleen
6 NLB-810, *Mastomys* B.marrow
7. NLB-813, *Lemniscomys* liver
8. NLB-326, *L. major* reference strain
9. NLB-926, *Mastomys* spleen
10. NLB-325, *L. donovani* reference strain
11. NLB-203, *S. schwertzi*
12. NLB-493, *L. adleri* reference strain



12g



12h

Fig. 5

Composite diagramatic representation of isolates from Masinga and Marigat examined by cellulose acetate electrophoresis of nine enzymes.

1. NLB-515, *Tatera* skin
2. NLB-496, *Arvicanthis* liver
3. NLB-706, *Mastomys* skin
4. NLB-326, *L. major* reference strain
5. NLB-926, *Mastomys* spleen
6. NLB-1029, *Acomys* spleen.
7. NLB-804, *Mastomys* B.marrow
8. NLB-813, *Lemniscomys* liver
9. NLB-507, *T. musculi* reference strain
10. NLB-979, *Helogale* spleen
11. NLB-1054, *Canis* spleen
12. NLB-325, *L. donovani* reference strain

Fig.5

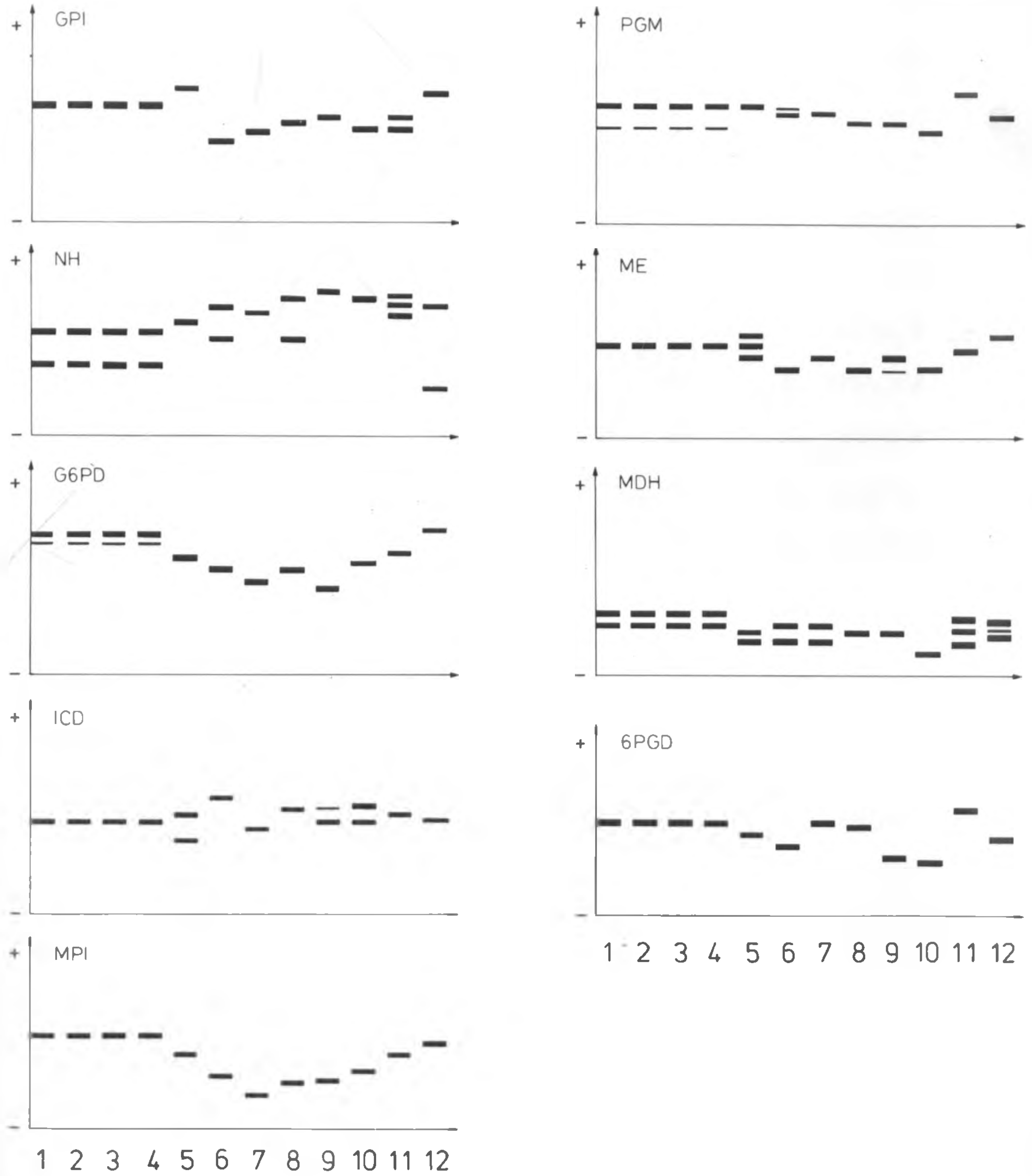


Table 12

A summary of isolates examined by cellulose acetate technique

Host	Location	No. examined	No. identified	Parasite ID
S. schwetzi	Marigat	24	6	*L. adleri*
S. antennatus	"	19	18	*L. adleri*
S. clydei	"	10	9	*L. adleri*
S. africanus	"	1	1	*L. adleri*
P. duboscqi	"	5	5	*L. major*
P. martini	"	4	3	*L. donovani*
Tatera	"	32	32	*L. major*
Mastomys	"	2	1	*L. major*
Arvicanthis	"	3	3	*L. major*
Latastia	"	10	10	*L. adleri*
Varanus	"	2	2	*L. adleri*
Man	"	3	3	*L. major*
Man	"	16	16	*L. donovani*
Acomys	Masinga	62	-	*Herpetosoma*
Mastomys	"	7	-	*Herpetosoma*
Lemniscomys	"	1	-	*Herpetosoma*
Latastia	"	1	1	*L. adleri*
Mongooses	"	3	-	*Herpetosoma*
Dog	"	1	-	*Herpetosoma*
Man	"	15	15	L. donovani

profiles to each other and to L. donovani reference strain. However, one isolate (NLB-616), showed variation in the enzyme MDH.

A summary of all the isolates examined by cellulose acetate electrophoresis is shown in Table 12.

4.4. DISCUSSION

Leishmania major from rodents in Marigat showed a striking degree of homogeneity regardless of source. The three isolates from man, five from P. duboscqi and the isolates from different rodent species were all indistinguishable by the nine enzymes examined, with variation in three enzyme profiles (MDH, G6PD, PGM) for NLB-544 and NLB-708 from Tatera. This indicates that there is a very low level of genetic variability in L. major strains, an observation that was also reported by Le Blancq et al. (1986). They observed that several strains of L. major from different parts of the Old World showed very little variation in 13 enzyme profiles despite the diverse ecological habitats where the strains were obtained. The rodents in Marigat, in particular, Tatera robusta, were shown to possess all the characteristics of a good reservoir (see 1.6.2) and repeated isolation of L. major from Tatera robusta indicates that this rodent is the main reservoir of L. major in Baringo District and that Mastomys natalensis

and _Arvicanthis niloticus_ play secondary roles in maintaining this parasite.

The failure to isolate _L. major_ from rodents in Masinga indicate that this parasite probably does not occur in this area. Earlier studies by Heisch (1963) in Machakos and Kitui districts did not indicate the presence of this parasite. The most common flagellates of animals in this area were nonpathogenic trypanosomes of the subgenus _Herpetosoma_. Heisch (1954) isolated trypanosomes from the spleen of a mongoose, (_Helogale parvula_) in Kitui District which was later identified as _Trypanosoma helogale_ by Grewal (1960). The dog isolate was found to be different from _Leishmania_ by isoenzyme analysis and failed to infect mice. The morphology of this isolate was similar to an isolate made from the spleen of a dog in Turkana District (Githure et al., 1986c).

The isolation of these trypanosomes from the skin, spleen, liver and bone marrow of different animals in this study, emphasises the importance of characterizing fully isolates before drawing conclusions about the epidemiology of leishmaniasis. This is the first report in Kenya that shows a very high infection rate of rodents with trypanosome flagellates. Some of these trypanosomes grew as epimastigotes in culture, a form that can easily be mistaken for promastigotes. The

unique characteristics of the nonpathogenic trypanosomes is their pointed posterior end and their host specificity. These were verified by examination under the microscope and by inoculating the trypanosomes into laboratory mice. Except for two Acomys and one Mastomys isolate, all failed to infect mice. In earlier studies, trypanosomes of the subgenus Herpetosoma were classified on the basis of their host specificity, as each species is reputedly restricted to a single host genus (Molyneux, 1976). The only way to infect a heterologous host is by manipulating the immunological system of the host with immunosuppressive procedures. Under natural conditions, despite the sympatric occurrence of the animal species, cross-infections do not occur. In addition, trypanosome infection in a specific host is cleared from the blood-stream after some time, an observation that was made in mice and laboratory reared Acomys infected with an Acomys isolate and a Herpetosoma reference strain, T. musculi.

The trypanosomes were found to be different from five trypanosome reference strains by isoenzyme analysis. For the first time, Mohammed et al. (1987) were able to identify these five rodent trypanosome strains on the basis of 13 enzyme systems. The trypanosomes in this study showed considerable intraspecific enzyme variations as demonstrated by the

numerous isoenzyme profiles obtained. The reasons for this great variation were not established. Six enzyme profiles were seen among the 72 isolates of trypanosomes examined. There were no suitable reference strains for comparison of trypanosomes from rodents, mongooses and a dog. The only well characterized strains which could have been useful were, *T. acomys* from an *Acomys cahirinus* in Jordan (Molyneux, personal communication) and *T. helogale* from a mongoose in Kenya (Grewal, 1960) neither of which was available for this study. Three of these flagellates (NLB-637, NLB-750, NLB-868) were sent to Prof. D.H. Molyneux of the University of Salford, UK who has a cryobank of mammalian trypanosomes. He compared them with *T. acomys* and *T. musculi* on 8 enzymes system (MDH, ME, PGM, 6PGD, MPI, GPI, NH, SOD) and observed that they were all different except for a mongoose isolate, NLB-868 which was similar to *T. musculi* in two enzymes profiles, PGM and ME.

Due to lack of proper trypanosome reference strains, flagellates isolated in Masinga were classified provisionally on the basis of host restriction (Molyneux, 1976), that is, *T. acomys* from *Acomys*, *T. mastomys* from *Mastomys*, *T. helogale* from *Helogale*, *T. herpestes* from *Herpestes*, *T. canis* from *Canis* and *T. lemniscomys* from *Lemniscomys*. However, this manner of identification requires objective analysis using several

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characterization techniques before conclusive identification can be made.

All 13 lizard isolates examined (12 from Marigat and one from Masinga) were found to be identical to each other and to L. adleri reference strain, NLB-493 by all the nine enzymes systems examined. Similarly, 34 of 54 (63%) sandfly isolates from the genus Sergentomyia were found to be identical to L. adleri reference strain. The remaining 20 isolates have not been identified but a few appear to have an undulating membrane, indicating that they are trypanosomes. However, they were found to be different from the rodent trypanosomes. Lizards are a major source of bloodmeals for some sandflies (Heisch, 1958), explaining a high prevalence of L. adleri in some sandfly species and lizards.

Of the 31 L. donovani human isolates from Masinga and Marigat, only 3 (2 from Marigat and one from Masinga) showed enzyme variation in the enzymes MDH and GPI, indicating that there is very little genomic variation in L. donovani from the two sites. This agrees with the finding of Peters et al., (1977) who showed that L. donovani isolates from Kitui and West Pokot Districts in Kenya were identical on isoenzyme, DNA buoyant density, and excreted factor serotyping.

The infection rate of L. major in rodents from Marigat was high but low in humans and sandflies. Most

of the isolates from the spleens and nasal swabs of patients were identified as L. donovani but the infection rate in sandflies was very low and no infected wild animals were found. The infection rate of L. adleri in lizards and sandflies was very high.

This is the first study in which an extensive number of leishmanial parasites has been examined by isoenzyme analysis. The result show that L. donovani, L. major and L. adleri, the most commonly found Leishmania parasites in Kenya, can be identified with accuracy by isoenzyme analysis of nine enzymes.

5.1. The current situation of animal reservoirs in Kenya

The previous three chapters have dealt with studies on animal reservoirs, susceptibility of wild and domestic animals to *L. donovani* and isoenzyme characterization of *Leishmania* species. This Chapter examines the overall situation regarding animal reservoirs in Kenya.

The immediate impression gained from these studies is the difficult situation faced by an epidemiologist studying leishmaniasis in Kenya and the extreme care with which he must interpret his results. Different types of flagellates were encountered during the survey, namely, *Leishmania*, *Crithidia*, *Trypanosoma* and other unidentified flagellates. This calls for a thorough identification of isolates before drawing any conclusion about their identity or the epidemiology of the disease. Prior to this study, leishmania isolates used to be sent to overseas laboratories for characterization. With the recent establishment of isoenzyme technique in KEMRI, all leishmanial parasites are now characterized locally. In this study, an extensive survey on leishmania identification was done by animal inoculation and isoenzyme analysis. These two methods were found to be adequate in differentiating the most commonly found *Leishmania* species in Kenya, namely,

Chapter 5

GENERAL DISCUSSION AND CONCLUSIONS

L. donovani, *L. major*, and *L. adleri*. However, differentiating flagellates other than known species requires additional techniques and reference strains. If all possible parameters examined are different from the known characteristics of a reference organism, the flagellate can be assigned to a new taxon. Flagellates from animals in Masinga showed different isoenzyme variations but remain uncharacterized due to unavailability of reference strains.

With the construction of Masinga Dam along the River Tana, there has been a significant migration of families into the Masinga Location. The greatest influx of migrants occurred between 1966 and 1970. Ho et al. (1982) showed that in spite of the presence of a susceptible population in this location, visceral leishmaniasis occurred with a low prevalence as evidenced by the small number of individuals with active disease (0.3%), a low leishmanin positivity rate (7.2%) and the presence of leishmanial antibodies in only 3.7%. The susceptible immigrants in Masinga should have allowed for an epidemic spread of the disease if a wild animal reservoir existed in the area.

To date, about 6,960 wild and domestic animals comprising 4,594 rodents, 1,181 canids, 172 bats, 61 bovids, 46 hyraxes, and 42 nonhuman primates have been examined for leishmanial parasites in different parts of

the country for the last four decades. Of these, only one dog has been found infected with L. donovani (Mutinga et al., 1980). This clearly suggests that wild animals do not play an important role in the transmission of visceral leishmaniasis in Kenya. However, Tatera robusta possess most of the criteria of a good reservoir (see section 1.6.2). This study confirms this rodent as the main reservoir of L. major in Baringo District, Kenya. Cutaneous leishmaniasis caused by this parasite has so far been reported in three individuals in Baringo District (Muigai et al., 1987). The most likely reason for the few cases of cutaneous leishmaniasis reported in this area as compared to countries in the Middle East may be a lack of awareness by the indigenous population. Those living in endemic areas are generally farmers or herders who do not have the inclination to deal with every bruise or lesion they may acquire in their occupation. Infected individuals suffer from a self-healing disease that they may feel does not require medical attention. At present, L. major is not a major public health problem in Kenya but the potential for an epidemic exists, particularly in Baringo District where the prevalence of L. major in rodents was found to be high. A similar situation existed in the neighbouring Sudan where the prevalence of cutaneous leishmaniasis was low for many

years until a major outbreak occurred in 1986 with more than 10,000 cases recorded in Khartoum (El-Safi et al., 1988). An increase in the population of vectors and reservoirs coupled with the immigration of a human population into the area may have brought about the epidemic.

Failure to isolate *L. donovani* from animals in Kenya was disappointing, as rodents and carnivores have reportedly been found naturally infected elsewhere (Hoogstraal & Heyneman, 1969; Mansour et al., 1970; Pozio et al., 1981). In the Sudan, follow-up studies to confirm the findings of Hoogstraal & Heyneman (1969) that rodents and wild cats are reservoirs of visceral leishmaniasis in that country have not been done. *Mastomys*, *Rattus*, *Arvicanthis*, and *Heterocephalus* are susceptible to experimental infection with *L. donovani* (Stauber et al., 1966; Githure, 1981; Shatry et al., 1987b; Nolan & Farrel, 1987; Githure et al., 1988). In this study, *Mastomys* and *Acomys* were found to be highly susceptible to *L. donovani* infection. The probable explanation for the absence of natural infection in these otherwise susceptible rodents could be that *P. martini*, the vector of the parasite, is strictly anthropophilic under natural conditions and does not feed on other animals. Further studies on host preferences will be necessary to test this hypothesis.

The work of Mansour et al.(1970) indicated that dogs were unlikely to be the reservoirs of visceral leishmaniasis in East Africa. In this study, an infective L. donovani strain inoculated into two dogs failed to cause an infection six months post-inoculation. Attempts to make isolation of L. donovani from suspected dogs in Kenya have met with little success, implying that the single reported infection (Mutinga et al., 1980) may have been accidental and that man could have been the source of infection for the dog.

5.2. Man as a reservoir of visceral leishmaniasis in Kenya

In epidemiological terms, asymptomatic individuals suffering from visceral leishmaniasis could also serve as reservoirs of the disease. This condition may contribute to positive leishmanin skin test reported in Kenya (Leeuwenburg et al., 1983), the Sudan (Heyneman, 1971), and the Mediterreanean region (Pampiglione et al., 1974). A spectrum of visceral leishmaniasis ranging from subclinical to clinical occurred in a ratio of 5:1 in Kenya (Ho et al., 1982) and in a ratio of 4:1 in Italy (Pampiglione et al., 1974). During serological screening of the population, 6 of 24 positive cases were asymptomatic, one of which had demonstrable amastigotes

in the spleen. The presence of *L. donovani* in the blood or skin of a reservoir host is an absolute requisite for transmission of the parasite by sandflies. In Kenya and India, post kala-azar dermal leishmaniasis is found in 4.5% and 20% respectively among patients who have been cured of visceral leishmaniasis (Thakur, 1984; Muigai, R. personal communication). Post kala-azar dermal leishmaniasis is a condition in which amastigotes disseminate to the skin of the entire body. This type of infection is very difficult to treat and the individuals may serve as reservoirs of infection for the sandflies. Past studies by Shortt et al. (1927) showed that *L. donovani* was present in the peripheral blood smears of 79% of patients suffering from visceral leishmaniasis in India. To demonstrate whether sandflies can pick infection from visceral leishmaniasis patients in Kenya, Manson-Bahr (1959) took skin snips from various parts of the body and showed that 11% of 94 patients had positive smears taken from normal looking skin on the legs, arms and shoulders. He concluded that there were large numbers of parasites scattered over the entire body of visceral leishmaniasis patients which were readily available to the sandfly during feeding. He also showed that 30% of patients suffering from the disease had positive blood cultures of *L. donovani*. More recently, Chulay et al. (1985) demonstrated

parasites in different fractions of peripheral blood in 15 (75%) of patients with visceral leishmaniasis.

In India, no animal reservoir has been reported to date. It has been shown that L. donovani in India and the Sudan can be transmitted mechanically through nasal discharge (Forkner & Zia, 1934; Hoogstraal & Heyneman, 1969). Hendricks et al. (1985) examined nasal and throat swabs from 24 patients suffering from visceral leishmaniasis in Kenya. They were able to demonstrate L. donovani in culture and smears of nasal swabs from 10 (42%) of the patients. In this study, 3 isolates from nasal swabs were identified as L. donovani. Thus there is a possibility of man to man transmission through nasal secretions which might explain the clustering and focality of visceral leishmaniasis in Kitui and Machakos districts (Wijers, 1963; Ho et al., 1982).

Indian visceral leishmaniasis caused by L. donovani and cutaneous leishmaniasis caused by L. tropica in the Middle East constitute the final stage of the evolution from a zonoosis to anthroponosis with only man involved in the transmission. The situation in Kenya may be anthropozoonoses in which man acts as the main source of infection for other vertebrates. The above mentioned facts and the failure to isolate L. donovani from animals in this and the previous studies suggest that transmission of visceral leishmaniasis in Kenya is

similar to that in India, that is, man to man, with no animal reservoir involved. However, it should be noted that this investigation was not an exhaustive search for animal reservoirs and further investigations should continue in other animals such as, nonhuman primates, goats, dogs, wild canids, bats, ant-eaters, etc. Since no animal except man, has been found repeatedly infected with *L. donovani* in the last four decades of search for reservoirs, it seems reasonable to conclude that man is the main reservoir of visceral leishmaniasis in Kenya.

5.3. Conclusions

1. It is concluded that *Tatera robusta*, *Mastomys natalensis* and *Arvicanthis niloticus* are the main animal reservoirs of cutaneous leishmaniasis caused by *Leishmania major* in Marigat area of Baringo District and that the infection is absent in the Masinga area of Machakos District.
2. Although visceral leishmaniasis occurs in humans in Masinga and Marigat locations, none of the 1900 animals examined from these areas were found to be infected with *L. donovani*. Attempts to infect domestic animals (dogs, cats, goats) with *L. donovani* were not successful while *Mastomys* and *Acomys* were found to be susceptible. It is concluded that visceral leishmaniasis has no animal reservoir and therefore that man is the only reservoir of the disease.

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Appendix 1

Names, geographic distribution and disease manifestation of *Leishmania* spp. infecting humans[@].

Species/subspecies	Clinical disease	Geographic area
L. donovani donovani	VL, PKDL	India, Asia, E. Africa
L. d. infantum	VL, CL*	S. Europe, N. Africa
L. d. chagasi	VL, CL*	S. and Central America
L. major	CL	Mid-East, Africa, Asia
L. tropica	CL	Mid-East, Africa, India
L. aethiopica	DCL, CL	Ethiopia, Kenya
L. m. mexicana	CL, DCL*	N. and Central America
L. m. amazonensis	CL, DCL	Brazil, Panama
L. m. pifanoi	DCL	Venezuela
L. m. venezuelensis	CL	Venezuela
L. peruviana	CL	Peru (Highlands)
L. b. braziliensis	CL, MCL	S. and Central America
L. b. panamanensis	CL, MCL	S. and Central America
L. b. guyanensis	CL	N. East of S. America

@ WHO, 1984.

VL = Visceral leishmaniasis

CL = Cutaneous leishmaniasis

DCL = Diffuse cutaneous leishmaniasis

MCL = Mucocutaneous leishmaniasis

PKDL = Post kala-azar dermal leishmaniasis

* = Rare manifestation

Appendix 2a

Rainfall in Marigat between January 1986 to December 1987*

Months	Rainy Days	Rainfall (mm)
January	0	0.0
February	0	0.0
March	3	16.7
April	20	163.5
May	18	50.7
June	14	98.6
July	8	40.8
August	7	42.7
September	5	50.3
October	2	6.5
November	1	12.4
December	1	1.9
January	2	2.6
February	3	64.2
March	6	16.6
April	10	133.5
May	16	92.3
June	12	128.5
July	5	11.2
August	10	31.5
September	1	1.3
October	3	7.4
November	16	79.4
December	0	0.0
		1052.6

* Obtained from the Metereological Department, Ministry of Transport and Communication.

Appendix 2b

Rainfall in Masinga between July 1986 to June 1988*

Months	Rainy Days	Rainfall (mm)
July	0	0
August	0	0
September	1	0.3
October	4	18.6
November	22	261.3
December	3	34.1
January	3	45.5
February	0	0.0
March	2	14.1
April	12	187.0
May	6	13.2
June	5	77.5
July	0	0.0
August	3	13.8
September	0	0.0
October	1	0.4
November	13	122.4
December	2	7.5
January	7	34.6
February	0	0.0
March	9	77.2
April	21	300.8
May	3	44.4
June	2	15.6
		1268.3

*Obtained from the Metereological Department, Ministry of Transport and Communication.

Appendix 3a

Animals isolates from Marigat identified by cellulose acetate electrophoresis.

Animal sp.	Animal number	Sex	Nairobi Leishmania Bank Number Spleen	Liver	B.marrow	Skin
Tatera	5	M	-	-	-	538
Tatera	6	F	-	-	-	515
Tatera	14	M	-	-	-	526
Tatera	17	F	-	524	-	518
Tatera	29	F	544	-	-	-
Tatera	30	M	-	-	-	540
Tatera	31	M	-	-	-	541
Tatera	35	M	560	-	-	-
Tatera	55	M	584	-	-	-
Tatera	61	F	586	-	-	585
Tatera	87	M	-	-	-	+L
Tatera	95	F	633	-	-	-
Tatera	99	M	629	-	630	-
Tatera	102	F	632	631	635	-
Tatera	114	F	628	634	-	627
Tatera	116	F	+L	-	-	-
Tatera	124	F	601	-	-	-
Tatera	143	F	649	-	650	642
Tatera	145	F	665	-	-	-
Tatera	146	F	662	-	-	-
Tatera	158	M	663	-	-	-
Tatera	165	F	-	-	-	666
Tatera	172	F	693	-	-	-
Tatera	214	M	-	-	-	708
Tatera	245	F	737	-	-	-
Tatera	251	F	741	-	742	+L
Tatera	349	M	-	-	878	-
Tatera	400	M	-	-	922	-
Tatera	401	F	-	-	929	-
Tatera	407	M	927	-	-	938
Tatera	452	F	-	-	-	+L
Tatera	464	M	-	-	-	+L
Tatera	469	F	964	-	-	-
Tatera	472	F	957	-	-	-
Tatera	474	F	958	-	-	965
Tatera	477	F	959	-	-	-
Arvicanthis	10	F	-	496	-	-
Arvicanthis	13	M	-	497	-	-
Arvicanthis	509	M	-	-	780	-
Mastomys	200	M	707	-	-	706
Mastomys	316	M	926	-	-	-

NB

L = Isolate lost through contamination.

F = Female.

M = Male.

Appendix 3b
Animal isolates from Masinga examined by cellulose acetate electrophoresis.

Animal sp.	Animal number	Sex	Nairobi Leishmania Bank Number Spleen	Liver	B.marrow	Skin
Acomys	21	F	643	639	641	-
Acomys	22	M	638	640	636	-
Acomys	30	F	715	724	-	-
Acomys	31	F	710	-	717	-
Acomys	32	F	711	718	-	-
Acomys	33	M	712	713	721	726
Acomys	34	M	714	719	727	720
Acomys	36	F	722	709	-	-
Acomys	37	M	725	-	-	-
Acomys	38	F	+L	-	-	+L
Acomys	39	F	716	-	-	-
Acomys	43	M	723	+L	-	-
Acomys	45	F	-	-	750	-
Acomys	46	M	781	-	-	-
Acomys	47	M	746	-	-	-
Acomys	48	F	743	774	-	-
Acomys	51	F	+L	-	-	-
Acomys	53	F	+L	-	-	-
Acomys	54	F	773	753	-	-
Acomys	56	F	787	-	-	-
Acomys	57	F	767	-	752	-
Acomys	58	F	+L	-	747	-
Acomys	60	M	775	-	-	-
Acomys	62	F	789	-	754	768
Acomys	63	F	756	-	-	755
Acomys	64	F	+L	-	-	-
Acomys	66	M	790	758	-	-
Acomys	68	F	761	-	-	802
Acomys	69	F	748	-	-	-
Acomys	70	M	751	-	-	-
Acomys	72	M	-	+L	803	-
Acomys	73	F	-	809	798	-
Acomys	78	F	836	825	-	-
Acomys	79	F	841	865	-	-
Acomys	80	F	831	826	-	-
Acomys	81	F	832	827	-	-
Acomys	82	M	-	833	-	-
Acomys	83	M	842	-	-	-
Acomys	84	M	-	856	-	-
Acomys	85	F	-	837	-	-
Acomys	86	M	839	834	-	-
Acomys	87	M	-	835	-	-
Acomys	88	F	853	840	-	-
Acomys	89	F	864	843	-	-
Acomys	93	F	911	904	-	-
Acomys	94	M	-	-	897	-
Acomys	97	M	-	903	899	-
Acomys	99	F	-	900	895	-
Acomys	101	M	-	898	-	-

Appendix 3b continued

Acomys	103	F	-	-	901	-
Acomys	105	F	-	+L	896	-
Acomys	108	F	944	931	-	-
Acomys	110	M	-	923	-	-
Acomys	111	F	930	-	-	-
Acomys	112	M	945	924	-	-
Acomys	113	M	941	925	-	-
Acomys	114	F	-	932	-	-
Acomys	119	M	+L	-	-	-
Acomys	123	F	969	-	966	-
Acomys	127	M	-	-	990	-
Acomys	129	F	980	-	-	-
Acomys	132	F	-	1033	-	-
Acomys	133	F	1029	1025	-	-
Acomys	134	F	-	1028	-	-
Acomys	135	F	-	1026	-	+L
Acomys	136	F	1027	1034	1030	-
Acomys	142	M	1055	1059	-	-
Mastomys	67	F	-	637	+L	-
Mastomys	95	M	686	687	691	-
Mastomys	185	M	702	694	703	704
Mastomys	234	M	749	-	-	-
Mastomys	245	M	-	-	810	-
Mastomys	248	M	-	+L	-	-
Mastomys	253	M	-	+L	-	-
Mastomys	258	M	-	808	804	-
Mastomys	268	F	-	+L	-	-
Mastomys	272	F	-	+L	796	-
Mastomys	288	F	-	+L	-	-
Mastomys	300	F	-	+L	-	-
Lemniscomys	22	F	814	813	812	-
Lemniscomys	41	F	-	-	+L	-
Helogale	1	F	979	-	-	-
Herpestes	3	M	868	-	-	-
Herpestes	7	F	1002	-	-	-
Canis	78	F	1054	-	-	-

NB
L = Isolate lost through contamination.
F = Female.
M = Male.

Appendix 3c

Lizards isolates identified by cellulose acetate technique

Lizard sp.	Number	NLB #	Locality
Latastia	3	517	Marigat
Latastia	5	535	Marigat
Latastia	7	532	Marigat
Latastia	9	533	Marigat
Latastia	11	542	Marigat
Latastia	14	614	Marigat
Latastia	16	661	Marigat
Latastia	22	698	Marigat
Latastia	23	728	Masinga
Latastia	27	974	Marigat
Latastia	28	973	Marigat
Varanus	1	534	Marigat
Varanus	2	731	Marigat

Appendix 4

Human isolates identified by cellulose acetate technique

Isolate number	Patient number	Sex	Age	Source	Location	Parasite ID
256	231	M	7	Spleen	Marigat	L. donovani
273	231	M	7	Nasal	Marigat	L. donovani
276	242	M	2	Spleen	Marigat	L. donovani
309	287	M	15	Spleen	Masinga	L. donovani
422	344	M	50	Spleen	Masinga	L. donovani
424	347	M	12	Nasal	Masinga	L. donovani
426	347	M	12	Spleen	Masinga	L. donovani
437	362	M	7	Spleen	Masinga	L. donovani
439	366	M	18	Spleen	Marigat	L. donovani
443	366	M	18	Nasal	Marigat	L. donovani
446	370	M	7	Spleen	Marigat	L. donovani
452	369	M	4	Spleen	Masinga	L. donovani
453	380	M	46	Spleen	Masinga	L. donovani
455	382	F	9	Skin	Marigat	L. major
462	397	F	5	Spleen	Marigat	L. donovani
470	401	M	6	Spleen	Marigat	L. donovani
473	409	F	11	Skin	Marigat	L. major
480	404	M	30	Spleen	Marigat	L. donovani
491	405	M	11	Spleen	Marigat	L. donovani
498	412	F	NR	Spleen	Marigat	L. donovani
499	410	M	4	Spleen	Masinga	L. donovani
564	457	F	9	Spleen	Marigat	L. donovani
566	460	F	8	Spleen	Marigat	L. donovani
567	462	F	36	Spleen	Masinga	L. donovani
573	461	M	6	Spleen	Masinga	L. donovani
578	466	M	8	Spleen	Masinga	L. donovani
582	476	M	5	Skin	Marigat	L. major
596	475	F	11	Spleen	Masinga	L. donovani
610	478	F	17	Spleen	Masinga	L. donovani
616	481	M	14	Spleen	Masinga	L. donovani
625	493	M	37	Spleen	Marigat	L. donovani
626	494	F	4	Spleen	Marigat	L. donovani
762	543	F	16	Spleen	Masinga	L. donovani
849	588	M	9	Spleen	Marigat	L. donovani

Appendix 5
Sandfly isolates identified by cellulose acetate technique

NLB #	Sandfly	Parasite ID
144	P. duboscqi	L. major
145	S. schwetzi	L. adleri
146	S. schwetzi	L. adleri
147	S. schwetzi	L. adleri
148	S. schwetzi	L. adleri
198	S. clydei	L. adleri
199	S. clydei	L. adleri
200	P. duboscqi	L. major
202	S. clydei	L. adleri
203	S. schwetzi	Crithidia sp.
204	S. schwetzi	Crithidia sp.
205	S. schwetzi	Crithidia sp.
207	S. schwetzi	Crithidia sp.
210	S. schwetzi	Crithidia sp.
211	S. schwetzi	Crithidia sp.
212	S. schwetzi	Crithidia sp.
217	S. schwetzi	Crithidia sp.
366	S. antennatus	L. adleri
375	S. antennatus	L. adleri
376	S. antennatus	L. adleri
384	P. martini	L. donovani
386	S. antennatus	L. adleri
399	S. clydei	L. adleri
403	S. antennatus	L. adleri
420	S. africanus	L. adleri
425	S. antennatus	L. adleri
431	S. antennatus	L. adleri
432	S. antennatus	L. adleri
434	S. antennatus	L. adleri
435	S. antennatus	L. adleri
436	S. antennatus	L. adleri
438	S. antennatus	L. adleri
441	S. antennatus	L. adleri
442	S. clydei	L. adleri
477	S. antennatus	L. adleri
479	S. antennatus	L. adleri
486	S. antennatus	L. adleri
501	S. clydei	L. adleri
503	S. clydei	L. adleri
511	S. schwetzi	L. adleri
529	P. martini	L. donovani
543	S. clydei	L. adleri
550	S. schwetzi	L. adleri
677	S. clydei	L. adleri
732	S. antennatus	L. adleri
765	P. duboscqi	L. major
769	P. duboscqi	L. major
794	P. duboscqi	L. major
838	S. antennatus	L. adleri
867	P. duboscqi	L. major
1017	P. martini	L. donovani

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