PRODUCTION OF SPECIFIC ANTISERA AGAINST SELECTED MAMMALS AND IDENTIFICATION OF BLOOD MEALS OF PHLEBOTOMISE SANDFLIES TRANSMITTING VISCERAL LEISHMANIASIS (VL) IN ETHIOPIA

A Thesis submitted to the School of

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In partial fulfilment of the

requirements for the Degree of

Master of Science in Biology (Biomedical Sciences)

By

Hassen Mamo

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ABSTRACT

In an attempt to know the feeding preferences of phlebotomine sandflies that transmit visceral leishmaniasis (VL) in Ethiopia, *Phlebotomus martini* and *P. orientalis*, and thus to point to the possible reservoir host(s) of the disease in the country, blood meals of the vectors were identified using the counter current immunoelectrophoresis (CCIE) technique. First, genus/species-specific antisera were raised against the immunoglobulin G (IgG) of ten potential hosts of the sandfly vectors: human, sheep, goat, donkey, horse, camel, cattle, squirrel, mongoose and hyrax. The IgG was fractionated from the whole sera of the mammals by the ammonium sulphate precipitation method followed by the ion-exchange chromatography technique. The antisera were developed by priming (subcutaneously) rabbits, in triplicates, with water-in-oil emulsion of equal volumes of IgG and Freund's Complete Adjuvant and then boosting with Freund's Incomplete Adjuvant. The potency of the antisera was determined by assaying each test bleed from each rabbit against the homologous IgG. Cross-reactivities were checked by screening each test bleed against heterologous IgGs. Human whole serum and IgG were cross-reacted with anti-cattle antisera and vice versa. All other antisera were specific. The titre of each test bleed was qualitatively determined by visualizing the conspicuousness of the precipitin line. Hyperimmune antisera were used in blood meal analysis. Eight blood meal samples (*5 Sergentomyia* spp. and 3 *P. martini*) were collected, by sticky and CDC light traps, from Aba-Roba in a total of 42 night catches. One hundred and six blood meal samples (94 *P. orientalis* and 12 *P. bergeroti*) collected, by CDC, from the Middle Awash valley and stored for about 4 years were used. Each blood meal sample was tested against commercial anti-dog and anti-rat IgGs

in addition to the 10 anti-host sera raised in the present study . Of 114 blood meals processed, hosts were identified for the 93 (81.6%): 2 *P. martini*, 79 *P. orientalis* and all *P. bergeroti*. None of *Sergentomyia* blood meals were identified. 37.6% of the blood meals detected were from single host sources: 20 from cattle, 10 camel, 2 squirrel, 1 donkey, 1 human and 1 from mongoose. The rest 62.4% was from mixed sources. Altogether, 80.6% of the meals identified were from cattle origin and 59% from camel, singly or in combination with other hosts. Thus, *P. orientalis* appears to be an opportunistic feeder with a preference for cattle and camel at least in the Middle Awash where these hosts occur in large numbers. The CCIE technique is specific and sensitive enough not only to detect mixed blood meals from closely related hosts but half-digested meals. The assay is technically simple and inexpensive in terms of reagents required. Thus, CCIE is a robust technique for identification of blood meals of haematophagous insect vectors in general and smaller flies such as phlebotomine sandflies and of biting midges in particular.

1. INTRODUCTION

1. 1. General

Leishmaniases represent a spectrum of disease manifestations acquired mainly by mammals as a result of infection by groups of flagellate protozoa that belong to genus *Leishmania* (Kinetoplastida: Trypanosomatidae). The etiologic agents are obligate intracellular parasites. They parasitize the reticulo-endothelial cells (mononuclear or phagocytic cells) in various organs and tissues of their hosts. Of the over thirty species and subspecies described for the genus so far, about twenty are known to cause disease in man.

Leishmaniases are reported from about 97 tropical and subtropical countries (3). A conservative estimate by the above authors shows that, world wide while 12 million people are suffering from leishmaniases 0.40 million cases occur per annum and 367 million people are at risk of acquiring it. Thus, WHO considers leishmaniases as one of the six major tropical diseases of public health importance.

1. 1. 1. Transmission cycle

Most types of human leishmaniases are mainly transmitted by the bite of blood sucking female sandflies of the genus, *Phlebotomus* and *Lutzomyia* (Diptera: Pschycodidae: Phlebotominae). The former is an Old World genera and the latter is confined to the New World. Species belonging to the genus *Sergentomyia* have got no medical significance, they are responsible for transmitting reptilian leishmaniasis.

Leishmania have two developmental stages. The stage found in a vertebrate host is oval

shaped and devoid of external flagellum thus is called amastigote or Leishman-Donovan (L-D) body (in honour of its discoverers, W.B. Leishman 1903 & Donovan 1903). A sandfly feeding on a blood meal on a host having the L-D bodies in its skin and/or blood picks up these stages of the parasite. In the mid gut lumen of an appropriate species of phlebotomine sandflies the L-D bodies transform into a slender flagellated stage which is infective to a susceptible host. When the sandfly visits another host for a second round meal the promastigotes get inoculated into the skin of a luckless victim. While in the skin (or blood) of their host the promastigotes transform into amastigotes and remain in the cells of the skin or visceralize depending on the species and keep on reproducining by binary fission. This cycle repeats itself.

Although very negligible there are records showing transmission of leishmaniasis among humans through blood transfusion, congenital and direct modes (Ayele, 1982).

1. 1. 2. Clinical Forms

In man at least three clinically distinct forms of the disease exist: cutaneous, mucocutaneous, and visceral (kala azar).

Cutaneous leishmaniasis (CL) is principally caused by *Leishmania major, L. tropica*, and *L. aethiopica* in the Old World and *L. mexicana* complex & *L. braziliensis* complex in the New World (WHO, 1996). Majority of CL cases are characterized by a cell-mediated reactions of varying intensity, at the site of parasite inoculation, later after recovery (by immunity or treatment) leaving a prominent scar which is disfiguring. Under such circumstances the disease is referred to as localized CL (LCL). However, *L. amazonensis*

(species of *L. mexicana* complex) and *L. aethiopica* occasionally tend to disseminate to various parts of the victim's skin far from the local site of inoculation resulting in diffuse CL (DCL) for poorly addressed reasons (WHO, 1990a).

Mucocutaneous leishmaniasis (Espundia) results from infection with *L. panamensis* and *L. guyanensis* being highly confined to South and Central America and a few cases due to L. aethiopica in Ethiopia and Kenya (WHO, 1990a). *L. major* and *L. donovani* strains are also reported to induce espundia in immunocompromized patients (Desjeux, 1996). The typical features of the disease are a progressive ulceration and erosion of the soft tissues of the mucosa of the nose, mouth, and pharynx occurring either soon after the initial infection or many years (even several decades) after apparent resolution of the primary lesions (Osman, 1998).

Visceral leishmaniasis (VL) (kala azar) is a disease of visceral organs especially the spleen, liver, bone marrow and lymphoid tissues. The causal agents are various species and strains of *L. donovani* complex.

Post-kala-azar leishmaniasis (PKDL) is a complication of unknown cause observed in ex-VL patients after a successful treatment. It is characterized by a chronic granulomatous infiltrations of the skin and patchy hypopigmentations that occur during or after treatment up to 27 years, usually without ulceration (Osman, 1998).

1. 2. Epidemiology of Old World VL

1. 2. 1. Scope of the problem

VL is the most severe form of leishmaniases which is nearly always fatal if left untreated (Desjeux, 1996). It accounts for an estimated 75,000 deaths annually worldwide (Ashford *et al.*, 1992). The disease is characterized by irregular fever, loss of weight (emaciation), splenomegaly, hepatomegaly and/or lymphadenopathy and anaemia (WHO, 1996). Pertaining to the clinical and epidemiological characteristics of the disease, Old World VL endemic area fall into three major nosogeographical subregions which are best dealt with separately. These are the Mediterranean-Middle Asian subzone, Indian subcontinent and East African subregion. The causes of VL in the Mediterranean and Indian subregions are *L. donovani infantum* and *L. donovani donovani* respectively (Ashford and Bettini, 1987). While *Phlebotomus major, P. perniciosus, P. chinensis, P. longicuspis,* etc. are among the principal vectors in the former subregion *P. argentipes* is a proven vector in the latter (WHO, 1990b).

With regard to the natural transmission dynamics of the disease, the zoonotic nature of VL is clearly evidenced in the Mediterranean-Middle Asian subregion in that domestic and wild canidae are responsible for maintaining the natural cycle of the parasite and man is infected only secondarily (WHO, 1990b). According to same report, the Indian subzone represents a typical anthroponosis. So far no reservoir host was identified to link the transmission cycle between man and the vector, the cycle is maintained between man and the synantropic sandfly vector.

The East African VL endemic foci are represented mainly by the Sudan, Kenya, Ethiopia and Eritrea although Djibouti, Somalia, Chad and Uganda are known to be included (WHO, 1981). The parasite strain recorded here is *L. donovani archibaldi* (Ashford and Bettini, 1987). In the Sudan *P. (Larrousus) orientalis* Parrot (Hoogstraal and Heynenan, 1969) and in Kenya *P. (Synphlebotomus) martini* Parrot (Minter, 1963; Johonson *et al.*, 1993; Wijers, 1963) are the proven vectors.

In Ethiopia, the VL cases dated back to the 1920's and the first report was from Omorate and Kelem at the northwest end of lake Turkana (Humber *et al.*, 1988). Additional case reports came into picture in the 1940s during the second World War by Cole *et al.*, in 1942, Ferroluzi in 1943 and Anderson in 1943 (reviewed in Gebre-Michael and Lane, 1996). The endemicity of the disease was recorded in the peripheral low lowland areas of the country. In northwest, in Metema-Humera lowlands; in southwest, Aba-Roba/Segen, Woyto rivers basin & Lower Omo river valley; in the south, Dawa, Moyale & Genale being parasitological confirmed foci the northeast & Middle Awash valley are highly suspected on the basis of leishmanin skin test positivity and presence & abundance of the presumed vector (reviewed in Gebre-Michael and Lane, 1996; Hailu *et al.*, 1995; Humber *et al.*, 1988; WHO, 1981).

In the Aba-Roba/Segen focus the disease is transmitted primarily by *P. (Syn.) martini* and *P. (Syn.) celiae* Minter plays a secondary role (Gebre-Michael and Lane, 1996). In the Lower Omo river valley, eventhough the vectorial status of *P. (L.) orientalis* awaits further confirmation natural *L. donovani* infection was demonstrated (Hailu *et al.*, 1995). This species is also strongly suspected in the north-western, Middle & Lower Awash valley and

other VL foci in the country (except Aba-Roba) (Gebrwe-Michael T., personal communication).

Unlike the epidemiology of VL in the other two Old World subregions, the issue in the East African subregion is far from being clear. Despite the comprehensive efforts made in an attempt to fill this gap to date no animal reservoir has yet been discovered in the subregion.

Indeed, the Indian VL and that of East Africa share some clinical features in common. For instance, parasites exist in the nasal mucous, peripheral blood and normal skin of patients and PKDL cases are also well observed especially in the Sudan (Osman, 1998; WHO, 1981; WHO, 1996). The epidemiological significance of the PKDL is that patients are thought to be the main reservoirs by easily presenting parasites to the sandfly vectors. From such perspectives some investigators regarded the East African VL as anthroponosis which is practically similar to the Indian Kala-azar. However, the zoonotic nature of the East African VL was suspected from early times because of the occurrence of the disease in almost uninhabited Sudanese, Kenyan and Ethiopian lowlands where military patrols camped during the second World War (Ashford and Bettini, 1987; WHO, 1981). Furthermore, the behaviour and ecology of the sandfly vectors augmented this suspicion. As opposed to the Indian species, *P. argentipes,* which lives solely in or around human habitations the East African vectors are entirely associated with *Acacia seyal-Balanites aegyptiaca* forests or Macroterms termite hills (WHO, 1996). Multiple epidemiological evidences point out that *P. orientalis* bites only in the forest and forest edges. Therefore, it is assumed that both in Sudan and Ethiopia contraction of infection occurs when man

encroaches on the vector's forest area. This has been further strengthened by additional facts such as occurrence of VL cases in migrant labourers who spent nights in the forest area during the high prevalence period of the vector. Occasional case detections among solitary woodcutters, honey hunters and monkey trappers wandering in the wild account for zoonosis. These and other epidemiological observations assure the existence of a natural zoonotic cycle in the East African subregion. Nevertheless, epidemic outbreaks that took place in Sudan and Central Kenya seem to be not attributed to zoonotic origin. In general, it is supposed that while sporadic cases and reports from endemic areas are zoonotic, man to man transmission takes place during epidemics.

Since the body of knowledge with regard to the natural transmission dynamics is central in designing prevention and control schemes for VL, which poses a serious menace to the economy and health of human being, several attempts were made to have a clear picture of the transmission patterns of the disease.

In the Sudan several animal species (domestic and wild) were examined for natural *L. donovani* infections. Parasites were isolated from a horse, donkey, monkey, dog and possibly from a fox (Ashford and Bettini, 1987; WHO, 1981). But these infections were said to be causal and parasite identities were not confirmed. Hoogstraal and Heyneman, 1969 suspected the domestic dog for its remarkable association with tribal people and village families. Nonetheless, extensive surveys conducted to test this suspicion came up with nothing. Rather experimental studies undertaken, by the above workers, to test the behaviour of *L. donovani* strains from Sudan, Kenya & the Mediterranean area in local dogs revealed a dissimilar clinical feature. Hence, it was suggested that the dog might not

act as a reservoir host for either Sudanese or Kenyan strains.

However, the above authors succeeded in detecting natural *L. donovani* infections from three rodent species (*Arvicanthis niloticus luctuosus*, the common Nile grass rat; *Acomys albigena*, the African spiny mouse; *Rattus rattus*, common black rat) and two carnivore species (*Genetta* sp., a genet and *Felis serval phillipsi*, Sudanese serval). Together with some results shown in experimental infections of the above rodents, led the authors to conclude that these rodents might be reservoirs of VL in the Sudan.

In Kenya, the appreciable efforts made at various times by several workers to isolate leishmania from various suspected rodents were unsuccessful. The workers only managed to isolate parasites from hamsters indirectly through intraperitoneal inoculation of biopsy samples from gerbils (*Tatera robusta*, *Taterillus* sp., *Gerbillus* sp.), mongoose (*Helogale* sp.), ground squirrel (*Xerus rutilus*) from different VL endemic areas in Kenya (Heisch, 1957; Heisch *et al.*, 1959; Heisch, 1963). However, later biochemical identification of the strains revealed that the isolates were *L. major* (Chance *et al.*, 1977, Peters *et al.*, 1977; cited in Mutinga *et al.*, 1980). Later isolates from *Genetta* sp., *Arvicanthis niloticus*, *Mastomys natalensis*, *Tatera robusta* and the mongoose also isotyped to be *L. major* (Githure *et al.*, 1986; Mutinga, 1986). Despite the extensive studies undertaken on the domestic dog only a few were reported to be positive for natural *L. donovani* infection (Mutinga *et al.*, 1980).

In Ethiopia, dogs had been the main suspected reservoirs of VL as it is the case with other countries. According to WHO, 1981, several dogs were examined by authors named Conti

1938 (near Asmara, formely province of Ethiopia) and Batteli *et al.*, 1934. The former author recorded one infected dog and the latter nine dogs eventhough no information is available on parasite strain identity. According to same report, the extensive study made on dogs at Humera did not shed light in this respect.

From Lower Omo river valley and Gonder - Belessa area leishmanial species were recovered from *Arvicanthis* sp. but both were *L. major* (Haile and Lemma, 1977). From that time onwards, VL reservoir host study has been an area of active research and relatively extensive studies were undertaken in the Aba-Roba focus to discover naturally infected animals. To mention, of about 280 animals (rodents, small & large carnivores and a few domestic animals) examined in one study only one ground squirrel (*Xerus rutilus*) was found infected with *L. aethiopica* rather than *L. donovani* strain (Afework, 1987). Recently also of about 280 animals from at least 10 species sacrificed and looked for *L. donovani* infections no single infection was documented (Gebre-Michael *et al.*, 1996). In this work not only conventional methods of culture and smear but xenodiagnostic and laboratory infection approaches were attempted. Currently, efforts are underway to find out natural infections in potential hosts at least in the Aba-Roba focus by combining traditional methods with more sensitive modern tools such as the polymerase chain reaction (PCR) technique (Hailu, A. and Gebre-Michael, T., personal communication).

The concept of a reservoir of a disease deserves careful definition and adequate description. To incriminate a given animal as a reservoir host it demands extensive ecological studies. Laboratory inoculation of natural hosts may give very misleading results. Several workers had difficulties in reinfecting parasites isolated from the same

rodent species, as reviewed by Ashford, 1996. In fact there are no hard and fast rules to elucidate a reservoir host but there are series of guidelines that a putative reservoir host should fulfil. According to Ashford, 1997, a reservoir of an infection is a system in which the infectious agent exists indefinitely and the parasites' suprapopulation is maintained. From the view point of this definition infected individual animals discovered in the Sudan and Kenya might be incidental, liaison, or secondary hosts that are irrelevant for the long term maintenance of parasite population. Thus, for a long term existence of leishmanial parasites the following criteria should be met (Ashford, 1996; Bray, 1983; WHO, 1990a).

1. A reservoir host should be abundant and live gregariously in the climatic condition where the sandfly prevail so that there is an intimate host-fly contact and an ease of parasite transmission from one reservoir to the other. In addition there must be a contact between man and the reservoir via the sandfly vector.

2. Fairly a large proportion of individuals should be infected (infection rate greater than 20%) chronically without overt pathological effects for a long period of time, at least to the next transmission season.

3. Parasites must be in the skin and/or peripheral blood of the reservoir host so that they can easily be presented to the sandfly vector.

4. Parasite isolation should be made from naturally infected animals repeatedly and the isolates should be indistinguishable from human and sandfly isolates and a precise identification & classification of the isolates should be made through

reasonably sensitive and reliable tools.

5. A "good" reservoir host should provide a major bloodmeal source for the sandfly vectors.

Determination of whether a given host animal conforms to the fifth criterion or not calls for studies on the vectors' feeding preference.

1. 2. 2. Methods of host preference studies

1. 2. 2. 1. Bait-trap technique

In the bait-trap method, a commonly employed method to find out the feeding preference of vectors, a comparison is made between the catches in traps baited with various potential animals and a control cage without a bait. Accordingly, some workers tried to see the attractiveness of large domestic animals such as the domestic dog, sheep, goat and calves to *P. orientalis* and other sandfly species in the Sudan although their observations could not lead to any conclusive statements, as they pointed it out (Hoogstraal and Heyneman, 1969). The number of flies in baited traps was very few and the design of the trap and/or lack of interest in the flies to feed on large animals might have accounted for the low fly density, according to the authors. According to same work, however, rodent-baited traps were found to be successful in trapping many females of *P. rodhaini* and *P. heischi* (now *Sergentomyia heischi*) whereas data on other phlebotomine species could not be a representative of a natural feeding habit.

The above authors and various others insisted on using different types of live bait traps at various time in the Sudan but they testified the impossibility to generate reliable document (WHO, 1981). In Kenya similar investigations were practiced on various *Phlebotomus* and *Sergentomyia* species including *P. martini* but still the workers could not manage to firmly conclude about the vectors' host seeking behaviour (Johnson *et al.*, 1993; Mutinga *et al.*, 1986). In Ethiopia the issue appears unexplored and the information is meagre.

In general, data obtained by confining sandflies in cages with a single animal overnight and counting those which become engorged seems biologically or epidemiologically insignificant. When caged, sandflies tend to feed on the animal for which they do not show any predilection or even do not bite at all under natural circumstances (Guy *et al.*, 1984).

1. 2. 2. 2. Serological tools

Another approach in host preference studies is the application of serological techniques to trace the blood meal sources of field collected sandfly vectors. Included under these methods are haemoglobin crystallization, passive haemagglutination, the latex haemagglutination, precipitin ring & complement fixing tests, fluorescent techniques, etc.. Each of these techniques has its own merits and demerits (reviewed in Service *et al.*, 1986). Nonetheless, the precipitin ring test had been the method of choice for identifying the blood meals of haematophagous insects in general and of mosquitoes, tsetse flies and triatomine bugs in particular (Rurangirwa *et al.*, 1986; Zarate *et al.*, 1981) until enzyme-linked immunosorbant (ELISA) and the CCIE assays were adopted and tended to replace them in relatively recent times (Blackwell *et al.*, 1994; Blackwell *et al.*, 1995; Colmenares *et al.*, 1995; Guy *et al.*, 1984; Service *et al.*, 1986).

Owing to several peculiar features of phlebotomine sandflies the precipitin ring test has a limited significance for blood meal analysis of these flies. Thus the subject had been a challenging task and the feeding habits of sandflies through serological methods has also been poorly elucidated. Of diverse points that may account for this the main one is the very small size of the flies and thus the volume of the blood meal they engorge which can be as little as 0.01- 0.1 mg (Service *et al.*, 1986). According to same report, the small volume of the sandfly blood meal provides sufficient proteins only for four or five precipitin tests and the system is not sensitive enough to identify all of the feedings of the insect. Another factor is that the tests are usually performed on blood meals that have deteriorated antigenically, at least to some extent, due to digestion process and that is why continuous efforts have been in place to develop simple, sensitive and reliable assays or to refine already established ones. Admittedly, there are referable research outputs on host preference, through blood meal analysis, of the Indian kala-azar vector by using both relatively older techniques and recent adoptions (Ghosh *et al.*, 1990; Mukhopadhyay and Chakrvarty, 1987).

In the East African subzone, blood meal identification studies were undertaken on *P. martini* by using the precipitin ring test (WHO, 1981) and ELISA (Ngumbi *et al.*, 1992) in Kenya. In Ethiopia the single mentionable blood meal related work was the work done on *P. longipes* and *P. pedifer*, vectors of CL, by the precipitin ring test by Foster in 1972 and Ashford in 1977 (reviewed in WHO, 1981). Other than these no comprehensive blood meal tests were performed in East Africa and the host feeding patterns of both proven and suspected vectors of VL in this subregion is not well known.

Another important point of the test condition in blood meal identification is the assay's specificity to differentiate closely related host species on the basis of minute but unique antigenic determinants. But this attribute of an assay is determined by the specificity of high titre antisera prepared against the sera of potential hosts. Although multipurpose antisera can be supplied by commercial sources, antisera raised against specific marker proteins for a wide range of potential hosts of VL vectors are not easily obtainable in both quantity and quality for multiple practical reasons. This exacerbated the problem and hampered the research in the area of sandfly blood meal identification.

1. 3. Aims of the study

This project was designed to develop species/genus specific antisera against various potential hosts of VL vectors in East Africa in general and in Ethiopia in particular. And to evaluate the potency of the antisera to recognize blood meals of the vectors through the CCIE assay thereby providing some preliminary information on the host preferences of the proven and/or suspected vectors of VL in the country. Since host preference studies are decisive not only in the study of reservoir hosts but they have a significant epidemiological importance in understanding the risk of VL transmission to man, this study is envisaged to contribute to this end.

The specific anti-species antisera produced in this study would be essential in all immunochemical procedures besides their use to determine the blood meal indices of sandflies and other haematophagous arthropods in succeeding studies.

In addition the specific IgG fractionated in the present study can be a useful initial material

for various immunization schemes and other immunoassay related works.

Furthermore, the study was aimed at confirming the sensitivity and specificity of the CCIE assay to detect sandfly bloodmeals and to recommend it for further more comprehensive epidemiological investigations along this line.

2. MATERIALS AND METHODS

2. 1. Animal trappig

Various nocturnal and diurnal rodents were captured by the Sherman and other live, conventional wire mesh (Tomahawk) traps. The traps were baited with peanut butter, crushed sunflower seeds, etc. and set on near or around termite hills, animal borrows, within and around homesteads, rock cliffs, along animal tracks and human footpaths around small farms, near the mouths of rodent burrows and rodent runs under thorn bushes, etc.. The animals were the ground squirrel (*Xerus* sp.), the mongoose (*Helogale* sp.), African grass rat (*Arvicanthis* sp.), African spiny rat (*Acomys* sp.), the black rat (*Rattus* sp.), gerbils (*Tatera* sp.), the multimammate rat (*Mastomys* sp.) and *Praomys* species. Some rodents, especially the mongooses and ground squirrels were also caught by hand catches. Rock hyraxes (*Heterohyrax* sp.) were trapped by using locally designed traps by indigenous farmers. Almost all collections were from the Aba-Roba VL focus (Afework, 1989; Ayele and Ali, 1984) with the exclusion of the African grass rats that were from 'Gofa Sefer' (Addis Ababa) and Sebeta area (Jimma Road).

2. 2. Collection of blood samples and sera separation

In the field each animal caught was placed on a flat surface, ventral side up. The thorax and abdomen were made wet with alcohol and wiped with a clean gauze. The position of the beating heart was located by feeling with index finger. Cardiac puncture was performed by affixing a 22 g 1.5 needle to a 1, 3, 5, or 10 cc syringe depending on the size of the animal.

Blood sample was collected in sterile labelled test tubes and placed at room temperature. Besides the cardiac puncture method, retroorbital bleed was performed for the smaller mammals. Furthermore, a small amount of blood was collected from the heart of animals which have died during handling after opening the thoracic cavity using scissors or surgical blades.

From the clotted blood samples, sera were separated by centrifugation and aspirating by Pasteur pipette with a rubber bulb. Sera of individuals of a species were pooled and stored at 4°C in Aba-Roba Clinic till they were transported in cool boxes to Addis and kept frozen at -70°C until used.

Serum from domestic dog (*Canis familiaris*) was collected by bleeding the femural vein of the hind leg of the animal. Horse (*Equus cabalus*), donkey (*Equus asinus*), and cattle (*Bos* sp.) sera were obtained from Faculty of Veterinary Medicine, Addis Ababa University, Debre-Zeit. Sera of sheep (*Ovis aries*), goat (*Capra hireous*), camel (*Camelus ferus bacterianus*) and man (*Homo sapiens*) were collected previously and stored at -20°C in the laboratory of IPB. Chicken serum was collected around Addis Ababa during public holidays.

2. 3. Immunoglobulin G (IgG) Fractionation

IgG was purified from the whole sera by the ammonium sulphate precipitation method followed by the ion-exchange chromatography technique (Harlow and Lane, 1988)

2. 3. 1. Ammonium sulphate precipitation

Sera were centrifuged at 7000 rpm for 21 minutes (at 4°C) before the precipitation process to remove debris and any red blood cell contaminants. The supernatant was transferred to a clean beaker, a stirring bar was added and placed on a magnetic stirrer. An equal volume of 50% saturated ammonium sulphate (313.0 g in 1.0 l distilled water) at neutral pH was gradually added to a sample under constant stirring at room temperature. The resultant suspension was allowed to stand, at room temperature, for 30 minutes with occasional stirring after adding all the salt solution. The suspension was poured into centrifuge tubes and centrifuged at 7000 rpm for 21 minutes at 4°C. The pellet was drained and resuspended in 0.50 volume of the starting volume in phosphate buffer saline (PBS). The antibody solution was transferred to a dialysis tubing and dialysed against three changes (each for overnight) of twice the resuspended volume of PBS. The solution was transferred to centrifuge tubes and centrifuged to remove any remaining debris.

2. 3. 2. DEAE-Ion exchange chromatography

DEAE-celullose (anion exchanger) was extensively washed, by gentle centrifugation, with 0.5N HCl and then 0.5N NaOH prior to use. The acid-base was suceeded by a washing with 20 volume of 5mM sodium phosphate (monobasic), pH 6.5. The washing was continued until the pH of the matrix took that of the salt.

To the washed matrix about half its wet volume of ammonium sulphate precipitated protein sample was added while stirring on a magnetic stirrer. After complete addition of the sample the suspension was let to stand for 1 hour under continuous stirring at room temperature. Then, the suspension was transferred to centrifuge tubes and centrifuged at

7000 rpm for 21 minutes at 4°C. The supernatant was transferred into sterile 50 ml falcon tubes and stored at 4°C. The pellet was discarded.

2. 4. Protein assay

The Bicinchoninic Acid Protein Assay Kit (Sigma procedure No. TPRO-562) supplied by the manufacturer was used to determine the concentration of the ammonium sulphate-DEAE-cellulose purified IgG samples. The supplied reagents were bicinchoninic acid solution (B-9643): a 100 ml solution containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1 N NaOH (pH 11.25); A 25 ml solution containing 4% (w/v) copper (II) sulphate pentahydrate solution (C-2284) and a protein standard solution (P-0914) in package of 5 flame sealed glass ampules containing 1.0 ml of 1.0 mg/ml bovine serum albumin in 0.15 M NaCl with 0.05% sodium azide as a preservative. The procedure of the assay is described below:

1. About 100 ml of protein determination reagent was prepared by adding 1 part copper (II) sulphate pentahydrate 4% solution to 50 parts bicinchoninic acid solution.

2. Eighteen sterile test tubes were labelled. In the first tube 0.10 ml distilled water, in the second 0.08 ml water & 0.02 ml protein standard, in the third 0.06 ml water & 0.04 ml standard, in the fourth 0.04 ml water & 0.06 ml standard, in the fifth 0. 02 ml water & 0.08 ml standard and in the sixth tube 0.1 ml standard were added. Each combination was done in triplicate. To each of the 18 tubes 2 ml protein reagent was added and vortexed. Additional tubes were arranged for the

IgG samples (in triplicate). IgG samples at, various dilutions, were mixed with 2 ml protein reagent and vortex mixed.

3. The tubes were incubated at 37°C (in hot water bath) for 30 minutes.

4. The tubes were allowed to cool to room temperature and the absorbance was determined at 562 nm in spectrophotometer, using water to zero the instrument. The absorbance of the blank was subtracted from the remaining assay tubes to obtain the net absorbance due to the sample/standard.

5. The eventual net absorbance of was determined by adding the absorbance of the three tubes in the group and dividing by three.

6. A standard curve was prepared by plotting the average net absorbances at 562 nm vs the known added protein standard. This standard curve was used to determine the amount of IgG in each sample.

Dilute samples were concentrated to the desired quantity by adding the samples in dialysis sacs (8/32") on to which dry polyethylene glycol (PEG) 40000 (mol. wet. 6000) was sprinkled and left for a few hours (Osmotic Pressure Method) (Johnstone and Thorpe, 1987).

2. 5. Production of anti-IgG antisera

2. 5. 1. Rabbit purchase and screening

A total of 33 adult male and female rabbits (New Zealand whites) were purchased from institutional and individual suppliers. The rabbits were closely examined for the presence of ecto-parasites such as ticks, mites, fleas, fungi, etc.. By assigning three rabbits per IgG sample (triplicate) cages were allotted and coded for each rabbit. Prior to launching the immunization regime all rabbits were bled, from the marginal ear vein. About 5 ml blood was drawn from each rabbit and sera were separated. The whole serum of each rabbit was tested against the purified IgG of each vertebrate species by the CCIE technique. Procedural details for both bleeding and the assay are indicated in succeeding pages.

2. 5. 2. Rabbit immunization

From each sample 3 mg/3 ml IgG solution was suspended in physiological saline for every three rabbits grouped (1 mg/ml per rabbit). Equal volume of Freund's complete adjuvant (FCA) was mixed with the antigen solution after resuspending the mycobacterium in the adjuvant with vortex mixing. A very stable water-in-oil emulsion was prepared by repeatedly emptying and refilling a syringe (using 25 g needle) with the mixture vigorously for a long period of time. The integrity of the emulsion was tested simply by adding a few drops to a beaker containing cold water or physiological saline (when emulsions are ready for immunization drops remain as intact white drops). The freshly prepared emulsions were collected from beakers to labelled syringes and subcutaneously injected into multiple sites at the back of the respective rabbits. Physical observations (e.g. in search for granulomatous reactions at the sites of inoculation) were made from the time of primary

injection onwards. Booster injections were administered at three week intervals in a similar manner except that the antigen dose was increased (6 mg/3 ml/3 rabbits) and Freund's Incomplete Adjuvant was used. A total of five booster injections were delivered to each group of rabbits throughout the course of immunization until the animals were hyperimmunized.

2. 5. 3. Test bleeds

Between 7-14 days after each injection, test bleeding was carried out on each rabbit. This was done by shaving the hair on the dorasl view of the ear and heating the shaven ear for about one minute with a heater lamp. The vein was nicked with a scalpel blade and about 10 ml of blood was collected into a glass vessel. The collected blood samples were incubated at 37°C for one hour and then left at 4°C overnight. Sera were collected after dislodging the clotted blood by wooden applicator and then by centrifugation. A total of five test bleeds were made.

Each test bleed of each rabbit was assayed against the corresponding (homologous) IgG by the CCIE assay as outlined in the following way (Guy *et al.*, 1984):

Micro-slides/glass plates (7.5 x 5 cm) were cleaned by absolute ethyl alcohol , dried and pre-coated with 1% agar in sodium barbital buffer (pH 8.6). The pre-coated slides were coated with 3% agar prepared in same buffer. In the coated gel pairs of wells (a maximum of 9 pairs) were punched, nearly at the centre, by a 1.5 mm wide and 2 mm apart puncher. 5 μl immunogen (IgG) and anti-species rabbit antisera were added in the cathodic (-) and anodic (+) wells respectively. The electrophoresis tank was filled 3/4 full with the sodium

barbital buffer (pH 8.6). Sample-loaded glass plates (maximum number of 4 plates per run) were placed over the tank bridge. Pieces of paper wicks (chromatography papers) were used to bridge the buffer in the tank and the gel in the glass plates. The tank was closed and a current of 100 V and 5 mA per plate was maintained for 30 minutes. Plates were washed for 4 hours in two changes of physiological saline and then for 2 hours in distilled water, covered with damp filter papers and incubated at 37°C overnight. The plates were stained with amido-black (1% in 7% acetic acid) for 20 minutes and destained with 5% acetic acid. After drying (at 37°C) the plates, fine precipitin lines (arcs) were looked for, macroscopically, between well pairs.

By following the same procedure attempts were made to see the cross-reactivity between vertebrate IgGs and heterologous rabbit antisera for each test bleed for each rabbit. The titers of the antisera were qualitatively determined by looking at the thickness of the precipitin lines formed. After fully assuring that the required antisera titer was achieved, large volumes of blood (up to 50 ml) were drawn from each rabbit by the heart puncture method. Such antisera were used in blood meal analysis.

2. 6. Assay standardization

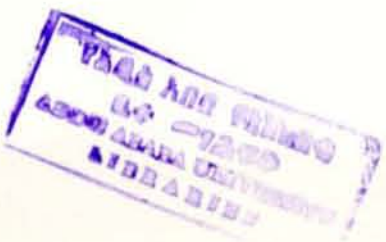
To determine the potential of the assay to detect sandfly blood meals, first human whole blood (neat and diluted as 1:10, 1:25, 1:50, 1:100, 1:200) was tested against undiluted anti-human rabbit sera. It was after this that testing blood meals from known host sources was begun.

Laboratory-reared phlebotomine sandflies (*P. orientalis* and *P. sergenti*), from IPB, were

allowed to feed on human volunteers, guinea pigs and rabbits. After full engorgement, individual flies were soaked in 100 μl physiological saline and kept at 4°C overnight. While human fed flies serving as positive controls, guinea pig/rabbit fed ones were negative controls for the test battery. Unfed laboratory-reared female flies were also used as negative controls. The sandfly blood meals/the abdomen of unfed ones were squashed, thoroughly homogenized, loaded in the cathodic well and tested against anti-human antisera. In a similar way, samples were also assayed against anti-host (other than man) antisera and cross-reactions, if any, were assessed.

2. 7. Blood meal collection and analysis

Three field trips (in February, May and November, 1998), each lasted for 2 weeks, were organized to Aba-Roba in an attempt to collect blood meal samples. A total of 42 sampling nights were passed. The CDC light trap powered by 12 volt batteries and sticky traps made from white polythene sheets (21 x 29 cm) smeared with castor oil were used as sampling techniques to collect sandflies. The light traps were set near termite hills, in bushes, near human dwelling and other selected trapping sites. The sticky traps were placed at the ventilation shafts of termite hills, animal burrows, tree holes, rock cliffs and around human habitations. Both traps were set from dusk to dawn and checked for trapped sandflies early in the morning. Blood-fed flies were distinguished from unfed ones. The ones that were caught by the sticky traps were washed in 2% savlon and rinsed in distilled water. The head was severed and mounted in gum chloral for species identification. The rest of the body of each specimen was kept in labelled gelatine capsules containing cotton and silica gel. Daily collections were stored at 16-18°C in the Aba-Roba Clinic until they were taken to the IPB laboratory and stored at -70°C. Species identification was carried



out by Ato Meshesha Balkew at IPB.

P. orientalis and *P. bergeroti* blood meals collected by Dr. Teshome Gebre-Michael and Ato Meshesha Balkew, in January 1995, from Middle Awash Valley and deep freezen at IPB were used for analysis.

Each field collected blood meal was transferred to sterile eppendorf tubes and soaked in 100 μl physiological saline and kept at 4°C overnight. The eluate was homogenized and allowed to react with the rabbit antisera against human, sheep, goat, donkey, horse, camel, cattle, ground squirrel, hyrax and mongoose. Commercial anti-dog (Sigma D-8650), anti-rat (Sigma R-5005), and anti-monkey (Sigma M-0278) IgGs were tested against the blood meals in a similar experimental set up after reconstituting and checking their reactivity against homologous and heterologous IgGs in the experiment.

2. 8. Data analysis

Data were analysed, qualitatively, by tallying the number of individual flies those fed on each test animal.

3. RESULTS

3. 1. IgG yield obtained

The IgG yield obtained from the whole sera of various mammals through the ammonium sulphate precipitation method accompanied by the DEAE-cellulose ion exchange technique is presented in table 1 below.

Table 1. The total volumes (ml) and concentrations (mg/ml) of IgG obtained from the indicated volumes of whole serum used.

Mammal	ml whole serum	ml IgG obtained	mg/ml IgG
Human	20	3	11
Sheep	42	20	32
Goat	36	10	4
Donkey	45	36	10
Horse	52	29	4
Camel	50	30	16
cattle	27	14	18
Squirrel	60	37	2
Mongoose	37	22	8
Hyrax	45	33	4
Grass rat	45	21	0.68*
Dog	38	26	1*
Chicken	42	14	5*

* Not used for immunization. The sera of *Acomys* sp., *Rattus* sp., *Tatera* sp., *Mastomys* sp. and *Praomys* sp. were so small in volume that fractionation proved impossible.

3. 2. Screening pre-immunization rabbit sera

The pre-immunization sera drawn from each rabbit did not show any reaction when screened against the IgG of each potential host species involved in the study. This confirmed the eligibility of each rabbit to immunization.

3. 3. Post-immunization physical observations

At the third week of primary injection a swelling (granuloma) was developed at a single site of immunogen inoculation in one of the rabbits. Ten days later the first booster injection granuloma were noticed at multiple sites of at least four of the rabbits under observation. At the third week of the second booster injection granuloma of larger sizes were observed in most of the rabbits. At the third week of the third booster injection the granulomatous reactions were very intense and evident in all rabbits that were under observation.

3. 4. Test bleeds

3. 4. 1. Homologous tests

All rabbit sera collected six days following priming were nonreactive with the respective immunogen delivered except four rabbits, one from each group, of those injected with horse, mongoose, squirrel and hyrax IgGs. In fact, in these the response was so weak that very faint precipitin lines were viewed. In the second test bleed, which was conducted at the tenth day of the first booster injection, almost all rabbits responded. Conspicuous precipitin lines were observed when rabbit undiluted antisera reacted with the respective undiluted IgG. Of the three rabbits boosted with sheep IgG only one rabbit showed a

response at this time of bleeding and this held true for the goat antigen too. Furthermore, anti-human serum from one of the three rabbits in the group had shown no reactivity on the third test bleed.

During the fourth test bleed (ten days post the third booster injection) nearly all rabbits responded strongly. Through qualitative observation the potency (or titre) of most antisera were grouped as very good (very thick precipitin line) and a as few good (thick precipitin line).

In the fifth test bleed (on the tenth day of the fourth booster injection) all antisera showed a very thick precipitin line.

3. 4. 2. Heterologous tests: cross-reactivity

The specificity of anti-species antisera was determined by testing the antiserum of each rabbit against the IgG of each host species. Anti-human sera from all the three rabbits cross-reacted with both IgG and whole serum of cattle and vice versa. Other than these there were no cross-reactions between the heterologous antigen and antisera. Generally, the antisera were specific thereby confirming the purity of the antigen used.

The anti-dog IgG from commercial source was screened against the IgG of all test animals and no cross-reaction was noticed.

Whole sera of *Mastomys* sp., *Rattus* sp. and *Tatera* sp. reacted with the commercial anti-rat IgG and that of *Arvicanthis* sp., *Acomys* sp. and *Praomys* sp. failed to do so. With

heterologous host IgG, no reactivity was observed.

Table 2 shows the results of reaction between anti-species antisera and homologous & heterologous host IgG.

Table 2. Undiluted anti-host rabbit sera screened against undiluted homologous and heterologous host IgG by the CCIE assay

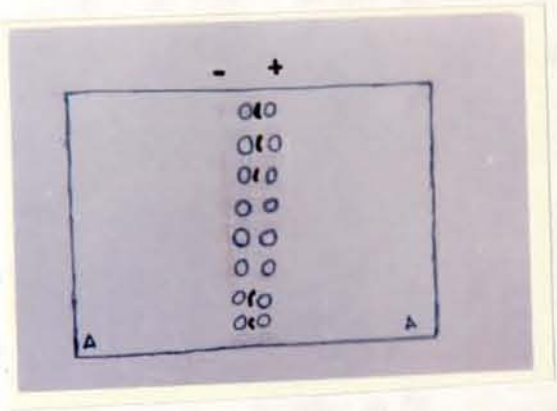
	Anti-host antisera/IgG											
Host IgG/whole serum	Human	Sheep	Goat	Donkey	Horse	Camel	Cattle	Squir-rel	Hyrax	Mon-goose	Dog **	Rat **
Human	++	-	-	-	-	-	++	-	-	-	-	-
Sheep	-	++	-	-	-	-	-	-	-	-	-	-
Goat	-	-	++	-	-	-	-	-	-	-	-	-
Donkey	-	-	-	++	-	-	-	-	-	-	-	-
Horse	-	-	-	-	++	-	-	-	-	-	-	-
Camel	-	-	-	-	-	++	-	-	-	-	-	-
Cattle	++	-	-	-	-	-	++	-	-	-	-	-
Squirrel	-	-	-	-	-	-	-	++	-	-	-	-
Hyrax	-	-	-	-	-	-	-	-	++	-	-	-
Mongoose	-	-	-	-	-	-	-	-	-	++	-	-
Dog	-	-	-	-	-	-	-	-	-	-	+	-
Rat*	-	-	-	-	-	-	-	-	-	-	-	++

++: denotes strong reactivity, +: weak reactivity and -: no reactivity
*: whole sera of *Mastomys* sp., *Rattus* sp. and *Tatera* sp.
**: commercial anti-host IgG.

3. 5. Assay optimization

Undiluted anti-human sera from all the three rabbits showed strong reactions with human whole blood (up to 1:50 dilution only). Anti-human antisera reacted with the positive control, but was not as strong as with the whole blood, and did not react with the negative controls (figure 1).

Figure 1. Strong and weak reactions observed in the CCIE test as determined by the degree of the thickness of the precipitin lines*.



* Antisera (in the anodic well) from top to bottom: anti-human (the first six wells), anti-cattle and anti-camel.

Antigens (in the cathodic wells) from top to bottom: human whole blood, human IgG, human fed sandfly (positive controls); unfed sandfly, rabbit fed sandfly and guinea pig fed sandfly (negative controls); field collected sandfly blood meal (in the last two wells)

3. 6. Blood meal collection

Of the total 42 nights at which traps were set, only eight blood meals (3 *P. martini* and 5 *Sergentomyia* sp.) were collected. All the three *P. martini* were caught by CDC light trap and the *Sergentomyia* sp. by sticky trap method. On visual inspection blood meals were brown and black/dark rather than pinkish in colour revealing that they were not fresh but at various degrees of digestion.

3. 7. Blood meal identification

A total of 114 blood meals (3 *P. martini*, 5 *Sergentomyia* sp., 94 *P. orientalis* and 12 *P. bergeroti*) were processed. Of these, 93 blood meals (2 *P. martini*, 79 *P. orientalis* and all *P. bergeroti*) were successfully identified (Table 3) . Both *P. martini* meals were from cattle source. 28 *P. orientalis* (14 cattle, 9 camel, 1 donkey, 2 squirrel, 1 human and 1 mongoose) fed on single hosts. 4 *P. bergeroti* fed on cattle and 1 on camel. Overall, 35 (37.6% of those identified) flies fed on single hosts (20 on cattle, 10 camel, 2 squirrel, 1 donkey, 1 human & 1 mongoose). The rest proportion (62.4%) was possibly from mixed sources (from two to six hosts) (Table 4). Nevertheless, in case of blood meals that were from multiple host sources, strong reactions were with anti-cattle antisera (figure 1) followed by anti-camel and anti-squirrel. The rest were weak or very weak to the extent that they would not have been detected if they were diluted further. Unlike the cross-reaction between

Hosts were not identified for 18.4% of the samples processed (1 *P. martini*, all *Sergentomyia* species, and 15 *P. orientalis*).

In summary, of the 12 possible hosts screened for, blood meals were identified from only the 9: cattle, camel, squirrel, donkey, horse, human, sheep, goat and mongoose although the rest of animals are known to occur in the trapping areas.

Unlike the crossing of anti-human antisera with whole serum and IgG of cattle and vice versa, blood meals from cattle source surprisingly failed to repeat that with anti-human serum.

Table 3. Blood meals of *P. orientalis*, *P. martini*, *P. bergeroti* and *Sergentomyia* sp. as identified by the CCEI assay

Sandfly sp.	Host sp. (blood meal source)														
	Total tested	Human	Sheep	Goat	Donkey	Horse	Camel	Cattle	Squir-rrel	Mong-oose	Hyrax	Dog	Rat	Mi-xed	Total identified
P. orientalis	94	1	-	-	1	-	9	14	2	1	-	-	-	51	79 (84 %)
P. martini	3	-	-	-	-	-	-	2	-	-	-	-	-	-	2 (66.67 %)
P. bergeroti	12	-	-	-	-	-	1	4	-	-	-	-	-	7	12 (100 %)
Sergentomyia sp.	5	-	-	-	-	-	-	-	-	-	-	-	-	-	0 (0 %)
Total	114	1	0	0	1	0	10	20	2	1	0	0	0	58	93 (81.6 %)

Table 4. *P. orientalis*, *P. martini*, *P. bergeroti* and *Sergentomyia* sp. blood meals from multiple host sources

Sandfly sp.	Blood meal sources*											
	a	b	c	d	e	f	g	h	i	j	k	total identified
P. orientalis	-	1	13	1	2	1	2	29	1	-	1	51
P. martini	-	-	-	-	-	-	-	-	-	-	-	0
P. bergeroti	1	-	1	-	1	-	-	3	-	1	-	7
Sergentomyia sp.	-	-	-	-	-	-	-	-	-	-	-	0
Total	1	1	14	1	3	21	2	32	1	1	1	58

* Multiple hosts. a: human/sheep/goat/camel/cattle/squirrel; b: human/sheep/camel/cattle; c: camel/cattle/squirrel; d: horse/cattle/squirrel; e: horse/camel/cattle/; f: sheep/camel/cattle; g: camel/squirrel; h: camel/cattle; i: donkey/camel
j: cattle/squirrel; k: sheep/cattle.

4. DISCUSSION

The two-step protein fractionation procedure using ammonium sulphate precipitation combined with the DEAE-matrix (batch) technique yielded a fairly high quality of IgG. Purity and appreciably high and powerful immunogenicity the IgGs was confirmed by the response of rabbits rendered when injected with a small dose (in μg) of it. The antisera developed in response to this soluble antigen are in line with the important features of a suitable antiserum: avidity, specificity and titre. The antisera did not suffer from cross-reactivity and thus very lengthy and tedious absorption procedures were avoided (Rurangirwa *et al.*, 1986). The specificity of the antisera further confirmed the already established notion that IgG is a marker protein for a species.

Of the total 114 blood meals processed, hosts were identified for the 93, assay sensitivity being 81.6%. This is a far better result in as far as blood meal analysis in general and that of sandfly blood meal in particular is concerned. Various immunoassays employed for blood meal analysis by various workers had lower sensitivity levels except the work of Guy and colleagues, on *P. ariasi*, who recorded an 89.4% sensitivity for the CCIE assay (Guy *et al.*, 1984). A work on the New World species, *Lutzomia peruensis*, revealed a sensitivity of 67.5% for the precipitin test (Perez *et al.*, unpublished data). Even for triatomine bugs, with large volume of blood ingested, the capillary precipitin test technique showed negative results for 71% of the specimens examined (reviewed in Zarate *et al.*, 1981). Furthermore, the refined ELISA technique has shown a sensitivity of 71.5% for blood meals of biting midges analysed (Blackwell *et al.*, 1994). Another work conducted on *P. perniciosus* showed a 59.5% success for 425 samples analyzed by the competitive ELISA Biotin/Avidin method (Colmenares *et al.*, 1995). Out of 224 *P. martini* blood

meals analyzed, by sandwich ELISA, hosts were identified for 63.8% (Ngumbi *et al.*, 1992).

Besides, our assay was sensitive enough to identify partially digested meals. Perhaps the best attribute of our assay was its capability to identify multiple feedings by determining minute antigens in mixed meals. 62.4% of the total bloodmeals identified were from 2 to 6 host sources. Although cross-reactions might be expected to some extent (since our antigen was not affinity purified) it seems very unlikely (because there were no cross-reactions between antisera and heterologous IgGs) to consider cross-reaction as a major factor for a single blood meal to to be reacted with more than one anti-host antisera.

Since the assay requires small volume of samples it renderes the advantage of testing each blood meal sample against 12 different anti-host antisera. A single blood meal can be screened against a wider (up to 20-25) range of hosts.

P. orientalis and *P. bergeroti* blood meals which we tested were stored for more than 4 years. Gradual degradation and deterioration, at least to some extent, of detectable proteins and hence reduction in antigen concentration was expected. Nonetheless, the sensitivity of the assay was not significantly affected by this parameter.

Thus, one need not be hastened to analyze blood meal samples without having enough collections fearing serious antigen degradation as a result of extended preservation.

Singly or in combination with others, cattle blood took a share of 65.8% followed by

camel 55.3% and squirrel 18.4% of all samples processed. Comparison of this result with previous work proved impossible for there are no comprehensive and reliable studies on the natural feeding patterns of *P. orientalis*. However, the role played by cattle as a major blood meal source for several sandfly species was remarkably indicated in various studies (Colmenares *et al.*, 1995; Desjeux, 1991; Ghosh *et al.*, 1990; Guy *et al.*, 1984; Mukhopadhyay and Chakrvarty, 1987; Perez *et al.*, unpublished data; WHO, 1981). It can be deduced that the feeding behaviour of the flies in this investigation may not greatly deviate from that of other sandflies.

Operating factors behind host selection in sandflies in general are poorly elucidated. The chemical, physical, etc. stimuli that drive sandflies to show a preference for one host and not the other (provided there is equal existence) are only presumably touched upon. The hosts' body heat and convection currents are supposed to play a role (Bray, unpublished document). In the light of this, larger animals such as cattle, camel, etc. could be more attractive than smaller animals because the larger the body the greater the volume of attractive space, the greater the production of carbondioxide and homoiothermic temperature, etc. Such characteristics together with specific contrast of scent should make larger animals innately more attractive than smaller ones. But some authors claimed that there were no statistically significant differences although larger animals seemed to attract higher number of flies (Christensen and Herrer, 1980; Johnson *et al.*, 1993).

On other hand, size *per se* does not appear to be a critical factor. For instance, in this study the blood of the ground squirrel was more frequently encountered than that of the horse and the donkey. Of course, this reinforces previous studies that reported the

recovery of different leishmanial species from the squirrel and urges for further parasitological studies to establish the status of this animal in the epidemiology of leishmaniases in general.

Multiple factors must come into play to determine the feeding selection of a given sandfly vector. The relative density of hosts that exist in a host-vector interaction play a great role in feeding and *P. orientalis* seems to be zoophilic mainly feeding on domestic or wild animals that are easily accessible as is the case with many Ethiopian sandfly species (Humber *et al.*, 1988). In the Awash valley, where the cattle and camel population is high, *P. orientalis* feeds by and large on these animals but in other localities the fly might prefer other hosts.

So far the role of cattle, camel and other larger domestic animals in the epidemiology of leishmaniasis in general and VL in particular is not known. And yet cattle and camel are the ones that are frequently bitten. This might be possible for the fact that some times non-reservoirs are the favoured hosts of the vectors. For example, the main host of *Glossina palpalis*, the transmitter of *Trypanosoma gambiense*, is the crocodile which is a non-reservoir (Bray, unpublished data). Furthermore, other animals rather than the two-toed sloth, the principal reservoir host of *L. braziliensis* in Panama, are attractive to the vectors (Christensen and Herrer, 1980). A similar explanation may be suggested for the negative results shown (in the present study) for the rat, dog and hyrax, animals that remained as primary suspects for natural VL infection in this country and elsewhere in East Africa. But this should not create a room for overlooking the role of cattle and camel in the epidemiology of VL and the role of these and other larger domestic

animals should be reappraised. Normally host preference studies point to the animal(s) to investigate. Suceptibility experiments should be carried out on these larger animals to unequivocally determine whether they are zooprophylactic, i.e, are prefereably bitten by the vector but refractory for the parasite.

Almost none of *P. orientalis* blood meals were from human origin and this may be interprated in different ways. It may indicate the unattractiveness of man to the flies, or man encroaches to the flies habitat only rarely or his activity in the forest areas is out of the flies' peak biting period. Or the ferocity or irritability of humans may not allow the flies to feed on them, etc.. In general, from the very low percentage of flies that fed on humans it can be said that the rate of parasite transmission is low in the focus where flies were collected and parasite population seems to be maintained elsewhere perhaps in animal sources rather than in man. In the absence of cattle and camel the chances of anthropophilly vis-a-vis transmission of infection may be possibly increased. Thus, the risk of human infection is lowered in the permanent presence of the favoured hosts: cattle and camel, for instance.

As it has already been outlined earlier, the great proportion of blood meals were probably from multiple host sources (mixed feedings): 40% on two hosts, 19.4% on three hosts, 1.06% on four and 1.06% on six hosts. Though it is difficult to forward exact justifications for multiple feedings, given the very poorly studied feeding pattern and ecology of *P. orientalis*, it is possible to raise some points that hold true for other haematophagous insect vectors. Multiple feeding may be due to interruption which may happen as a result of host defensive nature (ferocity/irritability) or when the fly

discovers that the animal is not its natural host. If a host is too defensive or unnatural host, a fly feeding on that animal may be obliged to interrupt and completes its feeding in other hosts. Under such circumstances that animal's blood is possibly expected in mixed feedings while infrequently being encountered as a single meal. Furthermore, the reactions between such mixed blood meal and antisera against the IgG of the "defensive" or "unnatural" host are expected to be weak due to low antigen concentration. However, in this study all mixed feedings were from cattle with other animals. This could not be due to frequent interruption because powerful reactions were observed only against anti-cattle IgG indicating the greater proportion of the blood was from cattle source. The time required for full engorgement does also matter. The longer the time a fly needs to probe its host's skin and to be engorged the more likely it can be interrupted as a result of irritation or other activities of the host or other natural factors such as wind. The time needed for many sandflies, including *P. orientalis* is not known but it is roughly 5 to 15 minutes for *P. martini* (Mutinga and Odhiambo, 1982). Whether the fly pierces painfully or silently may also affect the chance of full engorgement from a single host.

Apart from this, in nature some flies of *P. orientalis* species might show a gonotrophic discordance requiring repeated feeding before complete digestion of a previous meal. This increases the chance of feeding on various hosts. Although such report on *P. orientalis* is lacking, it has been demonstrated in *P. papatasi* (Said *et al.*, 1986), biting midges (Blackwell *et al.*, 1994) and tsetse flies (Rurangirwa *et al.*, 1986). This is a very momentous issue that mixed or double feedings are very essential in understanding vector-host interaction and vectorial potential and deserves special attention.

Of the samples analyzed hosts were not identified for the 18.4 per cent. Multiple factors could operate behind this. The anti-species antisera used were only against a few of the potential hosts on which the tested flies might have fed and this may account for the failure in identifying hosts for *Sergentomyia* species which prefer to bite reptiles to mammals (Muting and Ngoka, 1981). Likewise, the absence of antisera against large animals such as warthog and baboon which are among abundant animals in the Middle Awash (Gebre-Michael, T., personal communication) may also explain our failures to detect some of the blood meals of *P. orientalis*.

If anti-IgG against wide range of potential hosts such as cat, fox, birds, reptiles, etc. together with baboon and warthog were included in the test system, possibly host sources would have been traced for all blood meal samples that have been run.

Another possibility is that, depending on the degree of engorgement some samples might have been diluted above the detectble concentration when soaked in 100 μl diluent and gave negative results.

Still some samples, especially *Sergentomyia* sp., might have been well digested/half graved with traces of black, incompletely digested blood meal in which protein marker might have lost its identity and integrity.

The available information on the feeding habit of *P. martini* in Kenya illustrates divergent patterns. Studies using bait traps, in Kenya, showed that the species feeds on lizards, mongoose, rat, goat, dog, chicken with a preference for man (Mutinga *et al.*,

1986). On blood meal analysis 3 fed on bovidae, 1 on mammal, 3 on dog, 3 on reptile and 7 unidentified out of 17 samples analyzed (Mutinga and Ngoka, 1981). In a relatively wider survey of 224 blood meals analyzed a preference was shown for goat, rabbit and human in that order (Ngumbi *et al.*, 1992). These results do not tally with the findings in this study in which two of the three *P. martini* blood meals analyzed were from cattle source although the sample number was too small to put contrasting statements. Personal communications (Gebre-Michael, T.) point out that *P. martini* blood meals are only rarely encountered in Aba-Roba. Similarly, in Kenya, for instance, of 663 engorged flies collected only 8 were *P. martini* majority being *Sergentomyia* species (WHO, 1981). In the present study concerted efforts put in place to have a fair sample size were also given up in despair ratifying the complaints of previous workers. Indeed, the quite limited number of reports on blood meal identification of these flies is partly attributed to this difficulty in getting the engorged flies. Provision of certain explanation for this paucity of blood fed flies remained very challenging although some suggestions could be made. Since *P. martini* and *P. celiae* confine themselves to erodded termite hill microclimates, which are closed ecosystems which may also be inhabited by several mammals reptiles, etc., the majority of the flies may feed on the animals in that system visiting man and other domestic animals in the immediate vicinity only occasionally or at restricted periods of time. Many of the flies may not show even a slight movement after feeding rather they may enter a resting phase (thereby avoiding trapping) during which egg development proceeds just like some species of biting midges (Blackwell *et al.*, 1995). Thus, as long as sticky traps are not inserted very dip into the central chambers and ventilation shafts of the hills trapping of engorged flies seems very unlikely. In that case also the attempts that have

been made were proved to be fruitless for the reason that many of the smeared polythene sheets inserted were dragged by rodents in the hill.

5. CONCLUSION AND RECOMMENDATIONS

On the basis of the results of the study the following concluding remarks were made:

1. The ammonium sulphate precipitation method together with the ion exchange column chromatography yielded fairly good quality serum IgG which remains stable for at least one year being stored at 4°C.

2. To obtain reactive rabbit antisera at least one booster injection with increased antigen dose is required. To have hyperimmune serum 4-5 booster injections are needed. But all these are based on the method used in this study.

3. The antisera raised in the study were specific and stored well. So, attempts must be made to use them, in the absence of antisera raised against affinity purified IgG, for bloodmeal identification of other haematophagous insects.

4. The CCIE is a simple, sensitive, reliable and inexpensive technique which can be a method of choice for identification of blood meals of vector haemathophagous insects.

5. A prolonged preservation of sandfly bloodmeal specimens (more than 4 years at -70°C) has no serious deteriorating effect. One should not necessarily need fresh specimens and time should not be a constraint.

Although more extensive studies lie ahead to put conclusive statements about the feeding preference of *P. orientalis*, the present study implicates that the vector is an opportunistic feeder feeding on several mammals, on the nine out of the twelve potential hosts against which tests were made, with a preference for cattle and camel in the Awash valley.

A complete description of the feeding preference of the vectors of VL in East Africa in general and in Ethiopia in particular and thus the epidemiology of the disease in this subregion might be possible when the following recommendations, together with others points, are given due emphasis.

By employing the already established CCIE method, further studies with using fairly large number of blood meal samples from a number of localities in different VL endemic foci and using specific antisera against a wider range of potential hosts the findings in this study should be attested. The ELISA technique should also be applied to same samples used in the CCIE to compare the results.

In the laboratory, it is necessary find out if *P. orientalis* (and other vectors for that matter) need refeedings without the previous meal being entirely digested.

The relative abundance and species composition of all available potential hosts in blood meal sampling areas should be studied before concluding about the flies' host preference. The existence of a suspected host animal throughout the sandfly trapping period need to be assured. Ecological and taxonomic studies of the mammalian fauna

of VL endemic localities is needed.

The role of the ground squirrel, cattle and camel in the epidemiology of VL has to be investigated.

In general, detailed studies on the ecology of vectors of VL in Ethiopia with reference to their feeding habits and resting sites is demanded.

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DECLARATION

I, the undersigned, announce that this thesis is my own original work which has not been presented in any other University for a Degree. All sources of materials used for the thesis are justly acknowledged.

Name Hasen Mam

Signature

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