DETECTION OF *PLASMODIUM FALCIPARUM* SPOROZOITES IN NATURALLY INFECTED ANOPHELINE SPECIES USING FLUORESCEIN LABELLED DNA PROBE AND PCR PROCEDURES.

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ABSTRACT

A dot blot assay to detect *P. falciparum* sporozoites from naturally infected *An. gambiae* s.l was developed. Indoor resting blood fed female *An.gambiae* s.l were collected from Metehara, Zeway and Arbaminch. These mosquito specimens were assayed for *P.falciparum* sporozoites using the hybridization method with a fluorescein labelled PFR1 oligomer and the Polymerase Chain Reaction (PCR). A total of 198 field collected samples were assayed. *P.falciparum* sporozoites were detected from 4 samples; 2 from Zeway and the remaining 2, each from Metehara and Arbaminch by both hybridization and PCR methods. Background signal in the hybridization assay, generated from blood fed mosquitoes was cleared by keeping mosquito specimens in cages for 48 hrs after collection, so that the majority, if not all of the blood meal was digested before killing and preservation. Results of this study indicate that this method could be used for large scale epidemiological studies.

1. INTRODUCTION

Malaria is considered to be one of the oldest diseases affecting mankind (WHO,1987). It is speculated that the protozoan parasites first infected the *Homoerectus* a million years ago in the forests of Southeast Asia then spread eastwards to Africa and Europe, eventually following Colombus to the New World (Technical Bulletin, USN, 1967).

Today the disease still remains to be a scourge. According to the World Health Organization (1991) an estimated 2073 million people (over 40 % of the world's population), living in more than 100 countries are at risk. Of these, 270 million are infected with malaria parasites. Global deaths amount to 1-2 million. A total of 110 million clinical cases are reported annually and 90% of these are from Africa. Not long ago due to the advent of new drugs and potent pesticides the WHO in 1955 declared that the disease would be eradicated (WHO,1957). Unfortunately, mainly due to the appearance of resistant strains of the parasite to the once curative drug of choice chloroquine, and the development of resistance to various pesticides by different *Anopheles* species, this goal was unattainable (WHO,1987). To further complicate things, where large scale control measures had previously resulted in considerable reduction or even elimination of the disease, resurgence of malaria is now seen especially in zones recently affected by major ecological or social changes, mass migrations or socio-political unrest (WHO,1987).

Malaria is caused by the protozoan parasites grouped under the genus *Plasmodium* Marchiafava and Celli, 1885, which is wide spread in the tropics, subtropics and parts of the temperate zone. Four species are known to infect human beings :- *Plasmodium falciparum* Welch, 1897; *P. vivax* Grassi and Feletti, 1890; *P. ovale* Stephen, 1922; *P. malariae*, Laveran, 1881. Of the four species, *P. vivax* has the widest distribution with *P. falciparum* being the deadliest and prevailing in most tropical and subtropical zones. *P. malariae* is the only species in the group which infects other primates as well as humans. The least encountered species is *P. ovale*.

It is only the female mosquitoes grouped under genus *Anopheles* that could transmit Plasmodia species. There are about 400 species that are known to transmit the disease (WHO,1987) of which about 60 are important transmitters of the disease. A mosquito that is an important malaria transmitter in one region might not be an important vector in another region. Thus, the vector efficiency in female anopheline species is dependent on four general characteristics (Bruce Chwatt, 1980; Beaver *et al.*, 1984).

These are:-

1. The vector must feed on human beings frequently (anthropophilly).
2. It must be susceptible to gametocyte infection.
3. It must have a life expectancy (longevity) that should allow the complete development of the Plasmodium parasite.
4. It must be present in adequate numbers to maintain transmission.

Superimposed on these biological factors, are the effects of temperature, rainfall and humidity.

Natural transmission of malaria occurs when a female *Anopheles* mosquito carrying Plasmodial sporozoites in its salivary gland bites a person (Fig.1). Once the sporozoites are injected into the circulation they remain there for not more than an hour and then disappear. On leaving the blood, the asexual phase of the parasite's multiplication (schizogony) starts, the sporozoites enter fixed tissue cells namely the liver and develop into primary tissue schizonts which enlarge and segment, producing merozoites. The infected cells eventually rupture and the liberated merozoites are called exo - erythrocytic merozoites. In the case of *P. vivax* and *P. ovale*, where relapse of the disease is known to occur, exo - erythrocytic phase development is limited only to one generation, that is by pre - erythrocytic schizogony. Due to the presence of various types of sporozoites, some of which initiate dormant forms in hepatocytes (Lysenko *et al.*, 1977) the resumed activity of these plasmodia explains late recurrence and prolonged incubation in vivax and ovale malaria. Studies have shown the presence of small parasites inside hepatocytes in addition to normally developing schizonts (Markus, 1976). Several names have been given to these parasites like; x - body or hypnozoites. In the case of non-relapsing malaria, which is a feature of *P. falciparum* and *P. malariae*, exo-erythrocytic merozoites invade red blood cells, here erythrocytic schizogony takes place and the red blood cells burst open and release merozoites which quickley infect other red cells and multiply again (Bruce-Chwatt, 1980; WHO,1987). Few of the erythrocytic merozoites in the blood cells differentiate into gametocytes. Gametocytes are of two types, the macrogametocyte (female) and the microgametocyte (male). These are ingested by the *Anopheles* together with the host blood. The sexual reproductive phase (sporogony) of the parasite takes place in the mosquito, here, the macro and microgametocytes develop into gametes. After a series of developmenal stages starting with fertilization a motile ookinete develops which penetrates the stomach wall of the mosquito and forms the oocyst on the outer wall of that organ. By the process of sporogony a large number of sporozoites are produced. After undergoing this development the oocyst ruptures and thousands of spindle shaped sporozoites are released. The sporozoites migrate to the salivary gland, where they are ready to enter a new individual when the mosquito takes another blood meal (Bruce-Chwatt, 1980; Beaver, *et al.*, 1984).

The transmission of malaria is possible only when a combination of several factors are available, including sources of infection, vectors, susceptible population and favorable natural and climatic conditions (Communicable Disease Centre, 1963).

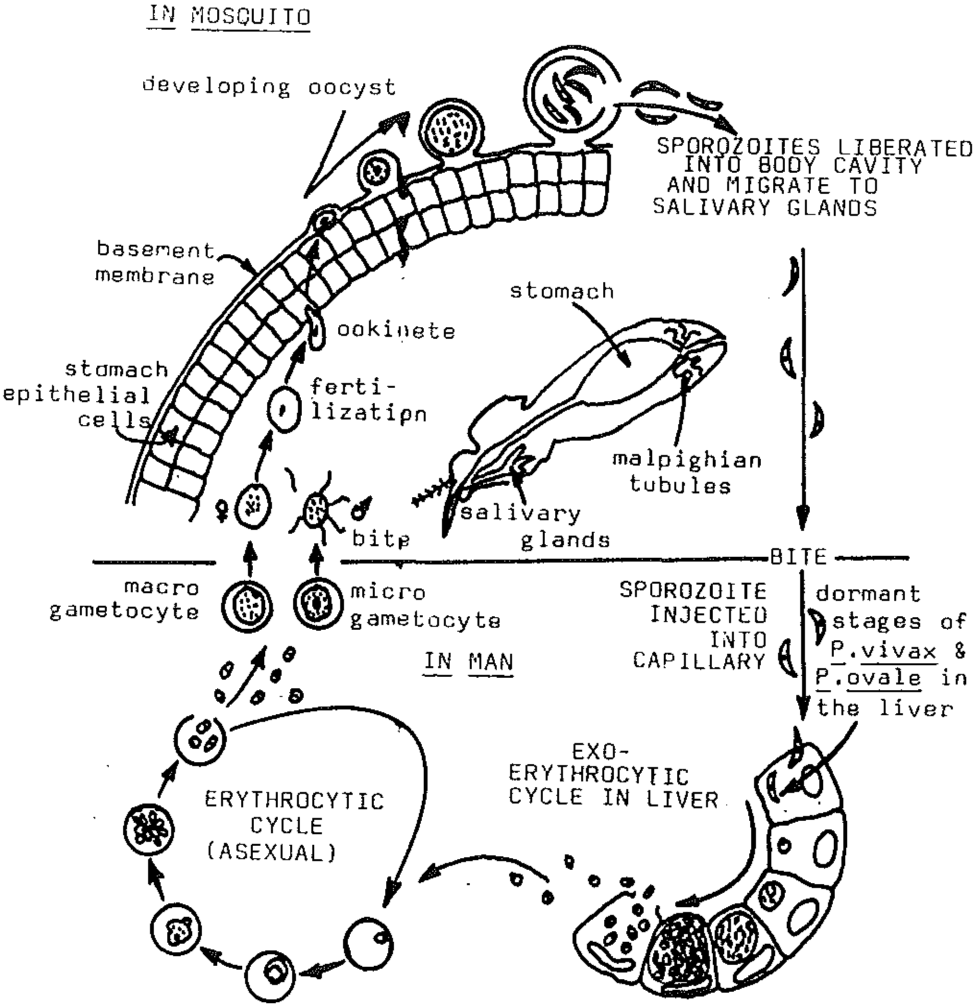

Fig. 1. Life cycle of *Plasmodium*
(Source: Adam *et al.*, 1971)

Malaria evokes a poor protective immunity in humans (Mendis *et al.*, 1991). Residents of endemic areas suffer repeated infections and it is only after several years of exposure to intense malaria transmission, that a partially protective immunity is acquired. This immunity is not sufficient to eliminate the parasite but leads at best to a state in which low *Plasmodium* densities prevail in the host without causing acute disease (Manson-Bahr and Apted, 1982). Despite the slow acquisition of protective immunity, potent immunity towards a specific strain has been shown to occur under experimental condition (Mendis *et al.*, 1991). The slow acquisition of immunity to natural malaria infections may, therefore, be partly due to the existence of immunological difference between parasite inocula that are prevalent in nature. In highly endemic malarial areas where the adult population possesses the acquired immunity, children present the major source of malaria (Technical bulletin, USN, 1963). Children have no acquired immunity, and consequently their parasitemia is usually heavy. Alternatively, parasite carriers in endemic areas may serve as sources of infection for a long time because malaria in such individuals is rarely identified and they receive no treatment (Loban and Polozok, 1985).

Malaria existed in Ethiopia for hundreds if not thousands of years (Pankhurst, 1955). Heavy tolls in life and extensive morbidity have been caused by malaria in the country. In the recent past, five major epidemics have been experienced, that is, in 1958, 1965, 1973 and 1981-1982 and 1987-1988 (Tulu, 1993). The regions severely affected by these epidemics were areas with altitudes ranging from 1600-2150m. The 1958 epidemics alone claimed the lives of 150,000 people (Fontain *et al.*, 1961) with about 3 million malaria cases being reported, the principal parasite responsible for this epidemic was *P. falciparum*.

According to the report of the Ministry of Health (1991), in 1989 malaria accounted for 4.3% of the outpatient cases in the country. 7.5% of all male hospitalizations were due to falciparum malaria. Hospitalizations due to by falciparum malaria for males and females in the country in general were 7.5% and 4.7% respectively. This disease was the second biggest killer only to be surpassed by tuberculosis of the respiratory system. Moreover 20% of the capital budget for health is allocated to malaria (Ministry of Health, 1986).

It has been known that climatic, altitudinal and topographic diversities in Ethiopia create micro- and macro-climatic conditions and result in a discontinuous and widespread malaria distribution (Gebremariam *et al.*, 1988). Based on the disease the country is classified into malarious and nonmalarious areas (Tulu,1993). The nonmalarious afroalpine zone with an altitude of above 2500 m is the area where no indigenous transmission occurs. This area comprises

15-20% of the total landmass and is inhabited by about 25% of the total population (Covell, 1957). The malarious zone, which refers to the land below 2000 m makes up 80 - 85% of the total land mass ; roughly 25 million people live here (Office of the Population and Housing Census Commission, 1991; Habtemariam and Kloos, 1993) and are at risk of malaria infection (Tulu, 1993). This zone is further classified into four epidemiological modalities; the sporadic transmission, occasional transmission, stable seasonal transmission and stable perennial transmission malaria zones (Mengesha and Ishii, 1992). Extreme altitudinal differences, which is a characteristic feature of the country, also further influences the endemicity of malaria. Parasitological surveys have verified the presence of all four human infecting *Plasmodium* species in Ethiopia. *P. falciparum* being the most dominant and *P. vivax* having the widest distribution (Gebremariam *et al.*, 1988). *P. ovale* has been so far only been identified from a few patients from Humera, Gambella and Tepi and *P.malariae* accounts for 15 % (Gebremariam, 1984).

Despite intence malaria transmission, information regarding the vectors of malaria in Ethiopia is scanty and inadequate. Giaquinto-Mira (1950) gives a summary of the work carried out by Italian and later on by British malariologists in few accessible areas of Ethiopia concerning identification and determination of *Anopheles* species. Based on this report 31 species and varieties of Anopheles mosquito with their geographical distribution and biology including their role in the transmission of malaria have been described. Pictorial keys for the identification of 34 anopheline and larvae were later on prepared by Verrone, (1962a and 1962b). A distribution map for the same number of anopheline species with the addition of two sub species was made by O'Connor, (1967) further more provincial distribution of these species was also outlined. Todate 42 species have been recorded in Ethiopia (Gebremariam *et al.*, 1988).

Data on the vectorial status of anophelines in Ethiopia based on information obtained from salivary gland dissections are few. Summary of these investigations is shown in Table 1.

Table 1. Summary of the Salivary Gland Dissections of Different Anopheline Species Conducted in Ethiopia by Different Investigators.

Investigator	Study area	Anopheles species dissected	No. dissected	No. positive for sporozoites
Giaquinto-Mira (1953)	Bari, Hararge	*An. ziemanni* Grunberg, 1902	not specified	1
		An. gambiae s.l Giles, 1902	not specified	"varying rates"
Fontain *et al.*, (1961)	Kobo-chercher	*An. gambiae* s.l	100	3
Rishikesh (1967)	Awassa & Adamitulu	*An. gambiae* s.l	4573	9
		An. pharoensis Theobald, 1901	2577	0
		An. funestus Giles, 1902	337	0
O'Conner (1967)	Showa & Sidamo	*An. gambiae* s.l	4594	9
		An. pharoensis	2694	0
		An. funestus	357	0
		An. demelloni Evans, 1933	95	0
Krafsur (1970, 1971 &1978)	Gambella town & banks of Baro R.	*An. gambiae* s.l	8348	156
		An. funestus	9052	111
		An. nilli Theobald, 1904	619	8
		An. pharoensis	155	0
		An. welcomi Theobald, 1964	47	0
		An. coustani Laveran, 1900	26	0

Although the traditional method for identifying sporozoites in mosquitoes requires the dissection of salivary glands followed by microscopic examination for sporozoites, other more recent methods have also been developed. These methods include the immunological methods like the Immunoradiometric Assay (IRMA) (Zavala *et al.*, 1982; Collins *et al.*, 1984), Immunofluorescent Assay (IFA) (Ramsey *et al.*, 1983), the Enzyme Linked Immunosorbent Assay (ELISA) (Burkot et al., 1984a and 1984b; Petros *et al.*, 1989) and the Immunohistochemical (IHC) methods (Golenda *et al.*, 1992). Besides the immunological methods, techniques have also been developed which use DNA probes to identify sporozoites in mosquito specimens (Holmberg and Wigzel, 1987; Delves *et al.*, 1989; Lee Sim *et al.*, 1989). Although not yet in use, RNA probes also have potential to be utilized in this regard (Wirtz and Burkot, 1991). Methods have also been deviced whereby sporozoites may be detected in mosquitoes using the polymerase chain reaction (PCR) (Schriefer *et al.*, 1991)

Even though such a battery of tools exist to identify sporozoite species in mosquitoes, their introduction in Ethiopia has not been materialized. It is only the work of Adugna, (1991) that stands out in which the ELISA method was used to determine the sporozoite rate (the percentage of anophelines of a given species whose salivary glands contain sporozoites). In this study, 29 *P. falciparum* infections (15 salivary gland and 14 abdominal oocyst - associated sporozoite infection) and 17 *P. vivax* infections (9 salivary gland and 8 abdominal oocyst - associated sporozoite infection) were detected in 10 different *Anopheles* species.

In spite of the fact that the dissection technique makes it possible to identify sporozoites particularly in the salivary glands, and hence still make it a method of choice to mark the vectorial status of a mosquito (Wirtz and Burkot, 1991), serious questions are posed concerning its practicability. It's a labor intensive technique requiring trained personnel; under condition of low sporozoite rate thousands of dissections might be necessary which could be unmanageable, and the method does not allow the discrimination of sporozoites into different *Plasmodium* species. In contrast the immunoassay techniques offer several theoretical advantages (Beier *et al.*, 1987) i.e., sporozoites can be identified by species, mosquitoes need not be fresh specimens (dry or frozen specimens can be used) and spozozoite load (number of sporozoite per mosquito) can be estimated. Investigations carried out by different individuals (Burkot and Wirtz, 1986; Wirtz *et al.*, 1987) show that among other things,the ELISA utilizes; stable reagents that are easy to dispose and to transport, requires less expensive equipment and provides rapid results which could be interpreted visually. These advantages have thus made it a method of choice and a widely used technique.

Recently however, since the ELISA detects the immunodominant circumsporozoite (CS) protein (Dame *et al.*, 1984; Enea *et al.*, 1984; Young *et al.*, 1985) and since the CS protein is known to be shed off while the sporozoite migrates through the haemocoel after being released from a ruptured oocyst (Ponnudurai *et al.*, 1988; Beier, 1993) or disperses extensively in the anophelines' body (Roberts *et al.*, 1988) overestimation of the sporozoite load as a result of the disseminated CS protein has been shown (Roberts *et al.*, 1988; Wirtz and Burkot, 1991). The implication of this fact besides causing exaggerations, is that the method fails to directly detect the sporozoite. This therefore, has been the main drawback of the method.

The DNA hybridization technique is a method by which a labelled short DNA fragment (probe) with a specific sequence is made to interact with its complementary sequence on a target DNA. Specificity of complementary base pairing along the double helix in the case of original DNA pairing or between a target DNA and a probe, lies in the size of the nucleotide bases and the position of the amino (NH_2) and carboxyl (C=O) groups on the rings. Because of the proper position of amino and carboxyl groups for H-bonding to occur, only purine-pyrimidine pairing can be incorporated into the double helix at a proper H-bonding distance. Hence, only guanine-cytosine or adenine-thymine pairing occurs (Kellar and Manak, 1989) (Fig.2).

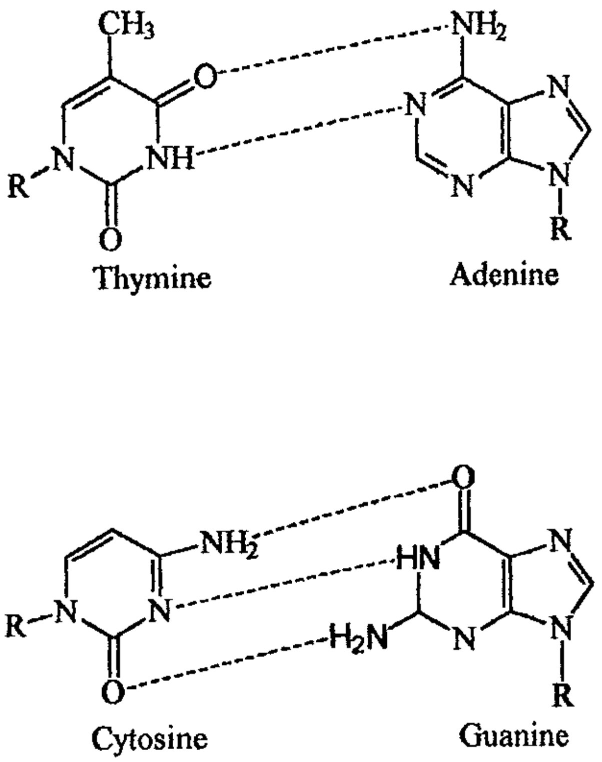

Fig. 2. H-bonding between purines and pyrimidines R-represents deoxyribose-phosphate backbone. (Source: Kellar and Manak, 1989)

DNA hybridization technology began by attempts to hybridize probe and target in solutions and isolating hybrids by gradient centrifugation (Hall *et al.*, 1961 in Kellar and Manak, 1989). This method was however slow, labor intensive, and inaccurate. The first simple solid phase hybridization was made by immobilizing denatured DNA in agar and hybridizing with a probe (Bolton *et al.*, 1962 in Kellar and Manak, 1989). Since then, hybridization procedures have been simplified significantly and presently hybridizations are commonly carried out by immobilizing DNA on nylon membrane (Holmberg and Wigzel, 1987). Means of detecting the interaction between the probe and the target DNA is by labelling the probe. The most widely used method for labelling of probes is with radioactive isotopes (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989; Ausubel *et al.*, 1989). Because of the problems associated with the use and disposal of radioactive isotopes, nowadays labelling of probes with non-radioactive labels is being widely practiced (Wilson *et al.*, 1991). The methodology for identification of sporozoites in mosquito vectors using DNA probes is still in its developmental stage (Wilson, 1991). Theoretically the technique has several advantages as compared to the ELISA (Homberg and Wigzel, 1987; Wilson, 1991). It detects the genomic DNA in sporozoites and therefore is a direct method for detecting sporozoites. It is rapid and accurate; there is minimal cross reactivity (that is in the case of differentiation during mixed infections). Additionally, more information may be obtained from a single dot representing a mosquito, like identity of the vector, the blood meal source, type of infection and age of the vector.

Limited information exists regarding the detection of sporozoites using DNA probes. In an experiment carried out by Delves *et al.*, (1989) an imperfect tandemly repeating 21 base pair DNA probe that was specific to *P. falciparum* and shown to be conserved in the asexual blood stage as well as in the sporozoites was labelled with $[\alpha\text{-}^{32}P]$ATP. The probe was used to detect *P. falciparum* sporozoites in laboratory infected *An. stephensi* Liston, 1901 squashed on nylon membrane. The sensitivity reported in this study was around 1700 sporozoites. Even though the problem of non-specific signal was not encountered in this study, cases of non-specific signal interfering with detection of sporozoites (Lee Sim *et al.*,1989) have been reported. Non-specific binding of DNA probes to mosquito squash blots has been correlated to the presence of chitin which happens to be the major component of the mosquito exoskeleton (Lee Sim *et al.*, 1989). Incubation of the nylon membrane in chitinase solution has been shown to remove the non-specific signal to a satisfactory level thereby improving the quality of signal obtained. This has enabled clear demarcation of positive and negative specimens. The sensitivity reported in this investigation was 20 picograms (pg) of DNA (Lee Sim *et al.*, 1989). Assuming the genome of a single *Plasmodium* parasite to be approximately 0.02pg of DNA (Goman *et al.*, 1982), the above amount would correspond to 1000 *Plasmodium* parasites.

The Polymerase Chain Reaction (PCR) is a technique that is used to amplify a segment of DNA that lies between two regions of known sequence. Two oligonucleotides that have complementary sequence that lie on opposite strands of the template DNA and that also flank the segment of DNA to be amplified are used as primers for a series of reactions that are catalyzed by thermostable *Thermus acquaticus* (Taq) polymerase. The process of synthetic reaction starts when the template DNA is denatured by heating in the presence of large molar excess of the two primers and the four deoxynucleotide triphosphates (dNTPs) namely deoxyadenosine triphosphate (dATP), deoxyguanidine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP). The reaction mix is then cooled to a temperature that allows the annealing of the two primers to their target sequence on the template DNA. The annealed primers are induced to extend by the help of the DNA polymerase by increasing the temperature once more. This cycle of denaturing, annealing and extension is repeated many times. Because the product of one round of amplification serves as a template for the next, successive cycles will produce DNA products at an exponential proportion (Sambrook *et al.*, 1989; Saiki, 1990). The major product of this reaction is a double stranded DNA whose termini is defined by the 5' termini of the primers and whose length is defined by the distance between the primers (Sambrook *et al*., 1989). During the process of amplification longer DNA molecules are generated, especially in the first round. However, in the second round and those that proceed, these molecules generate DNAs of defined length that will accumulate exponentially and form the dominant product. Although longer molecules continue to be generated from the original DNA template in each round, the accumulation is rather in a linear rate and hence do not constitute the bulk of the PCR product (Sambrook *et al*., 1989), (Fig.3).

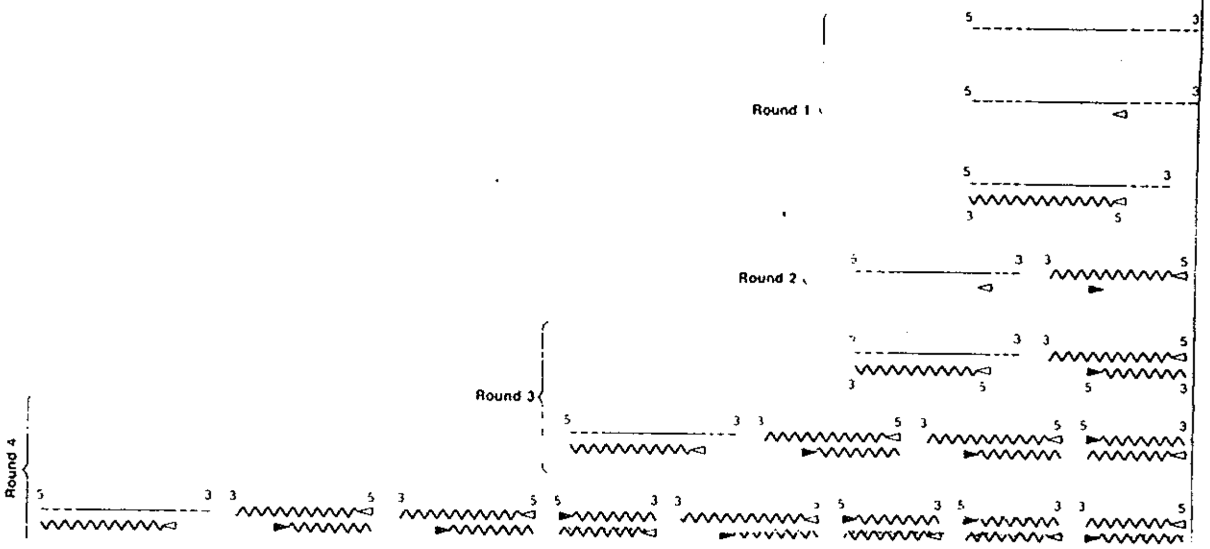

Fig. 3. Original template (top line) is single-stranded DNA and the leftward and rightward oligonucleotide primers are shown as < and ▸, respectively. The product of the first few rounds of the amplification reaction are heterogeneous in size. However, the tract of DNA lying between the two primers is preferentially amplified and quickly becomes the dominant product of the amplification reaction. If the original template is double-stranded, an equivalent set of reactions will take place using the complementary strand as template. (Source: Sambrook *et al.*, 1989).

There are few studies of gene amplification procedures used in the identification of parasitic infection in insects (Dissanayake *et al.*, 1991; Schriefer *et al.*, 1991). Probably, the reason for this might be the problem of isolating target DNA from specimens without going through the cumbersome and tedious procedure of phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989).

In a study carried out by Schriefer *et al.*, (1991) *Plasmodium* DNA was detected in infected blood and in individual mosquitoes after amplification by PCR. To deter the action of different inhibitors of the Taq polymerase enzymes in this study an iron chelating agent Chelex 100^{TM} was used. Inhibition during amplification, particularly of parasite DNA fragment in insect vectors has been reduced by alternative techniques which also include DNA binding slurry to capture parasite DNA from homogenates of mosquito tissue (Dissanayake *et al.*, 1991).

Although attempts have been made to detect *Plasmodium* parasites in laboratory infected anopheline mosquitoes using either the hybridization or the PCR techniques, no data exists regarding the application of these methods in naturally infected anopheline species. Additionally, in the hybridization assay, radioactive labelling of probes has been a widely utilized option (Holmberg and Wigzel, 1987). However, because of the problems associated with the short half life of most isotopes, safety and proper disposal system of radioactive materials (Wilson, 1991), their wide usage in the field has not materialized. This study, therefore, aims to fill the gap in knowledge concerning, the applicability of these methods in naturally infected anopheline mosquitoes and the use of nonradioactive labelling of probes in the hybridization assay.

The overall objective of this study is:

- -To develop a DNA hybridization method appropriate for large scale epidemiological studies for the detection of *P. falciparum* sporozoites in naturally infected *Anopheles* species.

The sub-objectives are:

- -To develop a DNA hybridization method based on a non-radioactive DNA probe labelling system.
- -To determine the sensitivity and specificity of the DNA hybridization method. An application of the PCR technique for detection of *P. falciparum* specific DNA will be used as the reference method.
- -To evaluate the DNA hybridization method on a collection of naturally infected *Anopheles* species.

2. MATERIALS AND METHODS

2.1 Sites of Mosquito Collection

Mosquitoes were collected from villages near the towns of Metehara (850m), Zeway (1800m), and Arbaminch (1145m). Selection of these sites was based on information obtained from the National Programme for the Control of Malaria and other Vector - borne Diseases (NPCMVD) and from the Medical Entomology Division of the National Research Institute of Health (NRIH).

The people inhabiting these sites are mostly pastoralists simultaneously practicing subsistence agriculture. The human activity of Metehara however, is predominantly nomadic pastoralism. The inhabitants of all the localities in the three collection sites are settled scattered in small collections of "tukuls". These "tukuls" are usually rounded and sometimes rectangular in shape. The walls are made of a network of poles and closely interwoven twigs, that may sometimes be plastered with mud or cow dung or a mixture of the two. The roof is often thatched and frequently conical in shape.

The cattle are usually kept for the night in a section of the house that is usually partitioned from the rest. As a result these dwellings are called "mixed dwellings".

2.2 Mosquito Collection and Handling

Indoor resting, blood fed female *Anopheles* mosquitoes were collected from the walls, roof and household objects using aspirators from 0500 -0800 hrs in the months of November 1992, May 1993 and December 1993. Collections were made twice, for six to seven days at each site.

After each collection mosquitoes were kept in cages and then transferred to unwaxed paper cups to be killed by suffocation with chloroform. Killed mosquito specimens were sorted and identified using a dissecting microscope. Mosquitoes collected on December 1993 were kept in cages for 48 hrs before killing, so that digestion of the blood meal already ingested may take place and mosquitoes would become gravid. The identification keys of Verrone (1962a) and Gilles and Coetzee, (1987) were used for mosquito speciation. Specimens were then divided into two equal portions. In the first portion, abdomens were severed off from individual mosquitoes and head - thorax parts were squashed on Hybond N+ nylon filters (Amersham, UK) after wetting the nylon membrane with a drop of phosphate buffered saline (PBS) (8.0gm NaCl, 0.2gm KH_2PO_4, 2.9gm $Na_2HPO_4.12H_2O$, 0.2gm KCl in 1 litre of distilled water, pH 7.4) at the spot where the specimen is to be applied. In the second portion, whole mosquitoes were kept in paper cups after killing and left for drying, the dried specimens were then packed in screw cupped vials and labelled.

Besides these, additional dried *An. tenebrosus* that were fed on *P. falciparum* gametocytes were obtained as gifts from Ato Nesibu Adugna (Medical Entomology Divisin, NRIH), and *An. gambiae* s.l that were maintained on rabbits in the laboratory were kindly provided from the Nazareth Insectary of the NPCMVD.

2.2.1 Mosquito Salivary Gland Dissection

Dissection of mosquito salivary glands was done in the field every other day. As the technique requires a lot of time, it was not possible to accommodate it in a days' work which included collection of mosquito, identification and taking blood smears from febrile patients.

Mosquito samples were first killed in paper cups with chloroform and then identified to the species level. Wings and legs were severed off and the mosquito was mounted with saline solution. Using a dissecting microscope the head was then cut off with a fine dissecting needle. A gentle pressure was applied by slightly pressing the thorax with a dissecting needle to expose the salivary glands. The exposed salivary glands were then pulled out and mounted with a drop of saline solution. Salivary glands were examined for gliding sporozoites using a compound microscope.

2.2.2 Collection of Thin and Thick Blood Smears from Febrile Individuals in the Three Study Sites

To ascertain malaria transmission at the time of mosquito collection and to determine the *Plasmodium* species responsible, blood films (thick and thin smears) were taken from febrile individuals.

2.3 Isolation and Purification of DNA from *P.falciparum*

To obtain a positive control for the hybridization assay, a small scale extraction of high molecular weight DNA from malaria parasites from blood was carried out following the protocols of Tungprachubkul and Panyim, (1986), with minor modifications.

Blood drawn from five febrile patients was centrifuged at 2000 revolutions per minute (rpm) for 5 min to separate the serum. After removing the serum the packed cells were washed with Rosewel Park Memorial Institute (RPMI) 1640 medium (Sigma, USA) three times. 200 ul of packed cells were transferred to a clean 1.5 ml tube and washed with 0.85% NaCl. Cells were retrieved after centrifuging at 10,000 rpm for 5 min and suspended in twice their volume in 1% cold acetic acid. After centrifugation, they were again suspended in twice their volume in 1% Triton X - 100 (BDH chemicals, England) and centrifuged for 5 min at 10.000 rpm. The pellet was then washed with 0.85% NaCl and 0.01M Tris (Sigma, USA), pH 8 and the supernatant was discarded after centrifugation. The pellet was then lysed with lysis buffer 1 [0.01M Tris, 0.01M EDTA, pH 8; 0.01M NaCl, 2% SDS (Sigma, USA) and 1mg/ml Proteinase K] vortexed for 5 sec and, incubated at 37^{0}C for 30min. An equal volume of phenol was then added and mixed by inverting the tube several times. After centrifugation for 2 min, the upper phase was transferred to a fresh tube and the phenolization step was repeated three times. An equal volume of isoamyl in chloroform was added, mixed, and centrifuged for 2 min. To the aqueous phase (upper phase) 100 ug/ml RNase was added and incubated for 30 min at 37^{0}C. After incubation, an equal volume of ether was added and the top ether phase was removed after centrifugation. Finally an equal volume of cold absolute ethanol was added and incubated at -20^{0}C overnight. DNA pellet was recovered after centrifugation at 14,000 rpm for 10 min. The pellet was washed with 70% ethanol and suspended in 0.1x Tris Ethylene diamine tetracetic acid (EDTA) (TE) [(1x TE): 10mM Tris HCl, 1mM EDTA pH 8] .

2.3.1 Gel Electrophoresis

A 0.7% agarose (SeaKem, FMC Bioproducts) gel was made by weighing an appropriate amount of agarose and suspending it in 1x Tris acetate EDTA (TAE) (4.84gm Tris, 1.14ml glacial acetic acid, 2ml 0.5M EDTA pH 8). The solution was heated until all the agarose had melted completely and kept at 65^{0}C. Ethidium bromine was added to a final concentration of 5 ng/ul to the hot gel (Maniatis *et* *al.*,1982). Samples were loaded in each well after mixing 1 ul of DNA with 9 ul of 1x loading buffer (1% glycerol, 10mM Tris/HCl pH 7.5, 1mM EDTA, 0.01% bromophenol blue). Electrophoresis was carried out at 100 V for 30 min. The DNA bands were visualized using a UV transilluminator. Permanent record of the gel was made by photography using a Polaroid camera.

2.3.2 Spectrophotometry

DNA samples were diluted 1:100 and the optical density at 260 nm was determined with a spectrophotometer (Beckman, DU-64). Conversions of Optical Density (OD) readings were made based on 1 OD = 50ug/ml double stranded DNA (Maniatis *et al.*,1982).

2.4 Dot Blots

2.4.1 Dot Blot of Purified *P.falciparum* DNA

Concentrations of purified *P.falciparum* DNA were adjusted to 5 ng using sterile distilled water. An appropriate size Nylon filter (Hybond N+) (Amersham,UK) was cut and samples were applied to the filter using a micropipette. Samples were allowed to dry and the nylon filter was baked at 80^0C for 90 min and stored at 4^0C until hybridization was carried out.

2.4.2 Dot Blot of Blood Obtained from Febrile Patients Infected with *P. falciparum*.

The protocol of Dayal-Drager, (1989) was adopted for the purpose of bloting lysed blood directly on nylon filter. Thin and thick films were initially made to determine parasitemia. Parasitized as well as blood obtained from a normal uninfected individual were centrifuged at 2000 rpm for 5 min to separate the serum. The blood from the normal uninfected individual was used as a negative control. After serum was removed, 1 ml of packed cells were lysed with lysis buffer 2 [10mM EDTA, 10 mM Tris, 0.1% Triton X-100 (BDH, chemicals England), pH 10 with 20ug/ml Proteinase K]. After mixing, incubation was performed at 42^{0}C for 1 hr. Serial dilutions were then made in the proportion of 1:20, an equal volume of phenol was added, and the mixture centrifuged for 5 min at 14,000 rpm. The aqueous upper phase was retrieved and added to 0.5N NaOH in a 96 well microtitre plate and incubated for 5 min at room temperature. Samples were then transferred to corresponding wells on a 96 well Bio - Dot apparatus (BIO - RAD, Richmond, CA), vacuum suction was applied to transfer DNA to the nylon filter following the manufacturers' instruction. The nylon filter was removed and air dried, and baked at 80^{0}C for 90 min to fix DNA.

2.4.3 Squash Blots

Squash blots from the field were treated with 10% sodium dodecyl sulphate (SDS) by soaking a Whatmann filter paper in the solution and then applying the nylon filter over it with the side containing the mosquito specimens on top. Applying the same procedure the filter was then soaked with 0.5M NaOH/ 2.5M NaCl for 5 min, and with 3M sodium acetate pH 5 for 2 min. After these steps the filters were incubated in 1.5M NaCl/0.5M Tris, pH 7.4 for 5 min, floated in 95% ethanol, washed in chloroform and rinsed in 0.3M Nacl. After each step the filter was air dried.

2.4.4 Dot Blots of Mosquito Specimens

Wings, legs and abdomens were first cut off from dried mosquito specimens brought from the field as well as from the laboratory. These include; *An. gambiae* s.l that were blood fed or gravid and those that were maintained by feeding on rabbits in the laboratory, *An. tenebrosus* that were fed on *P. falciparum* gametocytes. Head - Thorax portions were put in 1.5 ml tubes containing 200 ul of lysis buffer 3 [1mM EDTA, 10mM Tris, 0.5% Tween 20 (BDH Chemicals, England), pH 7.4. Containing 1ug/ul Proteinase K]. A metal pestle was used to homogenize the samples in the tubes. After each tube the metal pestle was heated over a flame to avoid any carry over of target DNA. Male anopheline mosquitoes were processed in the same way and used as negative controls. The homogenate was then incubated at 42^{0}C for 1hr. The Proteinase K (Boehringer Mannheim) present in lysis buffer 3 was later inactivated by incubating the homogenate at 98^{0}C for 15 min. An equal volume of phenol was added to 200 ul of homogenate and the aqueous phase was retrieved after centrifugation and added to a microtitre plate containing 0.5N NaOH and incubated for 5 min. Blotting was carried out following the steps outlined in section 2.4.2. *P. falciparum* DNA obtained from blood (section 2.4.2) was used as a positive control. Additionally, laboratory reared *An. gambiae* s.1 and gametocyte fed *An. tenebrosus* that were maintained by feeding on rabbits were processed for hybridization in the same fashion.

2.5 Hybridization Procedures

2.5.1 Labelling of Probe

A 21 base synthetic oligomer PFR1 (5'-AGGTCTTAACTTGACTAACAT-3') was purchased from Pharmacia, Sweden. The sequence of this oligo probe is complementary to a tandemly repeating sequence of *P. falciparum* and which represents 10^4-$2x10^5$ repeating units specific to *P. falciparum* (Franzen *et al.*, 1984; Aslund *et al.*, 1985; Pollack *et al.*, 1985 ; Oquendo *et al.*, 1986; Barker *et al.*, 1986; McLaughlin *et al.*, 1987) was labelled with a non-radioactive fluorescein-dUTP using the enhanced chemoluminescenct (ECL) 3'-oligolabelling and detection kit (Amersham, UK. Cat.# RPN 2130) following the manufacturers' instruction. Probe (PFR1) concentration corresponding to 100 x 10^{-12} moles was used for labelling. Corresponding amounts of fluorescein -11-dUTP, Cacodylate buffer and Terminal transferase enzyme were added (all are supplied in the kit). Sterile distilled water was used to bring the volume to the required amount. After mixing gently the reaction mix was incubated at 37^0C for 1 hr. Labelled probe was then stored at -20^0C until used.

2.5.2 Hybridization

All filters were hybridized following the 3'- oligo labelling and detection kit instructions. Hybridization temperature was optimized after considering the melting temperature (Tm) [(4x No. of G+C)+(2x No. of A+T)] of the probe and also conducting a series of trials at varying temperatures. Optimum results were obtained at 42^0C.

The principle behind the ECL 3'-oligolabelling and detection system is that; anti-fluorescein horseradish peroxidase (HRP) conjugate recognizes a specific epitope on the fluorescein label, tagged on the 3' end of the probe. The enzymatic reduction of peroxide (detection reagent 1) leads to the oxidation of luminol (fluorescein) in the presence of an enhancer molecule D-luciferin (detection reagent 2) (Whitehead *et al.*, 1983). As luminol breaks down it passes through an exited intermediate stage and as this falls to a ground state , light is emitted, that is detected on a blue-light sensitive film (Fig.4).

Nylon filters were first prehybridized for 45 min at 42°C in a prehybridization buffer [5x standard saline citrate (SSC) (1x SSC: 0.015M $Na_3C_6H_5O_7.2H_2O$, 0.15M NaCl, pH 7), 0.1% hybridization buffer component (supplied with the kit), 0.02% SDS, 0.5% blocking agent (supplied)] while gently rocking using a hybridization incubator (Robbins Scientific). Labelled probe, to a final concentration of 12 ng/ml was added to the prehybridization buffer. Overnight hybridization was carried out at 42°C. Filters were then removed from the hybridization buffer [5x SSC, 0.1 % hybridization component (supplied) 0.02 % sodium dodecyl sulphate (SDS) (Sigma, USA), 0.5% blocking agent] and placed in a clean container containing an excess of washing buffer (5x SSC, 0.1 % SDS). Washing was conducted twice for 5 min on a shaker. Stringency wash was conducted at 42°C using a low stringency wash buffer (1x SSC, 0.1 % SDS) for 45 min. The filters were then rinsed with buffer 1 (0.15M NaCl, 0.1M Tris HCl, pH 7.5) and placed in blocking solution [0.5% blocking agent (supplied in the kit) in buffer 1] the volume of which was equvalent to $0.25ml/cm^2$ and incubated for 30 min at room temperature. After the blocking step, filters were briefly rinsed in buffer 1, anti - fluorescein horseradish peroxidase (HRP) conjugate antibody diluted 1:1000 with buffer 2 (0.4M NaCl, 0.1M Tris HCl, pH 7.5)

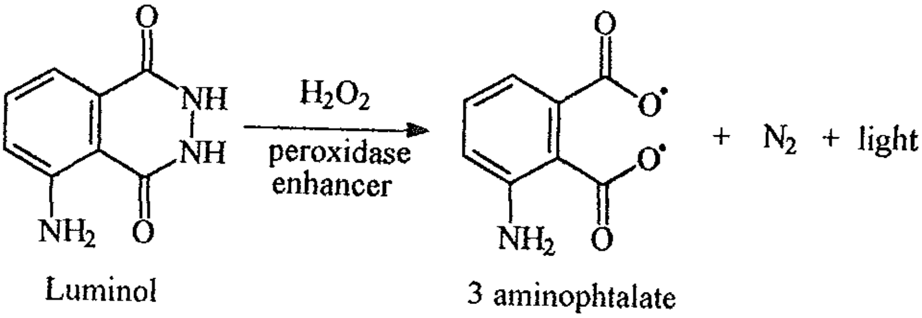

Fig. 4. The ECL detection reaction

containing bovine serum albumin (BSA) (fraction V) (Sigma, USA) was added and incubated for 30 min at room temperature. Next, washing was done with buffer 2 three times for 5 min, to remove all non - specifically bound antibody.

Signal generation and the detection steps were done in the dark room after mixing detection solution 1 and detection solution 2 (supplied) in a 1:1 ratio. Excess wash buffer was drained off from the filters and the detection solution was poured on the filters on the side containing the DNA. The filters were incubated for precisely 1 min, and excess detection buffer was drained off. Filters were then transferred to a carrier (cardboard or a discarded X - ray film), wrapped with a plastic sheet, and air pockets were gently smoothed out. The carrier containing the filters was then placed in an exposure film cassette (Amersham, UK) with the DNA side up. Autoradiograph film (Hyperfilm ECL, Amersham, UK) was placed over the filters, the cassette was then closed and exposed for 1hr, and developed. (Developing and Fixing solutions, Kodak).

2.6 Polymerase Chain Reaction (PCR) Procedures

2.6.1 Preparation of Blood Infected with *P. falciparum*

The basic protocol used for the preparation of blood infected with P. falciparum was that of Tirasophon *et al.*, (1991) and Barker *et al.*, (1992). Thin and thick smears were made from whole blood drawn from a febrile patient with

P.falciparum infection to determine the parasitemia. Serum part was removed and 80 ul of packed cells was taken and lysed in ten times it's volume in lysis buffer 4 (0.2% NaCl, 0.015% saponin and 1mM EDTA). The mixture was centrifuged at 10,000 rpm for 10 min and the pellet was washed with 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl, 0.01% gelatin). Pellet was then suspended in 1mM Tris and 10 - fold serial dilutions were made.

2.6.2 Preparation of Mosquito Specimens

Dried, female anopheline mosquitoes that have completely digested their blood meal and those mosquitoes that were fed on gametocyte were processed following the steps in section 2.4.4 i.e. the same homogenate was used for the hybridization as well as for the PCR assays. To optimize the volume of target DNA suitable for the PCR system 10 ul, 5 ul, 1 ul, 0.5 ul and 0.1 ul were taken from mosquito homogenates that were not phenolized.

2.6.3 Oligonucleotide Primers

Oligonucleotide primers K1-14-P1 (5'-CGCTACATATGCTAGTTGCCAGAC-3') and K1-14-P2 (5'-CGTGTACCATACATCCTACCAAC-3') were purchased (Pharmacia). These primers were used to amplify a 206bp DNA fragment that is part of the K1-14 gene of *P. falciparum* (Fucharoen *et al.*, 1988). The K1-14-P1 and K1-14-P2 primers are specific to *P. falciparum* (Tirasophon *et al.*, 1991).

2.6.4 Amplification Procedures

The reaction mix consisted of the 1x PCR reaction buffer in addition to 200 uM each of the nucleotides deoxyadenosine, -cytidine, -guanidine and -uridinetriphosphates and 0.1ng of primers. After addition of the appropriate volume of template, the reaction mix was overlaid with mineral oil and heated at 100^{0}C for 5 min 2.5 unit of (Taq) polymerase (Boehringer Mannheim) was added to each reaction mixture. 30 cycles were run using a thermal cycler (Hybaid Omnigene). The first cycle was done at a denaturing temperature of 94^{0}C for 2 min, annealing temperature of 45^{0}C for 1 min and an extension temperature of 72^{0}C for 3 min; subsequent cycles were done at 94^{0}C for 1 min, 45^{0}C for 1 min and 72^{0}C for 3 min; the last cycle was done at 94^{0}C for 1 min, 45^{0}C for 1 min and 72^{0}C for 10 min.

2.6.5 Analysis of PCR Products

The expected PCR product using the K1-14-P1 and the K1-14-P2 primers is at the 206 base pair position. An aliquote of 10 ul of the amplified mixture was analysed by 1.5% agarose gel and stained with ethidium bromide.

3. RESULTS

3.1 Anopheline Speciation

A total of 2071 indoor resting blood-fed female anopheline mosquitoes were collected from November 1992 to December 1993. Of these 1472 were collected during the malaria transmission seasons of November 1992 and May 1993 (Table 2). The *Anopheles* fauna of the collection in all three sites consists of *An.gambiae* s.l, *An.pharoensis*, *An.coustani*, *An.tenebrosus* Donitz, 1902, *An.funestus* and *An.ziemanni* Greunberg, 1902. These anopheline mosquitoes were directly killed after identifying them to the species level. Out of the 599 anophelines collected during the transmission season of December 1993, 317 were kept in cages for 48 hrs till they digest their blood meal and then killed and identified to the species level.

3.2 Dissection of Salivary Glands

Salivary gland dissections of 282 anopheline species was done in all three collection sites in December 1993. Anophelines belonging to 4 anopheline species were dissected for salivary glands altogether. Sporozoites were not observed in any of the salivary glands dissected in the three sites (Table 3).

Table 2. Indoor Resting Collection of Anopheline Mosquitoes From Metehara, Zeway and Arbaminch.

Species	Metehara		Zeway		Arbaminch	
	Nov.92 May 93	Dec.93	Nov.92 May 93	Dec.93	Nov.92 May 93	Dec.93
An.gambiae s.l	922	138	19	104	369	152
An.pharoensis	5	10	120	90	12	50
An.coustani	-	-	8	-	-	-
An.tenebrosus	-	-	-	-	-	4
An.funestus	-	7	17	-	-	31
An.ziemanni	-	-	-	-	-	13
Total	927	155	164	194	381	250
						2071

Table 3. Dissection of Salivary Glands of Anopheline Species Collected from Indoor Resting Sites in December 1993 from Metehara, Zeway and Arbaminch.

Col. Site	A.g		A.p		A.f		A.z		
	No. Dis.	No. Pos.	No. Dis.	No. Pos.	No. Dis.	No. Pos.	No. Dis.	No. Pos.	
Metehara	68	-	-	-	-	-	-	-	
Zeway	42	-	27	-	-	-	-	-	
Arbaminch	72	-	30	-	30	-	13	-	
Total	182 (64.5)[†]		57 (20.2)		30 (10.6)		13 (4.6)		282

[†] numbers in brackets indicate percentage of each species.

A.g (*An. gambiae* s.l) A.f (*An. funestus*)
A.p (*An. pharoensis*) A.z (*An. ziemanni*)

3.3 Malaria Infection in Humans in the Study Area.

Out of a total 177 thin and thick blood smears collected from febrile individuals, 62 (35%) had malaria infection (Table 4). The malaria parasites identified were *P.falciparum* and *P.vivax*.

3.4 Detection of *P.falciparum* Sporozoites Using the Hybridization Method.

3.4.1 Positive Controls

To determine the sensitivity and to obtain a cut off value for the assay, genomic DNA of *P.falciparum* was isolated and purified from blood drawn from five febrile individuals with *P.falciparum* infection. Varying amounts of DNA was obtained from each of the five specimens as seen from the agarose gel (Fig.5). DNA estimations done based on this ranged from 3 ug/ul to 0.2 ug/ul. Simultaneously, spectrophotometric readings of each DNA specimen gave DNA concentrations from 2.75ug/ul to 0.2ug/ul. The highest reading was obtained for sample C while the lowest was for E (Table 5).

Table 4. Parasite Positivity as Determined from Thin and Thick Blood Smears Taken from Febrile Individuals in the Study Sites (December 1993).

Collection site	Tot.# examined	*P.falciparum*	*P.vivax*	% inf.	
				P.f	P.v
Metehara	70	18	4	25.7	5.7
Zeway	50	-	10	-	20
Arbaminch	57	18	12	31.6	21.0
Total	177	36	26	20.3	14.6

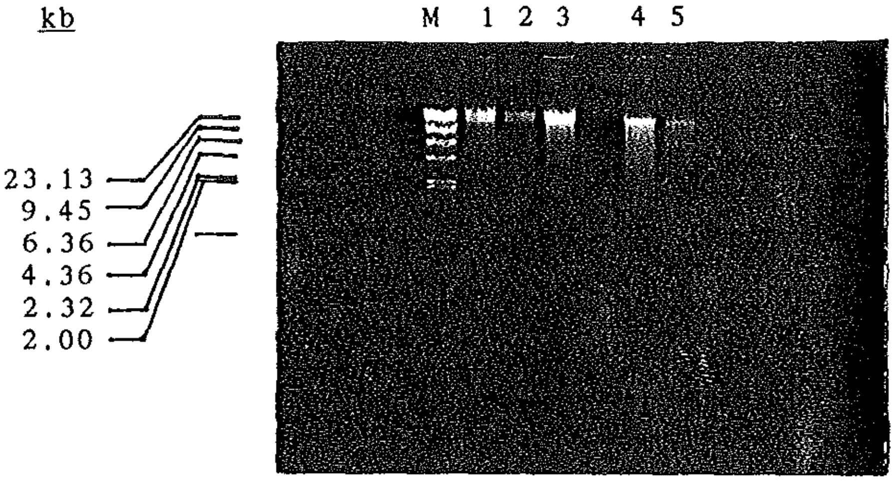

Fig.5 Agarose gel of *P.falciparum* DNA isolated from blood. M Lambda Hind III Marker. Lane 1 (A) 1ug/ul, Lane 2 (B) 0.5ug/ul, Lane 3 (C) 3ug/ul, Lane 4 (D) 2ug/ul, Lane 5 (E) 0.25ug/ul.

Table 5. Spectrophotometric Readings of Purified *P. falciparum* DNA at OD_{260nm}.

Sample Code	Optical Density (AU)	Concentration (ug/ul)
A	0.174	0.87
B	0.068	0.34
C	0.550	2.75
D	0.350	1.70
E	0.044	0.22

Even though the expectation was to obtain an equal signal intensity for 5 ng of purified DNA from each sample after an overnight hybridization using the PFR1 oligo probe, varying signals were obtained for each specimen (Fig.6). Dot blot of *P.falciparum* DNA from lysed blood drawn from febrile patients was made after determining the parasitemia from thick and thin smears. Autoradiograph showed a concentration gradient for successively decreasing parasitemia (Fig.7). Lysed blood from an uninfected individual on the same blot gave no signal. For the sake of simplicity, the signals were graded using '+' signs, therefore +++ corresponds to > 100,000 parasites, ++ to 10,000-50,000 and + to 100-1000.

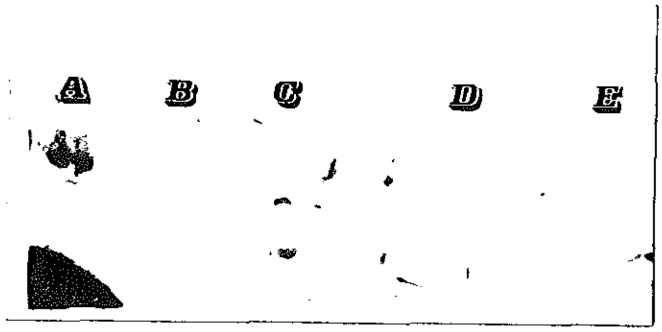

Fig.6. Hybridization of 5ng of purified *P.falciparum* DNA from five febrile patients.

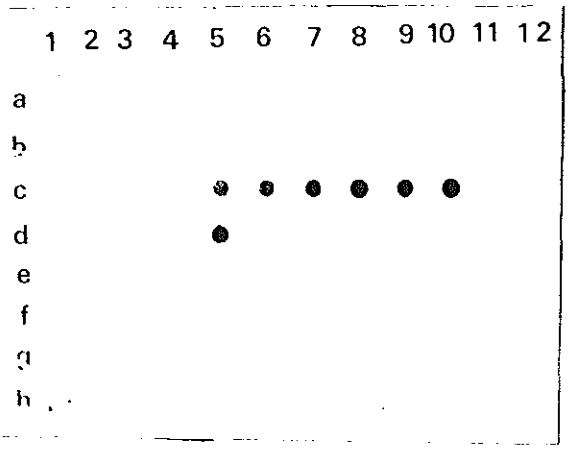

Fig.7. Dot blot of *P.falciparum* DNA from lysed human blood. c_5-c_{10} 180,000 parasites, c_{11} negative control (blood from uninfected individual). d_5-d_8 serial dilutions. d_5 180,000 (+++), d_6 8,960 (++), d_7 448 (+) and d_8 22.

3.4.2 Hybridization of Mosquito Specimens.

Overnight hybridization of squash blot of mosquitoes showed a high non-specific signal. Figure 8 shows the autoradiograph of the squash blot containing 120 anophelines together with a positive control and squashes of male anopheline mosquitoes that were used as negative control.

Dot blot of 60 field specimens of *An.gambiae* s.l that were killed just after identification also showed marked non-specific signal (Fig.9). In most cases strong non-specific signal was observed to be associated with mosquitoes containing blood meal in their gut. This was especially apparent when dot blot of 30 *An.gambiae* s.l that were maintained in the laboratory by feeding on rabbits was hybridized using the PFR1 probe (Fig.10 A). Clear difference of non-specific signal is seen between those with and without blood meal in their gut. Out of the 30, 10 contained blood in their gut, which was indicated by the dark appearance of their homogenates.

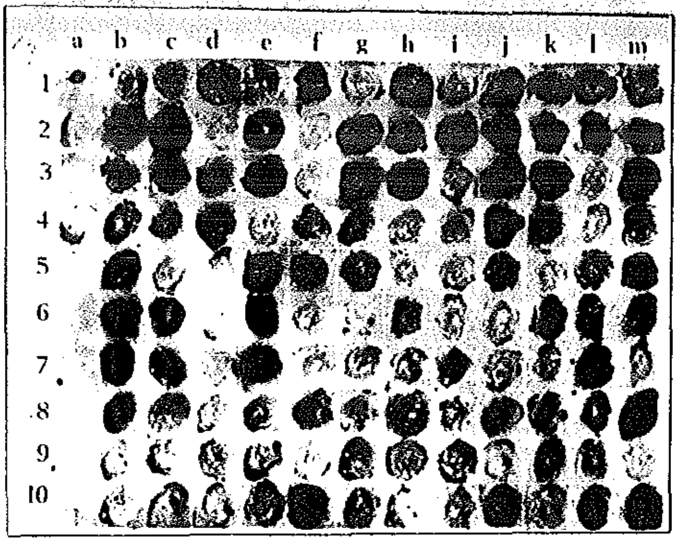

Fig.8. Squash blot of anophelines species. (a$_1$) positive control, (a$_2$, a$_3$, a$_4$) negative controls.

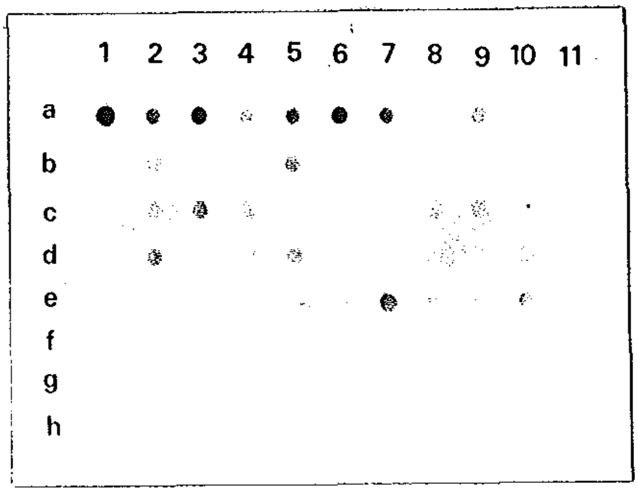

Fig. 9. Dot blot of 60 *An.gambiae* s.l containing blood in their gut. a$_1$ positive control, b$_1$ and c$_1$ negative controls.

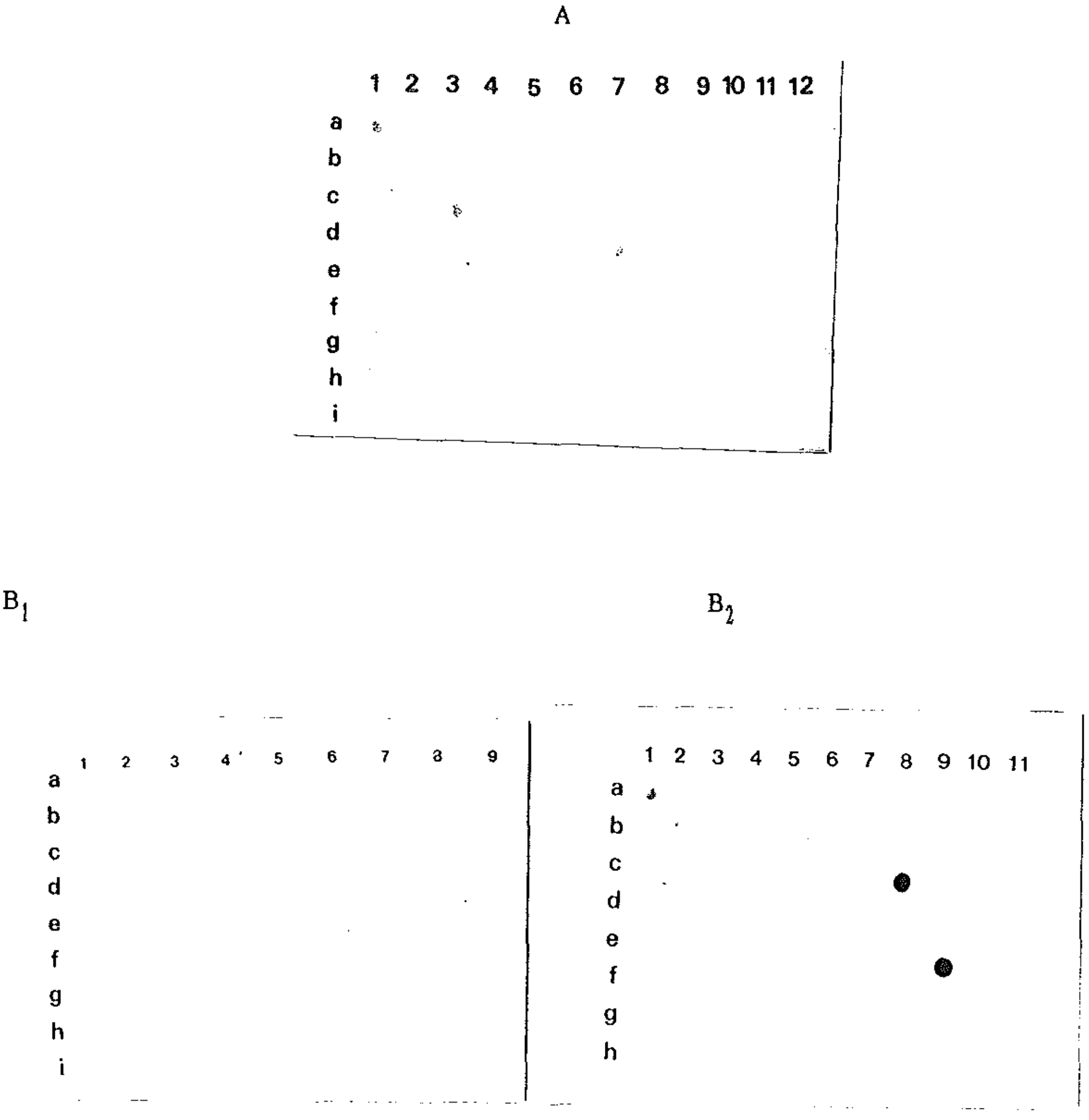

Fig.10. A. Dot blot of 30 non infected laboratory bred *An. gambiae* s.l a_1 positive control; b_1 and c_1 negative controls. c_2, c_3, c_7, c_9, d_7, d_{11}, e_2, e_3, e_6, e_8 correspond to those *An.gambiae* s.l with blood in their guts. B_1 & B_2 dot blots of gametocyte fed *An.tenebrosus* a_1 positive controls, c_5 (+), d_4 (++) & d_8 (+) in B_1. d_8 (+++) & f_9 (+++) in B_2 are positive specimens for *P.falciparum* sporozoites.

Gametocyte-fed *An. tenebrosus* that were maintained on sugar solutions for about 14 days after taking their first blood meal were assayed. A remarkably low background was obtained (Fig. $10B_1$ & B_2). 5 specimens out of 70 hybridized with the PFR1 probe. The presence of sporozoites of *P. falciparum* in these mosquitoes was later on confirmed by PCR (Fig.14).

Hybridization of blots of mosquitoes that were kept in cages for 48hrs in the field also resulted in a remarkably low non-specific signal. Consequently, these samples were used for the detection of *P. falciparum* sporozoites using the hybridization and the PCR methods.

A total of 198 samples of *An. gambiae* s.l were assayed by the hybridization and the PCR methods for the purpose of detecting *P. falciparum* sporozoites. 66 specimens of the afore mentioned anopheline species were assayed from each of the three collection sites.

In Metehara one specimen of *An. gambiae* s.l containing *P. falciparum* sporozoites was identified. Similarly, 2 specimens of *An. gambiae* s.l from Zeway and one specimen each, of the same anopheline species from Metehara and Arbaminch were found to contain sporozoites of *P. falciparum* (Figs.11 A & B). These same samples also showed characteristic bands on agarose gel with the PCR (Fig.15) indicating presence of *P. falciparum* sporozoites.

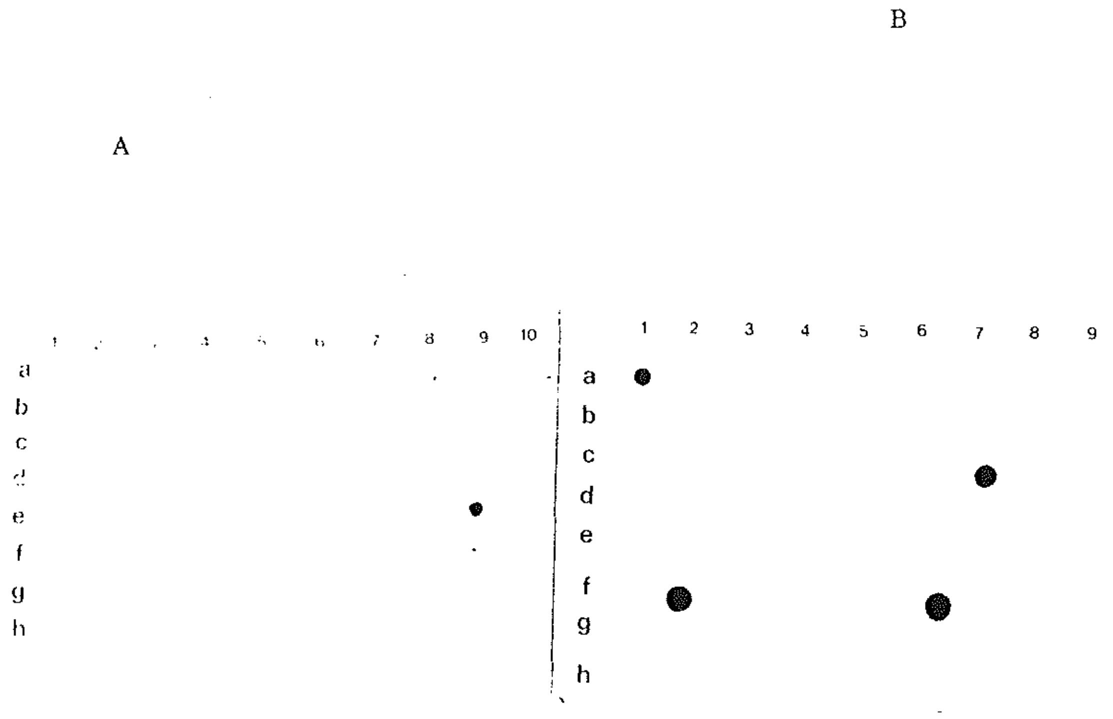

Fig.11. Dot blots of field specimens of *An.gambiae* s.l. A. dot blot of *An.gambiae* s.l from Arbaminch, e_9 (+++) & f_9 duplicates of the same positive sample (e_9:160ul f_9: 40ul) containing sporozoites of *P.falciparum*. B. Dot blot of *An.gambiae* s.l from Metehara and Zeway, c_7 (+++) positive specimen from Metehara, f_2 (+++) & f_6 (+++) positive specimens from Zeway.

3.5 Detection of *P. falciparum* Sporozoites Using PCR.

The expected 206 base pair (bp) product using the K1-14-P1 and K1-14-P2 primers was obtained after conducting PCR on blood drawn from a febrile patient (Fig. 12). Bands at the 206bp position were obtained for the first four serial dilutions which correspond to $4x10^5$, $4x10^4$. $4x10^3$ and $4x10^2$ parasites. 206bp bands were also observed for three ten fold serial dilutions made from a positive *An.gambiae* s.l specimen from Arbaminch. In comparison to the dot blot, the PCR shows a higher sensitivity level by a factor of 1000 (Fig.11a and 13).

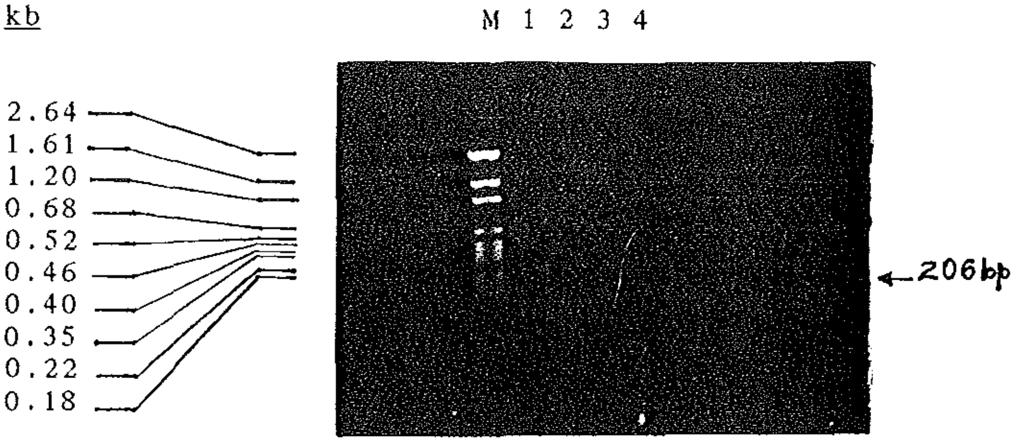

Fig.12. 206bp DNA fragment amplified from *P.falciparum* infected blood. M pGEM marker, Lane 1 4x10^5, Lane 2 4x10^4, Lane 3 4x10^3, and Lane 4 4x10^2.

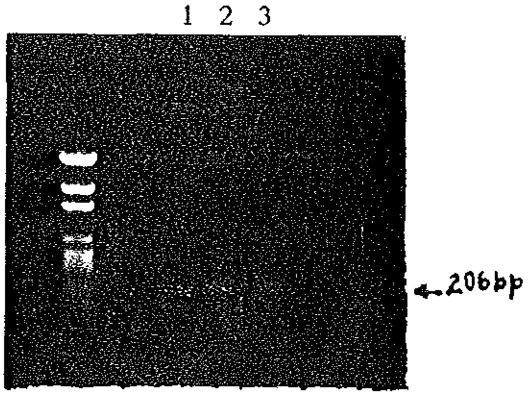

Fig.13. Serial dilution of *P.falciparum* sporozoites from *An.gambiae* s.l. Lane 1, 1:10; Lane 2, 1:100 & Lane 3, 1:1000.

3.5.1 Amplification of Sporozoite DNA Fragment from Mosquito Specimens.

Corresponding results to that of the hybridization assay were obtained by the PCR in general. The same gametocyte fed *An.tenebrosus* specimens that showed positive signals by the hybridization technique also showed 206bp bands on agarose gel after 30 cycles of amplifications (Fig. 14). Moreover, for the 4 field specimens of *An.gambiae* s.l that showed positive signals by the hybridization method, PCR products at the 206bp position were observed on agarose gel thus, confirming the presence of *P.falciparum* sporozoites in these mosquitoes (Fig.15).

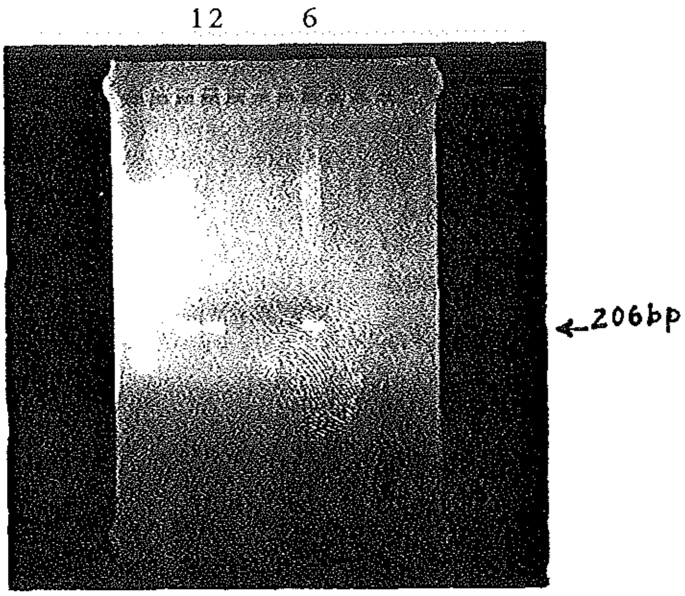

Fig.14. Characteristic bands at the 206bp position in gametocyte fed *An.tenebrosus*. Lane 1, 2 and 6.

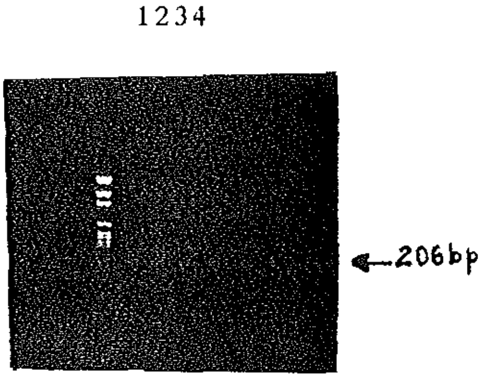

Fig. 15. 206bp DNA fragment amplified from *An.gambiae* s.l infected with *P.falciparum* sporozoites Lane 1, 2, 3 & 4.

4. DISCUSSION

Lysed blood spotted on nylon filter was used to obtain a positive control and determine the sensitivity of the hybridization assay using the fluorescein labelled PFR1 probe. Quantification of purified genomic *P. falciparum* DNA isolated from blood drawn from five febrile patients by separation on agarose gel as well as by spectrophotometer, gave comparable results (Fig.5 and Table 5). However, the filter containing 5 ng of DNA from each sample gave varying signal intensity instead of giving a uniform picture after autoradiography (Fig.6). The probable cause for this discrepancy can be the inclusion of human DNA while attempting to isolate genomic DNA of *P. falciparum* from the blood. Eventhough attempts were made to remove the majority of white blood cells by washing several times with RPMI initially, nucleated granulocytes which could still contaminate the red blood cells may serve as additional sources of genomic human DNA. This problem was later on circumvented by directly spotting lysed blood on nylon filter after determining the parasitemia from thin and thick blood smears. Autoradiography of this filter showed uniform signal intensity for an equal number of parasites (180,000) and a decreasing trend in signal intensity for a decreasing number of parasites (Fig.7). Signals were observed for as low as 500 parasites, which is comparable to other similar investigations (Pollack *et al.*, 1985; McLaughlin *et al.*, 1987; Barker *et al.*, 1989).

The PFR1 sequence is known to be conserved in the sporozoite stage (Delves *et al.*, 1989). Therefore it could be inferred that the probe detects sporozoites with the same efficiency as it does the asexual blood stages. Consequently, the sensitivity level for the sporozoite assay was extrapolated from the values observed in the hybridization experiment forlysed blood. Even though there exists a possibility of getting naturally infected mosquitoes containing less than 500 sporozoites in their salivary glands, majority of anophelines especially those that are associated with the active transmission of malaria contain more than 500 sporozoites in their salivary glands (Pringle, *et al.*, 1966; Burkot, 1988). Quite recently, however, it has been suggested that repeated or interrupted feeding rather than number of sporozoite in the salivary gland plays an important role in the transmission of malaria (Ponnudurai *et al.*,1991; Beier *et al.*, 1992). This view therefore, seems to instill the notion that mosquitoes containing few or large numbers of sporozoites in their salivary glands play an equal role in the transmission of malaria. Like wise, the concept that high sporozoite load being correlated with sporozoite inoculation, is still an accepted theory (Rosenberg *et al.*, 1990; Billingsley *et al.*, 1991). Because of these two contradictory views, an assay that is sensitive enough to detect very low amounts of sporozoite (< 50) would be desirable to provide information regarding the vectorial status that could help in obtaining a clear picture concerning the epidemiology of malaria in specified areas. Although such sensitivity levels have not been achieved by the hybridization assay in this study, hybridization assays with capture probes that have alternative complementary sequence other than the sequence of the labelled probe have been shown to produce very high sensitivity levels (Chen *et al.*, 1991).

The most commonly used method for detecting parasites or specified gene sequence in insect vectors by the hybridization technique is by squashing the insects on nitrocellulose or nylon filters (Anxoabehere *et al.*, 1985; Tchen *et al.*, 1985; Lee Sim *et al.*, 1989). However, this method produced non-specific signal with the fluorescein labelled probe used in this study and made it difficult to interprate the results (Fig.8). Hybridization carried out by other investigators using the same radiolabelled 21 bp probe on squash blots of anopheline species infected in the laboratory with *P.falciparum* sporozoites (Delves *et al.*, 1989; Lee Sim *et al.*, 1989) on the other hand produces no non-specific signal. It therefore seems that radioactive labelling produces a lower non-specific signal as compared to the non-radioactive labels (fluorescein). However, the problem associated with the short half life, disposal and safety of radioactive labels make it a very unattractive alternative for field work. Moreover, the sensitivity obtained through squash blots hybridized with radiolabelled probe was only around 1,000-1,700 sporozoites (Delves *et al.*, 1989; Lee Sim *et al.*, 1989). Low sensitivity values in these studies could have been associated with mosquito tissue, integument and other mosquito body parts covering target DNA and thus allowing only partial hybridization of the target DNA with the probe. The fact that better sensitivity levels where obtained when dried mosquito specimens where homogenized in the presence of buffers [Fig.10.B_1 c_5 (+) & d_8 (+)] might explain the low sensitivity in squash blots.

The problem of non-specific signal even after homogenization of mosquitoes in lysis buffers containing detergents as well as enzymes was not resolved originally. But in contrast to the squash blots, the negative controls (male anopheline mosquitoes) did not show any non-specific signal (Fig.9). All positive signals observed in this experiment however, could not be attributed to sporozoites of *P. falciparum* mainly since the number of positive samples were much higher than the sporozoite rate (4.8%) reported in naturally infected anophelines in Ethiopia (Adugna, 1991). Successive experiments carried out by using different concentrations of stringency wash buffers, extending the time for washes and blocking, using alternative hybridization temperatures and$├incubation times did not solve the problem of excessive non-specific signal. The non-specific signal however, was observed to be highly associated with mosquitoes containing blood meal in their gut. These mosquitoes were observed to give a homogenate with a dark appearance. Repeated experiments showed that non-specific signal was related to mosquitoes with undigested blood in their gut. This was further confirmed by the dot blot experiments performed on non-infected laboratory bred *An.gambiae* s.l that were maintained by feeding on rabbits. This blot contained blood fed and unfed *An.gambiae* s.l. All the blood fed mosquitoes produced a false positive signal while none of the unfed mosquitoes produced any signal. Paradoxically repeated hybridization carried out on lysed blood from a normal uninfected humans produced no non-specific signal. The non-specific signal observed repeatedly in mosquitoes with blood meal could be the result of chemical changes of the blood meal brought about by the process of digestion in the mosquito mid gut i.e. partial digestion. The explanation for the non-specific signal could lie in these chemical changes that could have interfered with the detection system of the hybridization assay. In the absence of DNA-DNA hybridization oxidative agents produced as a result of reduction of proteinatious residues during the digestion process of the blood meal, may have caused the oxidation of luminol. However, no work has been done in this line, in this or other studies to further clarify the issue.

Detection of sporozoites in gametocyte-fed *An.tenebrosus* that were maintained on sugar solution for about 14 days as of the day they were fed on blood containing *P. falciparum* gametocytes showed 5 specimens to contain *P. falciparum* sporozoites out of 70 mosquitoes assayed. According to unpublished data (Adugna, personal communication), the percentage sporozoite positivity of these mosquitoes was 15.8%. This figure was some what higher as compared to the 7.1% positivity obtained in this study. This disagreement could however be explained by the fact that those dried mosquitoes assayed by the hybridization method in this study include mosquitoes that were kept for more than 12 days and mosquitoes that have died before 8 days after taking blood infected with *P.falciparum* gametocytes. This means the mosquitoes in the later case, did not live long enough for the completion of the extrinsic cycle of the parasite . Thus the mosquito samples used for the hybridization assay during this study consisted of a mixture of mosquitoes in which complete and incomplete extrinsic cycle had occured. As a result of the inclusion of mosquitoes that were not likely to have developed sporozoites, the percentage sporozoite positivity was lower than the expected figure.

Removal of the non-specific signal for mosquito specimens from the field was achieved by allowing the mosquitoes to digest the majority, if not all, of the blood meal. This was accomplished by keeping the anopheline species collected each day for 48 hrs in a cage under humid conditions. Accordingly mosquitoes collected on December 1993 were handled in such a manner. Hybridization carried out on such mosquitoes gave satisfactory results and no non-specific signal was observed.

Sporozoite detection by the hybridization method carried out on a total of 198 *An. gambiae* s.l from Metehara, Zeway and Arbaminch revealed 4 specimens with *P.falciparum* sporozoites. Of these 2 were from Zeway and the remaining 2 were each from Metehara and Arbaminch. This result was confirmed by the PCR that is, out of the same 198 *An.gambiae* s.l specimens 4 samples (the samples that were positive by hybridization) produced characteristic bands on gel for *P. falciparum*. In comparison to the parasite rate determined from thin and thick blood smears taken from febrile individuals from the three sites, the identification of 2 *An. gambiae* s.l specimens with *P. falciparum* sporozoite from Zeway where no *P. falciparum* infection was found (Table 4) seems contradictory. Although no *P. falciparum* was identified from 50 blood smears taken during this study, data from the active case detection centre (ACD) of the area show 1.21% *P. falciparum* infection rate for December 1993.

Salivary gland dissections carried out on December 1993 on 282 anopheline specimens belonging to 4 species showed no sporozoites (Table 3). Eventhough a direct comparison couldn't be made between the hybridization and dissection methods because different mosquito samples were used in each case, the hybridization method was found to be much easier to perform, less labor intensive and more specific. Regarding sensitivity, sporozoite detection in anopheline species by the salivary gland dissection method is possible only for those anophelines with heavy sporozoite load in their salivary glands (Wirtz and Burkot, 1991). For anophelines with lower sporozoite load, the chances of finding sporozoites by this method markedly decreases (Holmberg and Wigzel, 1987). In case of the hybridization where squash blots were employed as low as 1,000-1,700 sporozoites were detected (Lee Sim *et al.*, 1989; Delves *et al.*, 1989). In this study mosquitoes were homogenized in lysis buffers better results were obtained (448 sporozoites ~ 500).

Ten-fold serial dilutions made to determine the level of sensitivity of the PCR to detect *P. falciparum* from an infected blood showed characteristic 206 bp bands for the first 4 dilutions (Fig.13). Amplification of the 206 bp DNA fragment was possible for as low as 400 parasites. Compared to similar investigations (Tirasophon *et al.*, 1991; Sethabutr *et al.*, 1992) this figure shows better sensitivity. Theoretically however, as low as 1 parasite could suffice for the amplification of a specific DNA fragment using PCR. However the achievement of this threshold is highly dependent on obtaining the target DNA in the simplest possible way, free of any inhibitors that could deter the action of the Taq polymerase. In the case of blood, haemoglobin among other things is known to have an inhibitory effect on the Taq polymerase (Higuchi *et al.*, 1989 in Barker *et al.*, 1992). The probable cause for not achieving higher sensitivity levels especially under lower parasite counts in blood could be highly related to inhibition of Taq with haemoglobin (Barker *et al.*, 1992). Because of this reason, direct extrapolation of the sensitivity level observed from the experiments done on blood for the sporozoite detection could be misleading. However, the sensitivity level observed for the blood could give a rough guideline to estimate sporozoite number especially if taken in relation to the result shown for three successive ten-fold serial dilutions of a homogenate of a naturally infected *An.gambiae* s.l (Fig.13). All three dilutions produced 206bp DNA fragments from *P. falciparum*. Moreover, as these mosquitoes do not contain bloodmeal, inhibition of the Taq polymerase is reduced significantly. As a consequence, the sensitivity for detecting sporozoites in mosquitoes without blood in their gut by the PCR is likely to be high. As compared to the dot blot the PCR shows a higher sensitivity level by a factor of 1000 (Figs.11A and 13). Eventhough this happens to be the case, additional positive mosquito samples were not detected from field specimens by the PCR. The probable explanation could be either the small sample size (66 mosquito samples from each collection site) or the absence of varying sporozoite rate in the mosquito population in the collection sites. The later case could have been induced by the poor summer rains which have markedly affected mosquito breeding and have resulted, at the time, in low sporozoite rate of the mosquito population.

For the same field collected as well as gametocyte fed mosquitoes that were assayed by hybridization, the PCR showed corresponding results (Fig. 14 and 15). This further confirms the presence of *P. falciparum* sporozoites in these samples.

5.CONCLUSIONS

Information obtained from this study indicate that it is possible to detect *P.falciparum* sporozoites in naturally infected *Anopheles* mosquito by using the hybridization method with a non-radioactive (fluorescein) labelled probe. Moreover, as a result of the simple procedure introduced to prepare mosquito specimens by homogenizing head-thorax portions in buffers that expose sporozoite target DNA, better sensitivity was obtained as compared to the squash blots. These properties provide suitable ground for it's application in the field for epidemiological studies .

Because of the high level of sensitivity of the PCR, mosquitoes with very low sporozoites in their salivary glands could be identified. Even though the high cost associated with the Taq polymerase might limit it's wide use for epidemiological investigations, it's application in areas where sporozoite rates are generally very low would be highly useful and the technique can also serve as a reference tool for confirmatory purposes. Further more, its accuracy and simplicity would make it applicable for research geared towards vector characterization and biology.

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