A HIGHLY SENSITIVE, NESTED POLYMERASE CHAIN REACTION BASED METHOD USING SIMPLE DNA EXTRACTION TO DETECT MALARIA SPOROZOITES IN MOSQUITOS

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Abstract. Dried *Anopheles farauti* mosquitos caught in Solomon Islands in 1990 were examined for malaria sporozoites by ELISA and nested polymerase chain reaction (PCR). Only heads and thoraxes were used. *Plasmodium* genus-specific nested PCR amplifications were carried out on all samples. Of the 402 pools of mosquitos that were processed, 30 were positive for malaria. Nest 1 products of positive samples were subjected to further PCR amplifications with species-specific primers for *P. falciparum* and *P. vivax*. Twenty pools were positive for *P. vivax* by PCR while only 7 were positive by ELISA. For *P. falciparum* 2 pools were positive by both ELISA and PCR, and one of these was a pool which was positive for *P. vivax* by PCR and ELISA. Thus the sensitivity of PCR for *P. vivax* was 100% while the specificity was 96.7%. For *P. falciparum* the sensitivity and specificity were 100%. The PCR technique is highly sensitive and can be used on dried mosquitos which makes it a valuable tool for determining sporozoite rates of mosquitos, even in remote areas.

INTRODUCTION

Malaria is still endemic in some tropical countries in the world. Evaluation of malaria campaigns is based not only on detection of parasites in patients but also on monitoring the infection of mosquito populations. Traditionally this has involved the dissection and microscopic examination of hundreds of mosquitos which is time consuming and labor intensive. With the advent of monoclonal antibodies specific to circumsporozoite (CS) antigens, an enzyme linked immunosorbent assay (ELISA), has been developed and shown to be a useful tool for the detection of *P. falciparum* and *P. vivax* in the mosquito vectors (Burkot *et al*, 1984; Wirtz *et al*, 1985).

An ELISA kit is now available and field evaluations demonstrated excellent correlation between ELISA positivity and salivary gland infection rates assessed by dissection (Wirtz *et al*, 1987). However ELISA fails to detect immature sporozoites present in oocystes (Beier *et al*, 1987) and it has been reported that circumsporozoite (CS) proteins in excess of those incorporated in sporozoites can be found in infected mosquitos (Boulanger *et al*, 1988) resulting in sporozoite number being overestimated.

As an alternative to ELISA, attention has been focused upon the development of diagnostic methods based on deoxyribonucleic acid (DNA) probes and polymerase chain reaction (PCR) for detection of parasites in mosquitos. The first demonstration of the application of DNA probes for diagnosis of the malaria parasite *P. falciparum* was by Franzen *et al* (1984). Following this technique other DNA probes for the detection of *P. falciparum* have since been developed (Oquendo *et al*, 1986; Baker *et al*, 1989; Zolg *et al*, 1987). Although DNA probes can be species specific and allow a large number of samples to be identified, sensitivity has been limited. Over 1,000 sporozoites per mosquito are required for reliable detection even with highly repetitive DNA probes (Delves *et al*, 1989).

The most recent and exciting development in the field of recombinant DNA technology has been the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). By using PCR, it is possible to synthesize *in vitro*, millions of copies of a specific target DNA sequence. It has been discovered that there are some regions of the sequences coding ssrRNA are specific to the parasite species from which they are derived (Walters and McCutchan, 1989; Goman *et al*, 1991). These regions were put to use for the designing of primers suitable for use in PCR amplification technique.

Snounou *et al* (1993) designed nested PCR which they felt was more sensitive. A PCR was

carried out with genus specific primers and this product was then used for the second round of PCR using species specific primers. However, Singh *et al* (1999) designed internal primers for genus specific *Plasmodium* for nest 1 and nest 2 amplifications and reuses the nest 1 amplification products of positive samples for species specific nest 2 amplifications.

Most of these studies have utilized infected blood samples or cultured blood stage parasites as a source of parasite DNA. Traditionally these samples require detergent lysis, proteinase K digestion, chloroform/phenol extraction and ethanol precipitation of parasite DNA prior to PCR. In some studies parasite DNA has been extracted from pools of mosquito vectors by similar methods (Rosenberg *et al*, 1989). In order to facilitate the analysis of malaria genes in small volumes of infected blood the utility of chelex treatment or Instagen of samples prior to PCR has been tested (Singh *et al*, 1996; Cox-Singh *et al*, 1997). Sample treatment with this cationic exchange resin has been shown to enhance PCR. Since malaria occurs in undeveloped areas, it is important to have simple method of DNA extraction.

However, very little work has been carried out using nested PCR on mosquitos especially wild caught mosquitos. Thus in this study we describe a simple method for the isolation of DNA from dried mosquitos and nested PCR using genus and species specific primers. This was compared with ELISA which was performed on the same mosquitos. The sensitivity and specificity of PCR and ELISA in diagnosis of malaria infection in field collected mosquitos was determined.

MATERIALS AND METHODS

Mosquitos were collected from Solomon Islands in 1990 using human landing catch. The mosquitos were identified and only *Anopheles farauti* were placed into capsules and stored in a dessicator. The abdomens were removed and only heads and thoraxes were preserved.

Preparation of mosquito pools

Each pool of 4 mosquitos was homogenized in 50 µl of blocking buffer and Nondiet P-40, 20 µl of this homogenate was used for the extraction of DNA and the remaining were kept at 20°C for ELISA.

Preparation of samples for ELISA

Enzyme linked immunosorbent assays (ELISA) were used to detect *Plasmodium falciparum* and *P. vivax* circumsprorozoite protein of malaria infected mosquitos. The method described by Wirtz *et al* (1985) was used. Briefly the sandwich ELISA technique was used, where 50 µl of monoclonal antibody (Mab) was added to each plate and incubated at room temperature for half an hour. Then 150 µl of blocking buffer was added to the frozen mosquito homogenate and centrifuged for 10 minutes at 2,000rpm. The well contents were aspirated and filled with 200 µl blocking buffer per well and was incubated for one hour. The well contents were aspirated and 50 µl of mosquito homogenate was added along with positive and negative controls and incubated for two hours. At the end of two hours the well contents were transferred to the second plate and the plate was washed twice with PBS tween. The monoclonal antibody peroxidase conjugate in blocking buffer was added to the wells and incubated for one hour. After incubation, the plate was then washed three times with PBS tween and then 100 µl of the enzyme substrate was added to each well. After one hour the optical density (OD) at 405 nm was measured using ELISA reader (MR 5000, DYNATECH). A sample was considered positive if it gave a visually detectable green color with an OD value at least twice the mean OD of 8 negative controls on that plate (Beier *et al*, 1988). All positive samples were reexamined to confirm the results.

Extraction of DNA from malaria parasites in mosquitos

Two hundred microliters of the Instagen (Bio Rad) was added to 20 µl of the mosquito homogenate. It was then incubated for 30 minutes at 56°C. It was vortexed at high speed for 10 seconds and placed in heating block at 100°C for 8 minutes. It was vortexed at high speed and then centrifuged at 12,000 rpm for 3 minutes at room temperature. The supernatant was then transferred to another tube. It was stored at -20°C and used in PCR.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out to detect the presence of sporozoite in mosquitos. Nested PCR was carried out using universal primers to detect the genus specific *Plasmodium*. The Nest 1 primer sequence was rPlu. 1 (TCA AAG AAT AAG CCA TGC AAG TGA CCT GTT GTT GCC TTG AAC TCC) and rPlu5. (CGT GTT GTT GCC TTA AAC TCC) (Snounou *et al*, 1993).

The Nest 2 primers were rPlu3 (TTT TTA TAA GGA TAA CTA CGG AAA AGC TGT) and rPlu 4

(TAC CCG TCA TAG CCA TGT TAG GCC AAT ACCA) (Singh *et al,* 1999).

The PCR amplification was carried out in Eppendorf Thermal Cycler. The 20 µl reaction mixture consisted of 9.6 µl of H_20, 2 µl of 10 x reaction buffer, 2.4 µl of 25nM $MgCl_2$, 1 µl deoxynucleotide triphosphate (dNTPs) to a final concentration of 200 µM and 10 pmol of each primer. Three microliters of DNA template was added and mixture was denatured for 5 minutes at 100°C and chilled on ice. Taq polymerase (2.5 units Fermentas) was added. Thirty five amplification cycles were completed with denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 1 minute. For the second nest, a similar PCR mixture was prepared but Nest 2 primers were used. To this, 3 µl of template from nest 1 was added and the PCR amplification was carried out as described except that the annealing temperature was 62°C.

Ten microliters of PCR product were loaded on a 2% agarose gel in 1 x Tris acetate buffer and gel stain and electrophoresed to determine the appropriate size target fragment. DNA bands were visualized by ultra violet (UV) light illumination.

PCR amplification for species (*P. falciparum* and *P. vivax*)

Samples which were positive for *Plasmodium*, were further analyzed with species specific primers. Three microliter of Nest 1 product was added to the PCR mixture as described above. The primer sequence used for *P.vivax* was 5′ CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC 3′; 5′ ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA; and for *P. falciparum* was 5′ TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT 3′; 5′ ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC 3′ (Snounou *et al,* 1993).

The PCR amplification was carried out for 45 cycles. The annealing temperature used for *P. falciparum* was 58°C and for *P. vivax* was 60°C. Ten microliters of this product were then electrophoresed as described earlier to determine the correct band size for each species. Positive and negative controls were included in all PCR reactions. For positive controls infected blood was used and for negative controls uninfected mosquitos were used.

RESULTS

Of the 402 pools of mosquitos examined for malaria parasites 30 were positive. Positive Nest 1 products were subjected to for determination of species namely *P. falciparum* and *P. vivax*. Twenty pools were positive for *P.vivax* by PCR and only 7 were positive by ELISA. For *P. falciparum* two pools were positive by both PCR and ELISA. Of these one pool was mixed infection being positive for both *P. vivax* and *P. falciparum*. Amplification of *P. vivax* and *P. falciparum* was demonstrated by the presence of a 120 bp and 205 bp respectively as shown in Figs 1 and 2.

Table 1
Sensitivity and specificity of PCR for detecting *P.vivax*.

PCR	ELISA as reference	
+VE	True +VE =7	False +VE =13
-VE	False +VE = 0	True +VE = 382

Sensitivity = 7/7 x 100 =100%
Specificity = 382/395 x 100=96.7%

Table 2
Sensitivity and specificity of ELISA for detecting *P.vivax*.

ELISA	PCR as reference	
+VE	True +VE =7	False +VE=0
-VE	False +VE=13	True +VE =382

Sensitivity = 7/20 X100= 35%
Specificity = 382/382X100=100%

The sensitivity of PCR for *P. vivax* was 100% and the specificity was 96.7%; while the sensitivity of ELISA was 35% and the specificity was 100% as shown in Tables 1 and 2. The sensitivity and specificity of PCR and ELISA for *P. falciparum* was 100%.

DISCUSSION

Over many decades plasmodial infection in vector population has been assessed using conventional dissection methods. Although, this method yields reliable results, it is time consuming and labor intensive, and does not distinguish between *P. falciparum* and *P. vivax*. Other techniques have been developed to overcome these difficulties. Monoclonal antibodies are used against the circumsporozoite protein in ELISA and this method has been widely used to detect the malaria parasites in mosquitos (Zavala *et al,* 1982; Burkot *et al,* 1984; Beier *et al,* 1987). However ELISA is not sensitive enough to detect the immature sporozoites present in the oo-

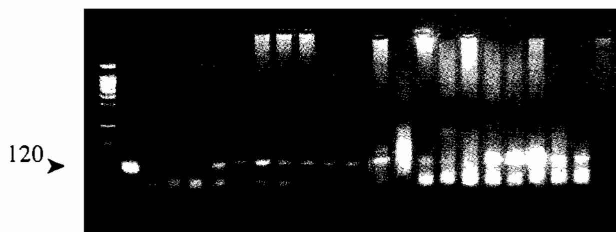

Fig 1–Electrophoresis in 2% agarose gel of *P. vivax*. Size of PCR product 120 bp. Lane 1:DNA marker, Lane 2: Positive control, Lane 3-22:*P. vivax* from mosquito, Lane 23:Negative control. Sample in Lane 11 positive for *P. vivax* and *P. falciparum*.

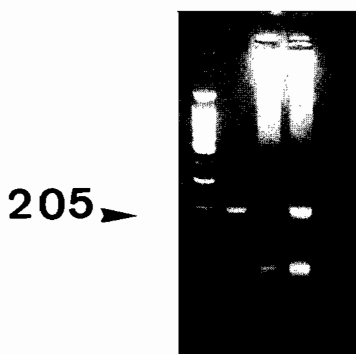

Fig 2–Electrophoresis in 2% agarose gel of *P. falciparum*. Size of PCR product 205 bp. Lane 1:DNA marker, Lane 2:Positive control, Lane 3 and Lane 4: *P. falcipurum* from mosquito, Lane 5:Negative control. Sample in Lane 4 positive for *P. falciparum* and *P. vivax*.

cysts (Beier *et al,* 1987). As an alternative method, DNA probes have been developed for the detection of mosquitos infected with *Plasmodium* (Barker *et al,* 1986; Delves *et al,* 1989). Despite being species specific and allowing a large number of samples to be identified, sensitivity of the DNA probes has been limited and require over 1,000 sporozoites per mosquito for reliable detection (Delves *et al,* 1989). Considering these practical difficulties, it was felt necessary to have more sensitive immunological/ molecular methods for the proper and accurate diagnosis of vector infection. In this context, PCR and ELISA have been developed and tested.

In the present study, ELISA and nested PCR were used to detect *P. falciparum* and *P. vivax* in wild caught *An. farauti*. The sensitivity and specificity of one method was validated using the results of the other method as reference. The sensitivity and specificity of these two methods for the detection of malaria parasites were compared. Although, there was not much difference between ELISA and PCR with reference to specificity, PCR had relatively higher sensitivity in detecting both *P. falciparum* and *P. vivax* when compared to the ELISA. In an earlier study, it was reported that in laboratory infected mosquitos, PCR could detect as few as 10 sporozoites of *P. falciparum* in dissected salivary glands and a single oocyst in a dissected midgut (Tassanakajon *et al,* 1993). The detection of *P. falciparum* DNA in wild caught mosquitos with low parasite infection indicated an advantage of the PCR over ELISA. The PCR technique was able to identify the presence of *P. falciparum* DNA in 4 or 5 wild caught mosquitos which were negative by ELISA, which requires at least 100 sporozoites per assay (Beier *et al,* 1987). In the present study it was found that nested PCR showed 100% sensitivity for both *P. vivax* and *P. falciparum*. While the sensitivity of ELISA for *P. vivax* was only 35% although specificity was 100%.

Recently, Tassanakajon *et al* (1993) demonstrated a highly sensitive detection procedure for *P. falciparum* in mosquito tissues, by increasing the number of amplification cycles to 40 and as few as 10 sporozoites, equivalent to 0.2 pg DNA, could be detected. In the present study, nested PCR was carried out to detect if *Plasmodium* species was present. Only if the correct band was obtained, then specific primers were used with nest 1 PCR product to determine the species present. This method has shown high sensitivity and specificity when tested with blood samples (Singh *et al,* 1999). Thus by using this method the cost can be reduced.

Overall, the present study indicates that PCR could detect malaria parasites in mosquitos with a high level of sensitivity. The PCR could be considered for processing large number of samples in operational programs, mapping areas with different levels of endemicity and stratifaction of areas, provided the technique is further refined to have higher specificity.

From this study it has been shown that a simple technique used to isolate DNA from dried mosquitos can be used for PCR amplification. This is especially useful in countries where malaria is occurring as samples can be transported dry to the main laboratory.

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