

MALARIA PARASITE DEVELOPMENTAL ANALYSES BY THE NESTED POLYMERASE CHAIN REACTION METHOD: AN IMPLICATION FOR THE EVALUATION OF MOSQUITO INFECTION RATES IN EPIDEMIOLOGICAL STUDIES

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Abstract. A malaria mosquito vector, *Anopheles soperi*, and a non-vector, *Aedes albopictus*, were allowed to feed on mice infected with murine malaria, *Plasmodium yoelii nigeriensis*, and were subsequently monitored for the development of parasites by the nested polymerase chain reaction (PCR) method, using *Plasmodium* genus-specific primer pairs. The mosquitos were divided into two parts, head/thorax and abdomen, for DNA analyses. The parasite DNA and murine DNA for each mosquito were examined in parallel. In both groups of mosquitos, murine DNA was detected up to 4 days post-blood meal in both the head/thorax and abdomen. After 4 days, the murine DNA fell below detectable limits. Murine DNA and parasite DNA remained undigested for the first 4 days post-blood meal. Parasite DNA was detected in the abdomen of 25% (3/12) of *Ae. albopictus* on day five and 10% (1/10) on day six, after murine DNA had fallen below detectable limits. Parasite DNA was not detected in the head/thorax of *Ae. albopictus* on those days or afterwards in either the head/thorax or abdomen, demonstrating that the parasite detected on days 5 and 6 in the abdomen degenerated and did not develop into mature oocysts or sporozoites. In the vector *An. soperi*, parasite DNA was detected continuously in the head/thorax and abdomen for many days after the murine DNA had fallen below detectable limits. The detection rate of parasite DNA in the head/thorax of *An. soperi* increased gradually from day 8 post blood meal until it reached a maximum level of 71.4% (15/21) 12 days post-infection. Parasite DNA in abdomen reached its maximum level of 81% (17/21) 10 days post-blood meal. The implications of these results for the design and interpretation of epidemiological surveys is discussed.

INTRODUCTION

Detailed knowledge of the incidence and transmission dynamics of malaria parasites is central to the design of effective measures in malaria control programs. The evaluation of malaria campaigns is based not only on the detection of parasites in humans, but also an identification of sporozoite-infected mosquitos, for effective vector control. Monitoring of sporozoite development is essential for determining the effect of antimalarial drugs on the development of parasites in

mosquitos (Li *et al*, 1993). To evaluate this and the effects of antimalaria drugs on the total parasite population, it is important to be able to identify the infected mosquito vectors in malaria endemic areas. The parameter most widely used by epidemiologists is the sporozoite rate, defined as the percentage of female anopheline mosquitos caught in the wild with sporozoites in their salivary glands (WHO, 1963). Measurements of sporozoite rates require simple, sensitive, specific and inexpensive methods for detecting and identifying *Plasmodium* species in mosquitos.

Traditionally, malaria parasites have been detected by dissection of freshly collected mosquitos and microscopic examination of the salivary glands or mid gut to obtain sporozoite and oocyst rates, respectively (Bruce-Chwatt, 1987). Microscopic examination of individual mosqui-

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tos for malaria parasites is labor-intensive, time-consuming and does not allow parasite detection in dried specimens. In anopheline mosquitos, the morphological features of the parasites do not permit reliable species identification (Pringle, 1966; Snounou *et al*, 1993).

Recently, monoclonal antibody-based methods have been developed to overcome the limitations of the microscopic dissection procedure (Wirtz and Burkot, 1991). An enzyme-linked immunosorbent assay (ELISA) kit is now available for epidemiological studies. A drawback of the ELISA method is that it cannot differentiate between the surface proteins of the sporozoites and circumsporozoites (CS) that may be deposited in mosquito tissue (Golenda *et al*, 1990; Beier and Koros, 1991). Hence, the CS antigen can be detected in the thorax of *Anopheles* without sporozoites in their salivary glands (Lombardi *et al*, 1987). In addition, problems of precision and the validity of the ELISA (Post *et al*, 1992; Stoffels *et al*, 1995) have complicated the use of monoclonal antibody-based assays, resulting in sporozoite rates being overestimated.

As an alternative to ELISA, attention has been focused on the development of diagnostic methods based on deoxyribonucleic acid (DNA) probes with the polymerase chain reaction (PCR) for the detection of malaria parasites in blood samples and mosquitos (Nitiavathy *et al*, 1999). Using oligonucleotide probes for the identification of malaria parasites is not suitable for implementation in field conditions because the probes are labeled with radioactive elements (Stoffels *et al*, 1995).

It is important to have a simple and affordable method for the detection and identification of malaria parasites in both humans and mosquito vectors. DNA amplification is thus becoming an important tool for the study of malaria parasites. As a result of the high sensitivity and specificity of the PCR method, DNA amplification is often observed in the absence of microscopically demonstrated parasites (Jarra and Snounou, 1998). Concern has, therefore, been expressed that in some cases, the target of PCR amplification might be circulating DNA derived from parasites ingested by peripheral phagocytic cells (Jarra and Snounou, 1998), or parasite DNA derived from

asexual parasites ingested with the blood meal (Snounou *et al*, 1993).

In this study, we describe a method of isolation of parasite DNA and murine DNA from experimentally infected mosquitos by the modified Chelex method and nested PCR amplification using genus and species-specific primers, where the development of murine malaria parasites in experimentally infected mosquitos was monitored. Murine DNA was monitored in parallel with the malaria parasite DNA in mosquitos as a function of time post-blood meal. The application of this technique in epidemiological studies of malaria is discussed.

MATERIALS AND METHODS

Mosquitos, mice, and DNA template preparation

Both infected and non-infected *Anopheles soperi* Bohart & Ingram and *Aedes albopictus* Skuse were used. These mosquitos were reared in an insectary at 25±1°C, 80% relative humidity and with a photoperiod of 16:8 (light:dark). Infections were obtained by feeding 5-7-day old *An. soperi* and *Ae. albopictus* with the blood of ICR mice infected with murine malaria parasite, *Plasmodium yoelii nigeriensis* (N67 strain). Parasitemia and gametocytemia in each mouse used to feed the mosquitos were checked by microscopy, and ranged from 5-10% and 0.01-0.1%, respectively. Fully engorged female mosquitos were maintained on a 2% sucrose solution until examination of the mid gut for oocysts and the salivary glands for sporozoites.

DNA was extracted from individual mosquito as follows: *An. soperi* and *Ae. albopictus* mosquitos were killed by exposure to cold (4°C) and the legs and wings were removed. To differentiate between infective mosquitos (with salivary sporozoites) and infected mosquitos (with mid-gut oocysts), the mosquitos were separated into two parts, head/thorax and abdomen. Chelex 100 (100 mesh, BioRad) was suspended in distilled water to yield a 5% (w/v) slurry stock (Singer-Sam *et al*, 1989). Mosquito samples (head/thorax and abdomen) were delivered into pre-heated (100°C in a heating water bath) Chelex as follows. Head/thorax and abdomen parts were

homogenized lightly in 20 µl of 0.9% NaCl in microtubes on ice and delivered into 200 µl of Chelex. The samples in Chelex were vortexed and heated for 10 minutes. The Chelex was pelleted by centrifugation at 8,000 rpm for 3 minutes. The supernatant was directly utilized as a source for the template in PCR amplification or stored at 4°C until PCR amplification.

PCR amplification of parasite DNA

PCR amplification was carried out in a DNA thermal cycler (PTC-100, MJ Research, USA). PCR reactions using parasite DNA as a template were carried out in a total volume of 20 µl. The reaction mixture contained 12.4 µl of Milli-Q H₂O, 2 µl of 10 x PCR buffer, 1 µl of 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.8 µl of 10 µM for each primer and 1 µl of 5U/6.6 µl *Taq* polymerase. Two microliters of DNA template were added and the mixture was denatured for 2 minutes at 92°C. Twenty-five amplification cycles were completed with denaturation at 92°C for 0.5 minute, extension at 60°C for 1.5 minutes and a final cycle with an extension time of 5 minutes at 60°C. For the second nested PCR, a similar PCR mixture was prepared, but nested two PCR primers were used. To this, 2 µl of primary PCR product was then used as a template in a second amplification reaction. Nested PCR amplification was carried out as described above, except the extension was at 62°C for 1 minute and the number of amplification cycles was eighteen.

Ten microliters of PCR product were loaded on a 2% agarose gel in 1 x Tris-acetate-EDTA buffer, and the gel was stained with ethidium bromide and electrophoresed to determine the appropriate band size of the target fragment. The DNA bands were visualized by ultraviolet (UV) transillumination. *An. soperoi* and *Ae. albopictus* fed with blood of uninfected mice or sugar solution were used as negative controls.

PCR was performed by the method of Kimura *et al* (1997) with modification. A complementary pair of primers P1 (5'-ACGATCA GATACCGTCGTAATCTT-3') and P2 (5'-GAACCCAAAGACTTTGATTCTCAT-3') to the SSUrRNA gene of *Plasmodium* was used in the primary PCR, and a pair of primers P1 and Y1 (5'-AAGGAAGCAATCTAAGATTCCCC GG-3'), the latter primer being specific for *P. y.*

nigeriensis was used for the nested PCR. The length of the targeted genome obtained by amplification was predicted to be 106 bp.

For the design of the primer Y1 on the murine malaria parasites, *P. y. nigeriensis* template DNA from infected blood was amplified with the P1 and P2 primers, then, the primary PCR product was sequenced. By comparing the sequences of the SSUrRNA genes for human *Plasmodium* (McCutchan *et al*, 1988; Goman *et al*, 1991; Arai *et al*, 1994; Quari *et al*, 1994) and *P. y. nigeriensis* in the present study, the Y1 primer was designed.

Murine DNA detection in the mosquitos

Detection of host murine (mouse) DNA in each individual mosquito was as follows: murine DNA obtained as described above was directly amplified in a total volume of 50 µl. The reaction mixture contained 4 µl of 2.5 mM dNTPs, 5 µl of 10 mM PCR buffer, 34 µl of Milli-Q H₂O, 2 µl of 10 µM each of the primers H14542; (5'-GCAGCCCCTCAGAATGATATTTGTCCTC-3'), L14230; (5'-GCTTCCATCCAACATCT CAGCATGATG-3'), 1 µl of 2.5U/2 µl *Taq* polymerase and 2 µl of template DNA. Amplification took place in the thermal cycler as follows: denaturation at 94°C for 2 minutes, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. Thirty-four cycles were carried out, with 1 minute extra for denaturation in the first cycle. Amplification with these primers targets a region of the cytochrome b gene for murine mitochondrial DNA. A concentration method was then used on the PCR product in order to obtain murine DNA as follows. One tenth volume of 5M NaCl and three volumes of 100% ethanol were added to the PCR product, then the mixture was vortexed and incubated at room temperature for 10 minutes. Then, the samples were centrifuged for 30 minutes at 12,000 rpm and the ethanol was carefully removed. The supernatant was washed with 75% ethanol and left to stand at room temperature for 10 minutes. Murine DNA was pelleted by centrifugation at 12,000 rpm for 30 minutes at 4°C. Ethanol was carefully removed and the pellet was re-suspended in 10 µl TE to re-dissolve the DNA. Murine DNA for each mosquito parts (head/thorax and abdomen) was identified by visualization of the appropriate band size in the agarose gel after electrophoresis. The gels were stained with

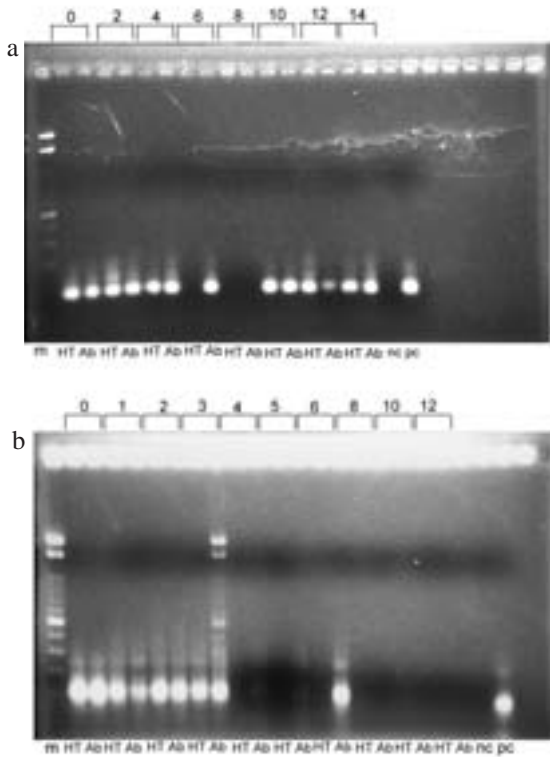


Fig 1—Example of the results from the nested PCR amplification of *Plasmodium yoelii nigeriensis* DNA in (a); mosquito vector *Anopheles soperoi* and (b); mosquito non-vector *Aedes albopictus*. Numbers above the tracks represent days post-infectious blood meal; m: DNA molecular marker; HT: head/thorax; Ab: abdomen; nc: negative control; and pc: positive control. The average number of mosquitos examined in each day was 20 for *An. soperoi* and 10 for *Ae. albopictus*. Electrophoresis was performed on a 2% agarose gel.

ethidium bromide and the band sizes were visualized by UV transillumination. Samples of mosquitos fed with sugar solution were used as negative controls.

RESULTS

Detection of *Plasmodium yoelii nigeriensis* and host murine DNA as a function of time in mosquitos

Mosquito specimens treated by a modified Chelex method, followed by boiling, resulted in a decreased concentration of *Taq* polymerase in-

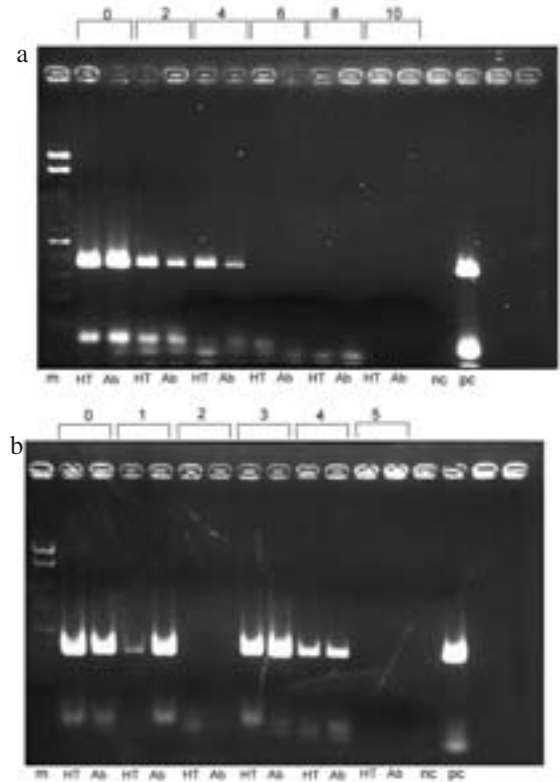


Fig 2—Detection of host murine DNA in (a): mosquito vector *Anopheles soperoi* and (b): mosquito non-vector *Aedes albopictus*, as a function of time post-infectious blood meal on a 2% agarose gel. m: DNA molecular marker, the numbers above the tracks represent days; HT: head/thorax; Ab: abdomen; nc: negative control; and pc: positive control.

hibitor, present in mosquitos, causing efficient PCR amplification. The nested PCR generated product of appropriately predicted size for *P. y. nigeriensis*. A 106 bp fragment corresponding to the predicted size of the primer-directed PCR was detected in the mosquito vector *Anopheles soperoi* and mosquito non-vector *Aedes albopictus* (Figs 1, 2). The courses of *P. y. nigeriensis* amplifications in vector and non-vector mosquitos were monitored as a function of time post-blood meal. On day 0, the detection rate of *P. y. nigeriensis* was 100% in head/thorax and abdomen for both vector and non-vector mosquitos (Figs 3, 5). These amplifications were most likely due to the presence of parasite DNA in the ingested blood meal, because analysis of the host murine DNA

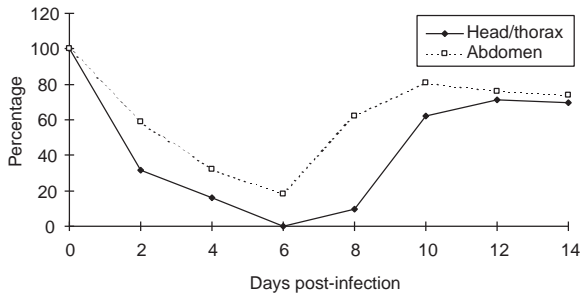


Fig 3—Percentage of head/thorax and abdomen parts of *Anopheles soperi* where *Plasmodium yoelii nigeriensis* DNA was detected by nested PCR.

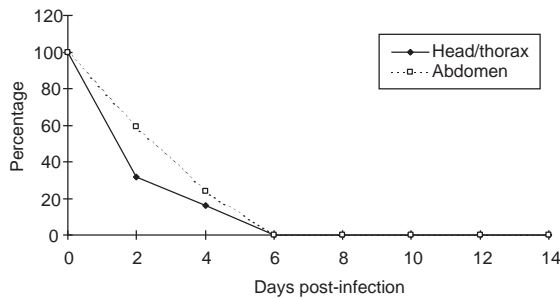


Fig 4—Percentage of head/thorax and abdomen parts of *Anopheles soperi* where host murine DNA was detected by nested PCR.

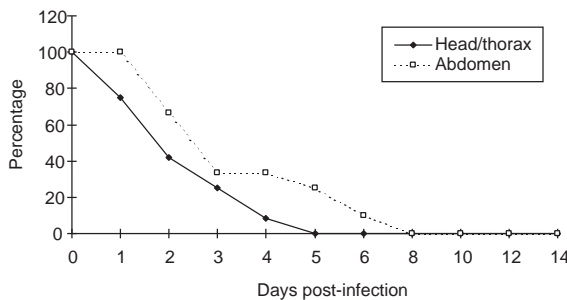


Fig 5—Percentage of head/thorax and abdomen parts of *Aedes albopictus* where *Plasmodium yoelii nigeriensis* DNA was detected by nested PCR.

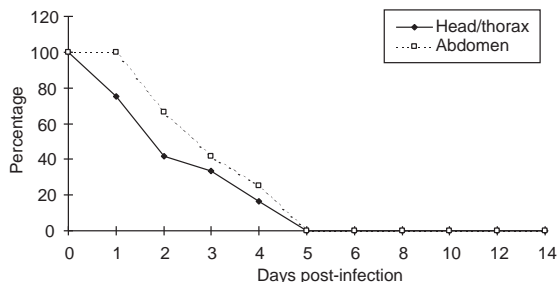


Fig 6—Percentage of head/thorax and abdomen parts of *Aedes albopictus* where host murine DNA was detected by nested PCR.

in each individual mosquito invariably confirms a detection rate of murine DNA in 100% head/thorax and abdomen for both the vector and non-vector mosquitos (Figs 4, 6). Surprising findings were the detection of parasite DNA in the abdomen of 25% (3/12) *Ae. albopictus* on day five and 10% (1/10) of on day six post-infectious blood meal, (Fig 5) even after murine DNA had fallen below detectable limits (Fig 6). Parasite DNA was not detected in the head/thorax and abdomen of *Ae. albopictus* 8-14-days post-infectious blood meal, suggesting that the parasite DNA detected on days 5 and 6 were in a degenerating stage, and did not develop into mature oocysts and sporozoites (Fig 5).

Parasite DNA was continuously detected in head/thorax and abdomens of mosquito vector *An. soperi* for up to 14-days post-infectious blood meal, even after 4 days, when murine DNA had fallen below detectable limits. The detection rates of parasite DNA in head/thorax and abdomen of *An. soperi* on day 14 post-infectious blood meal were 69.56% (16/23) and 73.91%, respectively (Fig 3). This demonstrates that there was a continuous presence and development of murine malaria parasites in the mid-guts and salivary glands of the mosquito vector *An. soperi*. Although murine DNA had fallen below detectable limits, the detection rate for parasite DNA in the head/thorax of *An. soperi*, increased gradually from day 8 post-infection until it reached its maximum level of 71.4% (15/21) 12-days post-infection. On the other hand, parasite DNA in abdomen reached its maximum level of 81% (17/21) 10-days post-infectious blood meal (Fig 3). In both vector and non-vector mosquitos, positive samples for *P. y. nigeriensis* on days 0-4 were also found to be positive for murine DNA (Figs 4, 6). Parasite DNA was not detected in vector and non-vector mosquitos fed with the blood of uninfected mice.

Murine DNA was analyzed in head/thorax and abdomen parts for each individual mosquito. PCR amplified products for *P. y. nigeriensis* from murine erythrocytes present in the blood meal gave the sign of predicted size. On day 0, the detection rate of murine DNA in the head/thorax and abdomen of both the vector and non-vector mosquitos was 100% (Figs 4, 6). In both *An. soperi* and *Ae. albopictus* mosquitos, murine

DNA was detected up to 4 days post-infectious blood meal. Murine DNA fell below detectable limits 4 days post-feeding, suggesting that the mosquito takes at least 4 days to completely digest an ingested blood meal present in the abdomen. PCR amplification products corresponding to murine DNA present in both the vector and non-vector mosquitos on day 0 post-infectious blood meal gave intensely-staining bands of bulky size, indicating the presence of a high content of blood meal in the mosquitos. However, the band intensity decreased gradually as a function of time post-infectious blood meal, suggesting continuous digestion of the blood meal in the mosquitos (Fig 2). Mosquitos fed only with sucrose solution in each assay consistently yielded negative results for murine DNA.

DISCUSSION

The measurement of sporozoite rates in malaria mosquito vectors is of fundamental significance in the design of malaria control programs. Detection of malaria parasites in mosquito vectors has traditionally depended on the dissection of mid-gut oocysts and salivary sporozoites. The problems of incriminating malaria vectors by dissection have been reviewed previously, and include the difficulty of sporozoite species identification and labor-intensive methodology (Zavala *et al*, 1982; Burkot *et al*, 1984). PCR amplification methods avoid these difficulties. Amplification of specific gene sequences by PCR has become increasingly utilized as a detection and identification tool of malaria parasites. (Schriefer *et al*, 1991). The PCR method has been used to detect parasites in blood samples (Snounou *et al*, 1993). However, few studies have addressed the detection and identification of malaria parasites in mosquito vectors. (Ranford-Cartwright *et al*, 1991). Recently PCR hybridization of specific DNA probes for detecting malaria parasites in mosquito vectors has been reported. Although the DNA probes are species-specific and can be used for a large number of samples, their sensitivity remains low and the use of isotope-labeled probes limits its application in field surveys (Li *et al*, 2001). Perhaps for these reasons, very few studies have addressed the detection and development of malaria parasites in mosquitos using isotope-

labeled probes.

The protocols that are currently in use for the extraction of malaria parasite DNA from infected mosquitos are cumbersome and favor pooled samples (Schriefer *et al*, 1991). We modified the Chelex method for extraction of DNA from infected mosquitos and subsequently performed PCR amplification of malaria parasites and murine DNA from the head/thorax and abdomen of infected mosquitos. Simple and affordable methods are essential for detection and identification of human malaria in epidemiological studies. In our study, parasite DNA products corresponding to the predicted band size for the primer-directed PCR were not detected in mosquitos fed with blood from uninfected mice or sucrose solution. The ability of our technique to detect the presence of parasite DNA in ingested blood meals and parasite DNA due to mosquito infection makes it very useful for the analysis of wild collected malaria mosquito vectors.

Inhibition of *Taq* polymerase-catalyzed PCR by the exoskeleton of mosquitos has been reported (Li *et al*, 2001). In our study, treatment of the mosquito samples with our modified Chelex method, followed by boiling the mosquito samples in a water bath prior to PCR amplification enabled us to remove the inhibitors from the exoskeletons of the mosquitos, and therefore enhanced the PCR amplification of the gene sequences. Our modified Chelex treatment of mosquito samples offers a simple alternative to traditional procedures for malaria parasite DNA extraction. When followed by DNA amplification of genes of interest, this method provides a highly sensitive means for detecting and identifying mosquitos infected with malaria parasites and a ready supply of DNA template for nested PCR analysis.

Electrophoresis detection of parasite DNA in mosquitos was parallel with the presence of either PCR amplifiable parasite DNA in the mosquito mid-gut and salivary glands or parasite DNA from murine erythrocytes present in the blood meal (Figs 3-6). Thus, it was possible to temporally follow the development of murine malaria parasites in the mosquito vector *Anopheles soperi*. Positive samples due to parasite DNA present in the blood meal were detected by the

analysis of murine DNA in each individual head/thorax and abdomen of the mosquitos. Analysis of murine DNA helps to differentiate false positive nested PCR amplification due to parasite DNA present in the blood meal from true positive PCR amplification due to mosquito infection.

In malaria endemic areas, female anopheline mosquitos have a chance to bite persons infected with malaria. Not all malaria mosquito vectors that bite humans infected with malaria become infected (with mid-gut oocysts) or infective (with salivary sporozoites). In this experiment, *An. soperi* infection rates for salivary sporozoites and mid-gut oocysts were 71.4% (15/21) and 81% (17/21), respectively (Fig 3). These observations are consistent with other studies reporting the sporozoite and oocyst infection rates of *An. soperi* infected with *Plasmodium yoelii nigeriensis* as 66.7% and 91.7%, respectively (Tsuzuki *et al*, 2001). A striking observation was the detection of parasite DNA in the abdomen of 25% (3/12) of the non-vector mosquito *Aedes albopictus* on day five and 10% (1/10) of on day six after murine DNA had fallen below detectable limits (Fig 5). It seems that the parasite DNA detected in the abdomen on days 5 and 6 was in a degenerating stage, because there was no detection of parasite DNA after day 6, indicating that the parasite detected on those days did not develop into mature oocysts and sporozoites.

A vital findings of the simultaneous PCR amplification of malaria parasite DNA and host murine DNA in the malaria mosquito vector is that parasite DNA was detected in the abdomen of 18.8% (4/22) of *An. soperi* on day 6 post-infectious blood meal, but it was not detected in head/thorax of the same mosquito (Figs 1a, 3). This observation enables us to distinguish between infective mosquitos with salivary sporozoites and infected mosquitos with mid-gut oocysts. This study also demonstrates that the sporozoites in *An. soperi* occur in the salivary glands 8 days post-infectious blood meal (Fig 3). The accuracy of measurement of sporozoite rates in the mosquito population is of fundamental importance, as it reflects the malaria transmission potential in a given location. We found that our technique was simple and may provide valuable tools for the estimation of malaria mosquito in-

fection rates in epidemiological studies.

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