POSSIBILITY OF FALSE-POSITIVE DETECTION FOR SPOROZOITES IN MOSQUITOS (DIPTERA: CULICIDAE) BY NESTED POLYMERASE CHAIN REACTION USING *PLASMODIUM YOELII* GENOMIC DNA

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Abstract. *Anopheles stephensi* Liston and *An. saperoi* Bohart and Ingram infected with the rodent malaria parasite *Plasmodium yoelii nigeriense*. They were examined 12 and 19 days after blood feeding for sporozoites in head with anterior thorax (HT) and oocysts in abdomen with posterior thorax (AB) by light microscopy and by the nested polymerase chain reaction (nested PCR-based on the amplification of the sequences of the small subunit ribosomal RNA gene). The detection rate of parasite DNA by nested PCR in HT samples 12 days after blood feeding was similar to that by microscopic method. However, in HT samples 19 days after blood feeding, the rate by the PCR method was higher than that by the microscopic method.

The incidence of sporozoites in salivary glands of infected mosquitos for 12 days after blood sucking was examined by the PCR method. Parasite DNA in HT of *Aedes albopictus* Skuse (a non vector for the rodent malaria) as well as *An. stephensi* and *An. saperoi* was detected for up to 4 days after feeding on mouse with the rodent malaria parasites. The results indicate that when the PCR method is used for detection of sporozoites of human malaria in mosquitos collected in the field, there are possibilities of including false-positive data for mosquitos that have just or recently fed on human blood infected with malaria (erythrocytic form).

INTRODUCTION

The oocysts of *Plasmodium yoelii* first appear on day 3-4 in midgut, and the sporozoites on day 8 in the salivaliry glands and heads of *Anopheles saperoi* Bohart and Ingram and *An. stephensi* Liston (Toma *et al*, 2001a). Microscopic examination has been used routinely to detects malaria parasites in human and rodent blood smears and infected mosquitos. This method depends on the skill of the observer and the time for examination is limited because parasites are not detected in dried specimens. Therefore, a simple, convenient, sensitive and species specific method has been developed for detection of malaria parasites in mosquito and human blood specimens.

Polymerase chain reaction (PCR) has been used to detect human malaria (Kain and Lanar, 1991; Snounou *et al*, 1993a,b, Singh *et al*, 1996) using a small subunit ribosomal RNA (SSUrRNA) gene (Snounou *et al*, 1993 c; Li *et al*, 1995; Kimura *et al*, 1997). Parasite DNA is amplified easily because the SSUrRNA gene is in a region of multicopy, and is highly sensitive for detection of the human malaria parasite species. Recently, the SSUrRNA gene nested PCR method has been established as a convenient, species specific and highly

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sensitive method for detection of the human malaria parasites from blood samples (Kimura *et al*, 1997). This application to blood samples has demonstrated that the prevalence of malaria parasites can be underestimated by microscopy (Toma *et al*, 2001a,b). Wilson *et al*, (1998) confirmed the sensitivity and specificity of the SSUrRNA gene-based nested PCR in the detection of sporozoites in the salivary glands of mosquitos.

We report here results comparing the sensitivities of microscopy and SSUrRNA gene-based nested PCR techniques in the detection of rodent malaria sporozoites and oocysts. To determine the incidence of sporozoites in salivary gland of infected mosquitos after blood sucking, mosquitos were separated surgically into 2 parts, and the PCR for these parts was performed separately.

MATERIALS AND METHODS

Mosquitos

Anopheles saperoi, *An. stephensi* and *Aedes albopictus* (Skuse) were maintained in an insectary at 25±1ºC, 80% RH and a photoperiod of 16:8 (L:D). *An. saperoi* and *Ae. albopictus* were collected originally from Yona River in Kunigami Village and Naha City, Okinawa, Japan, in 1999, respectively. The Beech strain of *An. stephensi* was originally derived from Delhi, India in 1947, and was maintained at London School of Hygiene and Tropical Medicine, England. The strain was obtained by Toyama Medical and Pharmaceutical School via the School of Medicine, Teikyo University, Japan.

Malaria parasite

The N67 strain of *P. yoelii nigeriensis* was isolated in Nigeria in 1967 and maintained at the London School of Hygiene and Tropical Medicine. The strain was obtained from Toyama Medical and Pharmaceutical School via Teikyo University.

Infected mosquito samples

P. yoelii nigeriense parasitemia and gametocytemia in each ICR mouse used to blood feed mosquitos ranged from 2-11% and 0.01-0.1%, respectively. *An. saperoi* were fed when 3-7 days old, while *An. stephensi* and *Ae. albopictus* were 3-5 days old. Fully engorged female mosquitos were maintained for 0-19 days after feeding.

Light microscopy method for the detection of oocysts or sporozoites in mosquitos and treatment of dissected samples for PCR amplification of parasite DNA

Mosquitos were anesthetized by ethylethel and separated into 2 portions under an aseptic condition: head with anterior thorax (HT) and abdomen with posterior thorax (AB). Parasite DNA was extracted from both portions. Salivary glands from HT samples were examined microscopically for sporozoites (x 400), and the midguts from AB samples were examined for oocysts. After examination, HT and AB samples were placed into a tube with 15 ml ethanol. Centrifugation was performed for 10 minutes at 2,000 rpm and the supernatant discarded. The sediment (about 200 µl) was dried several days at room temperature.

Parasite genomic DNA was extracted using the procedure of Martin *et al* (1991). The dried sample was homogenized lightly in 10 µl of 0.9% NaCl. 240 µl of preheated (100ºC in heating block) 5% Chelex 100 were added and mixed. After heating for 10 minutes, it was centrifuged at 6,000 rpm for 5 minutes. The supernatant was utilized directly as template in PCR or was stored at -20ºC until PCR was carried out. For dried and frozen mosquitos, the extraction of DNA was carried out in the same way as shown above.

PCR amplification of DNA and development of Y1 primer for detecting rodent malaria parasite

PCR was performed by the method of Kimura *et al* (1997) with modification. In our study, a pair of complementary primers P1 (5'- ACGATCAGATACCGTCGTAATCTT-3') and P2 (5'-GAACCCAAAGACTTTGATT

TCTCAT-3') to SSUrRNA gene of *Plasmodium* was used for primary PCR, and a pair of primers P1 and Y1 (5'-AAGGAAGCAAT CTAAGATTCCCCGG-3') was used for nested PCR (*P. yoelii nigeriense* specific).

For developing the primer Yl on the rodent malaria parasite, the *P. yoelii nigeriense* infected blood was amplified with P1 and P2 primers, then the primary PCR product was sequenced. By comparing the sequences of the SSUrRNA genes of the human plasmodia (Arai *et al,* 1994; McCutchan *et al,* 1988; Goman *et al,* 1991; Quari *et al,* 1994) and *P. yoelii nigeriense* in the present study, Y1 primer was designed as 5'-AAGGAAGCAATCTAAGATTCCCCGG-3'.

The component and volume of solution for PCR followed those of Kimura *et al* (1997). A 20 μl reaction mixture containing 2 μl template DNA was placed in a DNA thermal cycler (PTC-100, MJ Research, USA) at 92ºC for 2 minutes of initial denaturation. This was followed by amplification for 28 cycles of 30 seconds annealing at 92ºC, 90 seconds extension at 60ºC and a final cycle with an extension time of 5 minutes at 60ºC. Following the PCR, 10 μl of each sample was electrophoresed on 2% agarose gel and stained with ethidium bromide. The length of the targeted genome obtained by amplification was predicted to be 106 base pairs.

RESULTS AND DISCUSSION

DNA product corresponding to the predicted size of the primer-directed PCR was not detected in uninfected mouse blood samples in uninfected mosquitos maintained on sugar solution. The sensitivities of the nested PCR method for rodent malaria parasite DNA in mosquitos were compared with those by the conventional light microscope-based detection method (Tables 1, 2). In the first experiment, *An. saperoi* females fed on infected mice and maintained for 12 and 19 days were divided into 2 cohorts at random. The detections of oocysts and sporozoites by microscope were done in one cohort, and PCR was performed in the other cohort. At 12 days after feeding on infectious blood, the proportion of females with sporozoites in salivary glands detected by microscopic method was lower than that of HT detected with parasite DNA by PCR method. The detection rate of the parasite DNA by nested PCR was 66.7% in HT of dry and 91.3% of frozen mosquitos. By microscopic method, the rate (80.0%) of females with oocysts in the midgut was also lower than the rate (91.7% in dry and 95.7% in frozen mosquitos) of AB detected with DNA by PCR method. The sporozoite was not observed in the salivary glands of mosquitos on 19 days after infectious blood feeding by the microscopic method, but the parasite DNA was detected by the PCR method in 60% (3/5) of HT preserved at -20ºC (Table 1).

For direct comparison of microscopy and PCR, a total of 37 HT of *An. stephensi* on 12 days after feeding on mouse with malaria parasites was dissected and examined by light microscopy for the detection of sporozoites in salivary glands. After examination by microscopy, all mosquitos were analyzed by PCR. The parasite DNA was detected by PCR in all 15 HT found with sporozoites by microscopy. Furthermore, parasite DNA was amplified in 50% (11/22) of HT which was found to be negative for sporozoite by microscopy. In all 17 AB with or without oocysts as shown by microscopy, amplification products for parasite DNA were found in position of predicated size on agarose gel (Table 2). The results obtained in these 2 experiments indicated that 18SssrRNA gene-based nested PCR method is highly sensitive in the detection of sporozoites in HT samples.

At optimal temperature (24ºC), *P. yoelii nigeriense* sporozoites first invade the salivary glands of *An. stephensi* from 9 to 11 days after experimental infection (Killick-Kendric, 1973). The 1st oocysts have been observed on the outer side of midgut after 3 days of infection, and sporozoites are found on day 8 in the salivaliry glands and heads of *Anopheles saperoi* Bohart and Ingram and *An. stephensi* Liston at 25ºC (Toma *et al,* 2001a). These studies strongly suggest that

Table 1
Comparison of microscopic and PCR methods for the detection of *P. yoelii nigeriense* in *An. saperoi*.

Days after infectious blood feeding	Microscopic method			PCR method			
	No. of mosquitos examined	No. (%) of mosq with		No. of mosquitos examined	Preservation of mosq	No. (%) of	
		oocysts on midgut	sporozoites in salivary glands			AB[a] detected with parasite DNA	HT[b] detected with parasite DNA
12	40	32 (80.0)	26 (65.0)	24	Dry	22 (91.7)	16 (66.7)
				23	Frozen	22 (95.7)	21 (91.3)
19	8	7 (87.5)	0 (0)	5	Frozen	4 (80.0)	3 (60.0)

[a]AB: abdomen with posterior thorax, [b]HT: head with anterior thorax.

Table 2
Direct comparison of microscopic and PCR methods for the detection of *P. yoelii nigeriense* in the same samples of *An. stephensi*.

Head with anterior thorax (HT)				Abdomen with posterior thorax (AB)			
Microscopic method		PCR method		Microscopic method		PCR method	
No. of		No. of mosquitos				No. of mosquitos	
sporozoite observed	mosquitos examined	detected with parasite DNA	parasite DNA not detected	Oocysts	No. of mosq examined	detected with parasite DNA	parasite DNA not detected
Over 200	4	4	0	Observed	15	15	0
Up to 200	11	11	0				
None	22	11	11	None	2	2	0

sporozoite does not occur in the mosquitos at least for 4 days after infectious blood feeding.

We examined rodent malaria DNA in HT and AB portions of 2 species of mosquitos (*An. stephensi* and *Ae. albopictus*) 12 days after infectious blood feeding by PCR method (Tables 3, 4). In these experiments, it was found unexpectedly that some AB and HT samples of *An. stephensi* and even *Ae. albopictus* (a non-vector mosquito of rodent malaria), were positive for parasite DNA for 4 days after infectious blood feeding. These results demonstrated that some rodent malaria parasites were still present in undeveloped stage and not sporozoites in the esophagus and anterior midgut of mosquitos for several days after infectious blood feeding (the blood has been completely digested and matured eggs were found in ovaries of the abdomen).

We often have experienced that the females fed on a small amount of human blood and escaped before they were caught by human bait collection. Females collected in malarious areas indoor and outdoor include physiologically and parasitically different individuals, such as nuri- or prous, blood fed on malaria patient within or over 4 days after feeding and containing erythrocytic forms, oocysts and sporozoites of *Plasmodium* parasites or not. As shown in the paper, *Plasmodium yoelii* was still present as an undeveloped stages (erythrocytic form) even in the

Table 3
Detection of *P. yoelii nigeriense* in *Ae. albopictus* by PCR method.

Days after infectious blood feeding	No. of mosquitos examined	No. (%) of sample	
		AB[a] detected with parasite DNA	HT[b] detected with parasite DNA
0	15	15 (100)	14 (93.3)
1	12	12 (100)	8 (66.7)
2	10	7 (70.0)	4 (40.0)
3	10	3 (30.0)	3 (30.0)
4	10	2 (20.0)	1 (10.0)
5	10	0 (0)	0 (0)
7	5	0 (0)	0 (0)
9	7	0 (0)	0 (0)
12	15	0 (0)	0 (0)

[a]AB: abdomen with posterior thorax, [b]HT: head with anterior thorax

Table 4
Detection of *P. yoelii nigeriense* in *An. stephensi* by PCR method.

Days after infectious blood feeding	No. of mosquitos examined	No. (%) of sample	
		AB[a] detected with parasite DNA	HT[b] detected with parasite DNA
0	10	10 (100)	10 (100)
2	10	3 (30.0)	3 (30.0)
4	15	11 (73.3)	3 (20.0)
6	15	9 (60.0)	2 (13.3)
8	15	3 (20.0)	1 (6.7)
11	22	18 (81.8)	16 (72.7)

[a]AB: abdomen with posterior thorax, [b]HT: head with anterior thorax

non-vector mosquitos for 4 days after feeding infectious blood; after that they disappear completely in the esophagus and midgut. Usually in the salivary glands of excellent laboratory vector mosquitos, such as *An. stephensi* and *An. saperoi*, the sporozoites of the rodent malaria appear on day 8 after feeding (Toma *et al*, 2001a). If the PCR method is used for detection of human malaria sporozoites in the mosquitos collected in malarious area, it may detect the erythrocytic form of *Plasmodium* still present in a small amount of blood that remained in the head and anterior thorax, even though the abdomen with posterior thorax (blood meal in stomach and esophagus) has been removed. The PCR method has high sensitivity in detection of malaria parasites as reported by many scientists (Li *et al*, 1997; Thompson *et al*, 1999; Vythilingam *et al*, 1999). When it is used for detection of sporozoites in mosquitos collected in the field, we should bear in mind that there are possibilities of including false-positive data for mosquitos that have only recently fed on human blood infected with malaria (erythrocytic form).

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