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Abstract

We developed a sensitive polymerase chain reaction (PCR) method for the detection of Plasmodium falciparum DNA from mosquitoes collected in the field. Plasmodium falciparum was detected from 15.2% of 1-parous mosquitoes, Anopheles farauti, in the Solomon Islands through use of the PCR method. A novel mathematical model was developed to estimate the sporozoite rate based on the malaria-positive rate of 1-parous mosquitoes. Using this model, the sporozoite rate of Anopheles farauti in the Solomon Islands was calculated to be 0.09%. This method enables estimation of the sporozoite rate based on a relatively small number (100-200) of mosquitoes compared with the number needed for the ELISA method.

KEYWORDS: sporozoite rate, polymerase chain reaction (PCR), mathematical model, Anopheles, Plasmodium

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Estimation of the Sporozoite Rate of Malaria Vectors Using the Polymerase Chain Reaction and a Mathematical Model

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We developed a sensitive polymerase chain reaction (PCR) method for the detection of *Plasmodium falciparum* DNA from mosquitoes collected in the field. *Plasmodium falciparum* was detected from 15.2% of 1-parous mosquitoes, *Anopheles farauti*, in the Solomon Islands through use of the PCR method. A novel mathematical model was developed to estimate the sporozoite rate based on the malaria-positive rate of 1-parous mosquitoes. Using this model, the sporozoite rate of *Anopheles farauti* in the Solomon Islands was calculated to be 0.09%. This method enables estimation of the sporozoite rate based on a relatively small number (100-200) of mosquitoes compared with the number needed for the ELISA method.

Key words: sporozoite rate, polymerase chain reaction (PCR), mathematical model, *Anopheles, Plasmodium*

Molecular biological methods (1-5) instead of the microscopic examination of thin blood films have been reported as a means to improve the specificity and sensitivity of the diagnosis of human malaria. On the other hand, it is rather difficult to detect the malaria parasite in blood-engorged mosquitoes using the polymerase chain reaction (PCR) method. More complex systems (2, 3, 6-8) are therefore required to detect PCR products. Sporozoite detection methods using RT-PCR have recently been reported (9-11), but these methods have not yet been applied to field studies.

The aim of this study was to apply molecular biological methods to the ecological study of malaria vectors. In tropical areas, a great effort is needed to collect a sufficiently large number of mosquitoes for estimating the sporozoite rate of the vector. In this paper, we describe a sensitive PCR method for detecting *Plasmodium falciparum* (*P. falciparum*) DNA from blood-fed mosquitoes. We then present a method for estimating the sporozoite rate from a small number of mosquitoes that uses a combination of the PCR method and a novel simulation model. Using this model, the sporozoite rate of *Anopheles farauti* (*An. farauti*) in the Solomon Islands was estimated.

Materials and Methods

PCR. *Anopheles stephensi* were fed on cultured *P. falciparum* (FCO-1 strain) by membrane feeding (12) in experimental infection. One day after feeding, the blood-fed mosquitoes were individually crushed onto autoclaved filter paper and dried. Mosquitoes, *An. farauti*, collected by net sampling at a village near Honiara in the Solomon Islands were also treated using the same method. The mosquitoes together with the filter paper were cut into pieces and put into microtubes containing 240 μl 5% Chelex solution (2, 13), and the tubes were then boiled for 10 min. The supernatant was used as template DNA. PCR was performed using the specific primers designed from *P. falciparum* DHFR-TS gene (4) (Fig. 1). The products were analyzed by agarose gel electrophoresis. One PCR product was sequenced to ascertain the precise amplification achieved.

Primers: Primer 1 (5'-TGT TAG CGA TGT ATA TAC TAG-3') and primer 2 (5'-TTC GAT CAC TTT GTT TAT TTC CAT T-3') amplifying 410-bp nucleotides were used in methods 1, 2, and 3. Primer 3 (5'-AAT GAT GAC AAA GAT ACA TG-3') and

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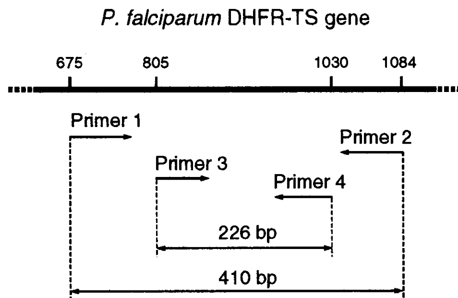

Fig. 1 Primers and amplification regions of *P. falciparum* dihydrofolate reductase (DHFR) - thymidylate synthase (TS) gene (4) (based on the nucleotide sequences of GenBank Accession number J03028).

primer 4 (5'-ATT GGT ATT CAG GAT GAT AT-3) amplifying 226-bp nucleotides were used in the 2 nd PCR of methods 1 and 3.

Method 1: Nested PCR using both sets of *P. falciparum*-specific primers (4). The PCR mixture (total 50 μl) contained 200 μM dNTP, Taq polymerase (Amplitaq, Perkin-Elmer Cetus, Norwalk, CT) 1.25 units, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 μM of each primer in the first PCR (1 μM in the 2nd PCR), and 2 μl template DNA (1 μl in the 2nd PCR). Twenty-one reaction cycles were performed as follows: 94 °C for 1.5 min, 60 °C for 1.5 min, and 72 °C for 2 min using primers 1 and 2. The 2 nd PCR was performed for 35 cycles of 94 °C for 1.5 min, 58 °C for 1.5 min, and 72 °C for 1.5 min using primers 3 and 4. After the last cycle the sample was stored at 4 °C.

Method 2: PCR with a longer extension period (6). The PCR mixture (total 50 μl) contained 200 μM dNTP, Taq polymerase 2.5 units, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 1 μM of each primer (primers 1 and 2), and 1 μl template DNA. Thirty-five cycles of PCR were performed as follows: 94 °C for 1.5 min, 58 °C for 2 min, and 72 °C for 4 min. After the last cycle the sample was stored at 4 °C.

Method 3: Nested PCR combining methods 1 and 2 with a longer extension in the first PCR. The PCR mixture (total 50 μl) contained 200 μM dNTP, Taq polymerase 2.5 units (1.25 units in the 2nd PCR), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 0.2 μM of each primer in the first PCR (1 μM in the 2nd PCR), and 2 μl template DNA (1 μl in 2nd PCR). Thirty PCR cycles were performed under the conditions of 94 °C for 1.5 min, 60 °C for 2 min, and 72 °C for 4 min using primers 1 and 2. The 2nd PCR was performed under the conditions of 35 cycles of 94 °C for 1.5 min, 58 °C for 1.5 min, and 72 °C for 1.5 min. Following the last cycle, the sample was stored at 4 °C.

Samples (10 μl) of the PCR products were electrophoresed in 2% agarose 1600 gel, stained by ethidium bromide, and photographed.

Model. We constructed a model of malaria infection in mosquitoes in order to estimate the sporozoite rate in a population of mosquitoes. For construction of the model, the following entomological assumptions and data regarding *An. farauti* and *P. falciparum* (*Pf*) based on references in Papua New Guinea or the Solomon Islands were adopted for practical reasons:

1. The age distribution in the mosquito population is stable.
2. The mosquito survival rate per day is stable after emergence.
3. Mosquitoes start to feed 4 days after emergence (14).
4. The oviposition cycle is 2 days (15).
5. Engorged DNA (malarial DNA except gametocytes) is detected within 4 days (16).
6. Gametocytes require 13 days for full development to sporozoites after ingestion (17).

The following 2 epidemiological assumptions were also made:

7. The ingestive rate of *Pf* in biting mosquitoes is the same as the parasite rate of *Pf* in humans.
8. The ingestive rate of *Pf* gametocytes in biting mosquitoes is the same as the parasite rate of *Pf* gametocytes in humans.

The following entomological and epidemiological parameters were used:

p : the survival rate of mosquitoes per day
α : the biting rate per day per mosquito
ε : the parasite rate of *Pf* in humans
λ : the parasite rate of *Pf* gametocytes in humans having *Pf*.

The number of days that have passed since emergence is denoted by t, and the number of oviposition times is denoted by s. In this malaria infection model, the mosquito population is divided into the following classes:

$a(s,t)$: having the ability to bite
  $a(s,t)$ is divided into the following 2 classes.
  $an(s,t)$: *Pf*-negative
  $ap(s,t,i)$: *Pf*-positive, *Pf* gametocyte-negative ($s \geqq 1,\ i = 2,3$)

$b(s,t)$: being unable to bite

$b(s,t)$ is divided into the following 2 classes.

$bn(s,t,i)$: *Pf*-negative, $(i = 0,1)$

$bp(s,t,i,j)$: *Pf*-positive, *Pf* gametocyte-negative $(i = 0,\cdots,3;\ j = 0,1)$

$opg(s,t)$: having developing *Pf* (gametocyte or ookinete or oocyst)

$opg(s,t,i)$: *Pf*-positive $(s \geqq 1,\ i = 2,\cdots,12)$

$cs(s,t)$: having sporozoites $(s \geqq 1)$,

where i denotes the number of days that have passed since the mosquito acquired *Pf* or *Pf* gametocytes, and j denotes the number of days that have passed since biting a human. As the values of $opg(s,t,i)$ and $cs(s,t)$ are much smaller than the other values, we use in the formulation of our model the sums over all stages s instead of $opg(s,t,i)$ and $cs(s,t)$, *i.e.*,

$$opg(t,i) = \sum_{s=1}^{\infty} opg(t,i)\ ,\quad cs(t) = \sum_{s=1}^{\infty} cs(t).$$

Our malaria infection model can be determined using the definitions and assumptions described above. The scheme of the model is shown in Fig. 2. The solid arrows in Fig. 2 represent the items of summation of $an(s,t+1)$; *i.e.*,

$$an(s,t+1) = p(1-\alpha)\{an(s,t) + bn(s-1,t,1) + ap(s-1,t,3) + bp(s-1,t,3,1)\}.$$

The broken arrows (1) represent the items of summation of $bn(s,t+1)$; *i.e.*,

$$bn(s,t+1) = p\alpha(1-\varepsilon)\{an(s,t) + bn(s-1,t,1) + ap(s-1,t,3) + bp(s-1,t,3,1)\}.$$

The broken arrows (2) represent the items of summation of $bn(s,t+1)$; *i.e.*,

$$bn(s,t+1,1) = p\{bn(s,t,0) + bp(s,t,3,0)\}.$$

bp, bpg, ap, opg, and *cs* are summed likewise, and they are expressed in the following recurrence formulae:

for $t = 0,1,2$,

$$an(0,t+1) = p\ an(0,t).$$

for $t \geqq 3$,

$$an(s,t+1) = p(1-\alpha)\{an(s,t) + bn(s-1,t,1) + ap(s-1,t,3) + bp(s-1,t,3,1)\}$$

$$bn(s,t+1,0) = p\alpha(1-\varepsilon)\{an(s,t) + bn(s-1,t,1) + ap(s-1,t,3) + bp(s-1,t,3,1)\}$$

$$bn(s,t+1,1) = p\{bn(s,t,0) + bp(s,t,3,0)\}$$

$$bp(s,t+1,0,0) = p\alpha\varepsilon(1-\lambda)\{an(s,t) + bn(s-1,t,1) + ap(s,t,2) + ap(s-1,t,3) + bp(s-1,t,1,1) + bp(s-1,t,3,1)\}$$

$$bp(s,t+1,i+1,0) = p\ bp(s,t,i,0) \qquad (i = 0,2)$$

$$bp(s,t+1,2,0) = p\alpha(1-\varepsilon)\ bp(s-1,t,1,1)$$

$$bp(s,t+1,3,0) = p\ ap(s,t,2)$$

$$bpg(s,t+1,0) = p\alpha\varepsilon\lambda\{an(s,t) + bn(s-1,t,1) + ap(s,t,2) + ap(s-1,t,3) + bp(s-1,t,1,1) + bp(s-1,t,3,1)\}$$

$$bpg(s,t+1,1) = p\ bpg(s,t,0)$$

$$ap(s,t+1,2) = p(1-\alpha)\ bp(s-1,t,1,1)$$

$$ap(s,t+1,3) = p(1-\alpha)\ ap(s,t,2)$$

$$opg(t+1,2) = p\sum_{s} bpg(s,t,1)$$

$$opg(t+1,i+1) = p\ opg(t,i) \qquad (2 \leqq i \leqq 11)$$

$$cs(t+1) = p\ cs(t) + p\ opg(t,12).$$

We denote the parasite rate of *Pf* in the whole population of mosquitoes by $R_{Pf,Total}$, the parasite rate of *Pf* in 1-parous mosquitoes (feeding on blood at least once and laying eggs once) by $R_{Pf,1P}$, and the sporozoite positive rate (sporozoite rate) in the whole population of mosquitoes by $R_{Sporozoite}$. In the above notations, those parasite rates are given in the following formulae:

$$R_{Pf,1P} = \sum_{t=6}^{\infty}\left[\sum_{j=0,1}\left\{\sum_{i=0}^{3} bp(1,t,i,j) + bpg(1,t,j)\right\} + \sum_{i=2,3} ap(1,t,i) + \sum_{i=2}^{12} opg(1,t,i)\right] \Bigg/ \sum_{t=6}^{\infty}\left[an(1,t) + bn(1,t) + \sum_{j=0,1}\left\{\sum_{i=0}^{3} bp(1,t,i,j) + bpg(1,t,j)\right\} + \sum_{i=2,3} ap(1,t,i) + \sum_{i=2}^{12} opg(1,t,i)\right]$$

$$R_{Pf,Total} = \sum_{t=4}^{\infty}\sum_{s=0}^{\infty}\left[\sum_{i=2,3} ap(s,t,i) + \sum_{i=2}^{12} opg(s,t,i) + \sum_{j=0,1}\left\{\sum_{i=0}^{3} bp(s,t,i,j) + bpg(s,t,j)\right\}\right](1-p)$$

$$R_{Sporozoite} = \sum_{t=14}^{\infty} cs(t)\ (1-p).$$

Now we set the initial conditions:

$an(0,0) = 1$, all other terms are equal to 0 at $t = 0$.

The system of our malaria infection model has been programmed by FORTRAN 90, and it works on any FORTRAN 90 compiler platforms. The model was solved by a simulation with the iteration day by day.

Results and Discussion

PCR. Three methods for detecting *P. falciparum* DNA in mosquitoes were tested. Using method 1 (4), 226-bp nested PCR products were obtained from all the mosquitoes one day after experimental feeding on *P. falciparum* (data not shown). This method detected no products from mosquitoes collected from the Solomon Islands (Fig. 3a). Using method 2 (2), the expected 410-bp products were detected from all of the mosquitoes one day after experimental feeding on *P. falciparum* (data

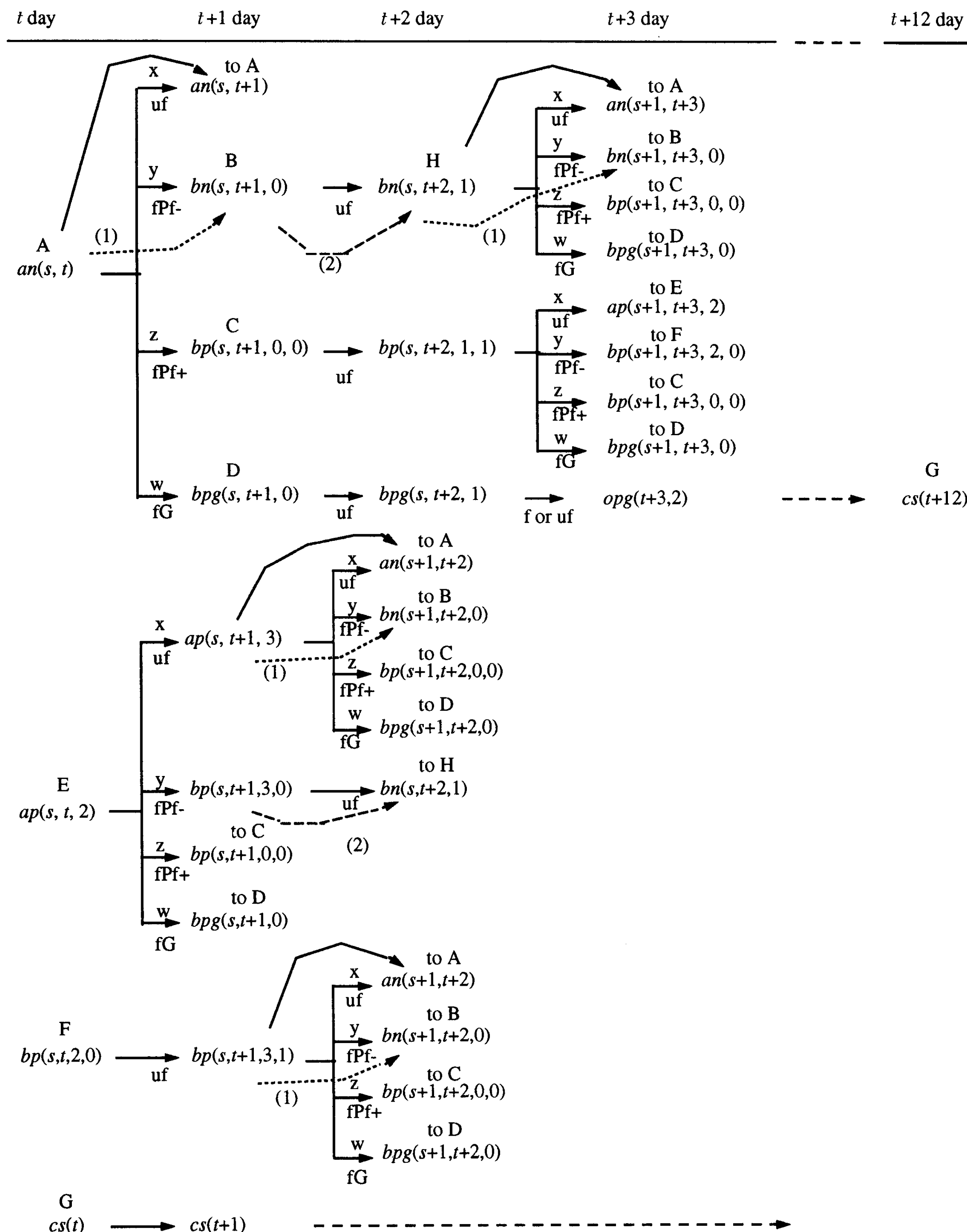

Fig. 2 A schema representing the mode in which mosquitoes feed on blood and ingest *P. falciparum* (*Pf*). Mosquito populations are divided into classes (See text for details on mosquito classes and parameters.). uf, non-feeding; fPf-, feeding on blood without *Pf*; fPf+, feeding on blood with *Pf* but without gametocytes; fG, feeding on blood with gametocytes. x, y, z and w represent each proportion on feeding, and $p = x + y + z + w$, $x = p(1-\alpha)$, $y = p\alpha(1-\varepsilon)$, $z = p\alpha\varepsilon(1-\lambda)$, and $w = p\alpha\varepsilon\lambda$. Each mosquito class with "to + capital letter" starts from the class with the same capital letter on the next day.

Harada et al.: Estimation of the sporozoite rate of malaria vectors using

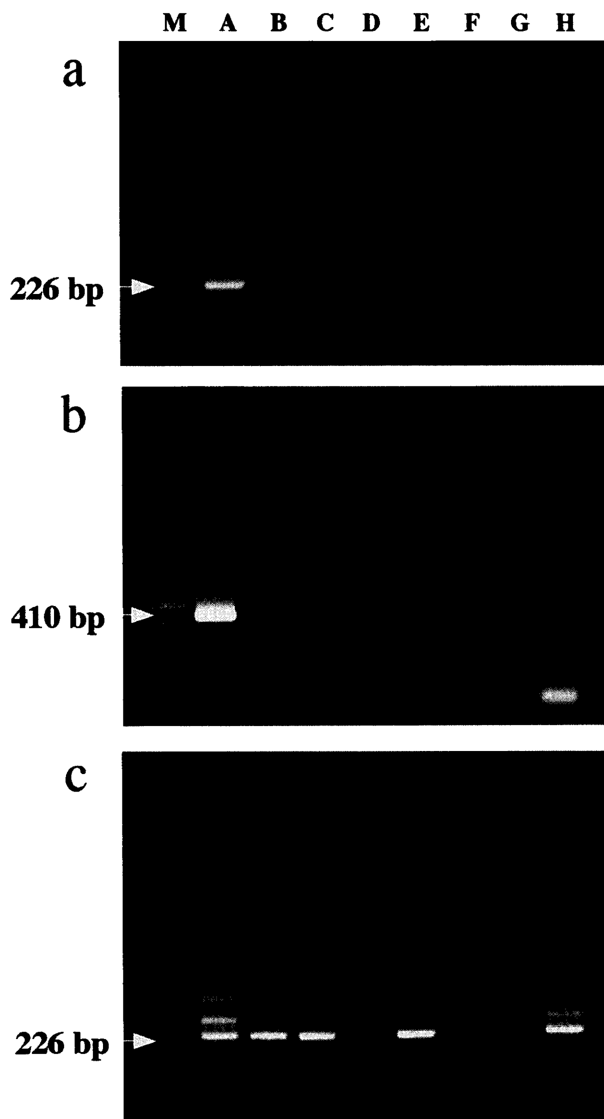

Fig. 3 Products of PCR using DNA from field-collected mosquitoes. **a**, results using simple nested PCR (method 1). Products were obtained from only the positive control. **b**, results using elongated PCR (method 2). Products were obtained from the positive control and 1 mosquito (lane G), though the 410-bp products in lane G were thought to be non-specific products. **c**, results using a combination of nested and elongated PCR (method 3). Products were detected from the positive control and 4 mosquitoes. No specific band was detected in lane G. M, marker (1000, 700, 525 + 500, 400, 300, 200, 100 and 50 bp); A, positive control; B-H, some of the mosquitoes collected from the Solomon Islands (the same individuals were examined in a, b and c).

not shown). One mosquito collected in the Solomon Islands produced the same 410-bp products (Fig. 3b). Using method 3, the expected 226-bp PCR products were detected from all of the mosquitoes one day after experimental feeding on *P. falciparum* (data not shown), from the positive control, and from 4 of 7 mosquitoes collected from the Solomon Islands (Fig. 3c).

One PCR product (lane B, Fig. 3c) was sequenced and this demonstrated that the primers were specific for the expected region of the DHFR-TS gene of *P. falciparum* (data not shown). The 410-bp products detected by method 2 (Fig. 3b, lane G) could not be sequenced by ordinary sequence procedures. In light of the results of method 3 (Fig. 3c) wherein that specific band was not detected in lane G, these products were thought to be non-specific.

Thus, although the sensitivities of these detection methods are difficult to determine precisely, the fact that the detection rate of *P. falciparum* from the same mosquitoes collected from the Solomon Islands was the highest by method 3 indicated that this method of detection has the highest sensitivity.

The limits of sensitivity in our experiments using DNA extracted from cultured *P. falciparum* were 40 pg (2,000 parasites in 2 μl; 0.02% parasitaemia) by method 1, 1 ng (50,000 parasites in 2 μl; 0.5% parasitaemia) by method 2, and 4 pg (200 parasites in 2 μl; 0.002% parasitaemia) by method 3. Our new method (method 3) was the most sensitive among the 3 methods and 10-times more sensitive than method 1. The sensitivity of method 1 was originally reported to be 0.000026% parasitaemia (10 parasites in 10 μl of blood) (4). The difference between the sensitivities of method 1 (0.02%) and the original method (0.000026%) (4) is thought to be due to the DNA extraction method and some modifications for its application to field-collected mosquitoes. If the PCR conditions in method 3 were applied after using the same sampling procedures as those of the original method, its sensitivity for the detection of malaria from human blood would be more than 0.000026% parasitaemia. The sensitivity of method 2 was almost the same as that originally reported (2).

The malaria-positive rate of all mosquitoes whose parities were determined (n = 127) were examined by PCR using method 3. Nineteen (15%) of the total 127 mosquitoes were determined to be *Pf*-positive, and 5 (15.2%) of 33 1-parous mosquitoes were *Pf*-positive.

Estimation of sporozoite rate. The parasite

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rate $R_{Sporozoite}$ of sporozoites is estimated on the basis of the parasite rate of *Pf*, which was determined by PCR. First, we determine the entomological and epidemiological parameters. For the daily survival rate p of mosquitoes, we take the mean parous rate (14):

$p =$ mean parous rate $= 1 -$ nulli-parous rate.

Then we estimate $p = 1 - 0.167 = 0.83$ from the data of parity determination of mosquitoes collected in the Solomon Islands. The distribution of the parasite in individuals was surveyed by Dr. Kumada in 3 villages in the Solomon Islands from January 1993 to August 1995. The overall parasite incidence at that location weighted for the age-specific ratio of the population can be estimated as follows (18).

P. falciparum: 21.3% ± 2.4%

P. falciparum gametocyte: 2.7% ± 0.9%

Thus, $\varepsilon = 0.21$ for the parasite rate of *Pf*, and $\lambda = 2.7/21.3 = 0.13$ for the parasite rate of *Pf* gametocytes in humans having *Pf*. To determine the entomological parameter α, we use the parasite rate ($R_{Pf,1P} = 0.152$) of 1-parous mosquitoes obtained by PCR. Since $R_{Pf,1P}$ depends only on α when all the other parameters are given as mentioned above, we solve the equation:

$R_{Pf,1P} = 0.152$.

Our model simulation was carried out using the computer in the Faculty of Environmental Science and Technology, Okayama University. The results showed that each class of mosquito reached a stable equilibrium after about 200 days. First various values of α were used in the $R_{Pf,1P}$ formula, and the value of $R_{Pf,1P}$ was calculated through simulation of the model. When it was nearest to 0.152, α was determined to be 0.125.

After the simulation with the iteration day by day, we finally obtained the parasite rate $R_{Sporozoite}$ of sporozoites in *An. farauti* in the Solomon Islands:

$R_{Sporozoite} = 0.0009$ (0.09%).

The sporozoite rate of *P. falciparum* is quite variable, *e.g.*, 1.86% (19) in Zaire and 9.6% (20) in Guinea Bissau in *An. gambiae*. Those values are probably overestimated because mosquitoes were collected in houses in Guinea Bissau and sporozoites were detected by ELISA using antibodies against CS proteins that react not only to sporozoites but also to oocysts expressing CS proteins. The sporozoite rate of *P. falciparum* is estimated to be 0.45-4.5% (21) in *An. farauti* in the Solomon Islands and 1.8% (22) in *Anopheles punctulatus* in Papua New Guinea. This difference may be partly due to the use of ecological parameters obtained at other sites in the Solomon Islands and Papua New Guinea. As indoor resting mosquitoes have often been used for the estimation of sporozoite rates, the values tend to be biased. For the present study, we used mosquitoes caught in the field, not in houses. Although the estimated sporozoite rate (0.09%) is only 1/5-1/50 of the above reported rates, one person would become infected within about a month (1,000 bites), since the human-biting rate (bites/day/person) in the Solomon Islands has been estimated to be 37 (23). The estimated sporozoite rate is therefore thought to be sufficiently high enough to maintain a high tranmission rate of falciparum malaria.

The application of this combination of PCR and our model to field studies for estimating the sporozoite rate may save considerable effort in the collection of mosquitoes. Moreover, this method for detecting parasites in blood-fed mosquitoes would be useful for the detection of pathogens in other vectors.

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References

1. Tirasophon W, Ponglikitmongkol M, Wilairat P, Boonsaeng V and Panyim S: A novel detection of a single *Plasmodium falciparum* in infected blood. Biochem Biophys Res Commun (1991) **175**, 179-184.
2. Schriefer ME, Sacci JB Jr, Wirtz RA and Azad AF: Detection of polymerase chain reaction-amplified malarial DNA in infected blood and individual mosquitoes. Exp Parasitol (1991) **73**, 311-316.
3. Snounou G, Pinheiro L, Goncalves A, Fonseca L, Dias F, Brown KN and do Rosario VE: The importance of sensitive detection of malaria parasites in the human and insect hosts in epidemiological studies, as shown by the analysis of field samples from Guinea Bissau. Trans R Soc Trop Med Hyg (1993) **87**, 649-653.
4. Wataya Y, Arai M, Kubochi F, Mizukoshi C, Kakutani T, Ohta N and Ishii A: DNA diagnosis of falciparum malaria using a double PCR technique: A field trial in the Solomon Islands. Mol Biochem Paraisitol (1993) **58**, 165-168.
5. Oliveira DA, Holloway BP, Durigon EL, Collins WE and Lal AA: Polymerase chain reaction and a liquid-phase, nonisotopic hybridization for species-specific and sensitive detection of malaria infection. Am J Trop Med Hyg (1995) **52**, 139-144.
6. Tassanakajon A, Boonsaeng V, Wilairat P and Panyim S: Polymerase chain reaction detection of *Plasmodium falciparum* in mosquitoes. Trans R Soc Trop Med Hyg (1993) **87**, 273-275.
7. Tirasophon W, Tassanakajon A, Boonsaeng V, Panyim S and Wilairat P: Sensitive detection of *Plasmodium falciparum* in blood and mosquito by DNA amplification. Parassitologia (1993) **35**, S117-S120.
8. Stoffels JA, Docters van Leeuwen WM and Post RJ: Detection of *Plasmodium* sporozoites in mosquitoes by polymerase chain reaction and oligonucleotide rDNA probe, without dissection of the salivary

glands. Med Vet Entomol (1995) **9**, 433–437.
9. Vernick KD, Barreau C and Seeley DC: *Plasmodium*: A quantitative molecular assay for detection of sporogonic-stage malaria parasites. Exp Parasitol (1995) **81**, 436–444.
10. Vernick KD, Keister DB, Toure A and Toure YT: Quantification of *Plasmodium falciparum* sporozoites by ribosomal RNA detection. Am J Trop Med Hyg (1996) **54**, 430–438.
11. Bouare M, Sangare D, Bagayoko M, Toure A, Toure YT and Vernick KD: Short report: Simultaneous detection by polymerase chain reaction of mosquito species and *Plasmodium falciparum* infection in *Anopheles gambiae* sensu lato. Am J Trop Med Hyg (1996) **54**, 629–631.
12. Harada M, Matsuoka H and Suguri S: A convenient mosquito mebrane feeding method. Med Entomol Zool (1996) **47**, 103–105.
13. Walsh PS, Metzger DA and Higuchi R: Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques (1991) **10**, 506–513.
14. Charlwood JD, Birley MH, Dagoro H, Paru R and Holmes PR: Assessing survival rates of *Anopheles farauti* (Diptera: Culicidae) from Papua New Guinea. J Anim Ecol (1985) **54**, 1003–1016.
15. Afifi SE, Spencer M, Hudson PB and Tavil NW: Biting prevalence and malaria transmission patterns in the *Anopheles punctulatus* complex (Diptera: Culicidae) in Papua New Guinea. Aust J Exp Biol Med Sci (1980) **58**, 1–17.
16. Sato C, Furuya Y, Harada M and Suguri S: Identification of human blood in mosquitoes (Diptera: Culicidae) using nonradioactive DNA dot blot hybridization. J Med Entomol (1992) **29**, 1045–1048.
17. Clements AN and Paterson GD: The analysis of mortality and survival rates in wild populations of mosquitoes. J Appl Ecol (1981) **18**, 373–399.
18. Ishikawa H: The prevalence of malaria in the Solomon and Vanuatu Islands: A comparative study based upon the malaria mathematical model; in Malaria Research in Solomon Islands, Ishii A, Nihei N and Sasa M eds, Inter Group Corporation, Tokyo (1998) pp152–155.
19. Coene J: Malaria in urban and rural Kinshasa: The entomological input. Med Vet Entomol (1993) **7**, 127–137.
20. Jaenson TG, Gomes MJ, Barreto dos Santos RC, Petrarca V, Fortini D, Evora J and Crato J: Control of endophagic *Anopheles* mosquitoes and human malaria in Guinea Bissau, West Africa by permethrin-treated bed nets. Trans R Soc Trop Med Hyg (1994) **88**, 620–624.
21. Kamei K, Shibuya T, Suzuki H, Kere N and Sasa M: Comparative study of the effect of using insecticide-impregnated bed nets and indoor residual spraying on malaria control in the Solomon Islands; in Solomon Islands and Malaria, Ishii A and Nihei N eds, Japan Association for Tropical Medicine, Tokyo (1995) pp.106–111.
22. Bockarie MJ, Alexander N, Bockarie F, Ibam E, Barnish G and Alpers M: The late biting habit of parous *Anopheles* mosquitoes and prebedtime exposure of humans to infective female mosquitoes. Trans R Soc Trop Med Hyg (1996) **90**, 23–25.
23. Harada M, Ikeshoji T and Suguri S: Studies on vector control by "Mosquito Candle"; in Malaria Research in Solomon Islands, Ishii A, Nihei N and Sasa M eds, Inter Group Corporation, Tokyo (1998) pp 120–125.

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