

## A GENUS- AND SPECIES-SPECIFIC NESTED POLYMERASE CHAIN REACTION MALARIA DETECTION ASSAY FOR EPIDEMIOLOGIC STUDIES

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**Abstract.** A nested polymerase chain reaction (PCR) assay that uses *Plasmodium* genus-specific primers for the initial PCR (nest 1) amplification and either genus- or species-specific primers for the nest 2 amplifications was tested on laboratory and field samples. With *in vitro* cultured *Plasmodium falciparum*-infected blood samples, it was capable of detecting six parasites/ $\mu$ l of blood using DNA prepared from 25- $\mu$ l blood spots on filter paper. The assay was evaluated on fingerprick blood samples collected on filter paper from 129 individuals living in a malaria-endemic area in Malaysia. Malaria prevalence by genus-specific nested PCR was 35.6% (46 of 129) compared with 28.7% (37 of 129) by microscopy. The nested PCR detected seven more malaria samples than microscopy in the first round of microscopic examination, malaria in three microscopically negative samples, six double infections identified as single infections by microscopy and one triple infection identified as a double infection by microscopy. The nested PCR assay described is a sensitive technique for collecting accurate malaria epidemiologic data. When coupled with simple blood spot sampling, it is particularly useful for screening communities in remote regions of the world.

Microscopy is the method of choice for the diagnosis of malaria in endemic areas because it is an inexpensive and rapid method of detection. Correct identification of the four species of *Plasmodium* causing human malaria and the level of detection by microscopy depends on a number of factors, including the experience of the microscopist, proper staining of the slides, appropriate maintenance of microscopes, and the time spent examining each slide. At best, the sensitivity of detection by microscopy is approximately 10–30 parasites/ $\mu$ l of blood.<sup>1</sup> However, this level of detection is normally not attained in malaria-endemic areas and particularly during epidemiologic studies when many samples need to be screened in a relatively short time. Thus, incorrect speciation is common and mixed infections and low levels of parasitemia may be missed.

To overcome some of the limitations of microscopy for detection of malaria, polymerase chain reaction (PCR)-based assays have been developed for the detection and identification of malaria parasites. These methods have proved to be more specific and sensitive than conventional microscopy and some are reported to be able to detect as few as one parasite/ $\mu$ l of blood.<sup>2–6</sup> However, to attain such a high sensitivity, blood samples collected from individuals have to be processed immediately or stored at low temperatures and the steps involved in DNA template preparation were multistep, often requiring biohazardous chemicals. Since malaria remains a problem of underdeveloped and often remote areas of the world, it is important to couple PCR-based assays with simple sampling and DNA extraction methods to maximize the value of PCR assays. In a previous study, we coupled the nested PCR assay of Snounou and others<sup>3</sup> to blood collection on filter papers and a simple DNA extraction method and found that the assay was able to detect more single and mixed malaria infections compared with microscopy.<sup>7</sup> This nested PCR assay involved sequential PCR amplifications for each blood sample screened. In the first PCR (nest 1) amplification, *Plasmodium*-specific primers were used. The product of this nest 1 amplification then served as DNA template for four separate second PCR (nest

2) amplifications with primers specific for each of the 4 human malaria species. Therefore, to screen a blood sample, a total of five PCR amplifications were required and four PCR products had to be analyzed by gel electrophoresis. For epidemiologic studies, it would be more cost-effective and faster if samples could be initially screened using *Plasmodium* genus-specific primers in both nest 1 and 2 amplifications and only positive samples subjected to subsequent speciation by further nest 2 amplifications.

In this study we describe a nested PCR assay that detects malaria in blood samples collected on filter paper. It uses *Plasmodium* genus-specific primers for the nest 1 and nest 2 amplifications and reuses the nest 1 amplification products of positive samples for species-specific nest 2 amplifications. The sensitivity of detection was determined and the assay was evaluated on field samples collected in a malaria-endemic area in Malaysia.

### MATERIALS AND METHODS

**Parasite culture.** The *P. falciparum* 3D7 clone, donated by Professor D. Walliker (University of Edinburgh, Edinburgh, Scotland) was maintained in continuous culture by the candle jar method of Trager and Jensen.<sup>8</sup> For sensitivity studies, cultures were synchronized using sorbitol<sup>9</sup> and allowed to grow for an additional 48 hr before Percoll density centrifugation<sup>10</sup> was done to remove any late trophozoite or schizont-infected erythrocytes. The resultant blood sample, containing only ring-stage parasites, was serially diluted five-fold using uninfected blood. Aliquots of 25  $\mu$ l were spotted directly onto 3 MM chromatography paper (Whatman International Corp., Maidstone, United Kingdom), allowed to air-dry, and stored at room temperature until required.

**Study area.** Individuals living in Bitoon, a malaria-endemic area located 200 km northeast of Kota Kinabalu, Sabah, Malaysia, were examined during a malaria survey by officers of the Sabah State Vector-Borne Diseases Control

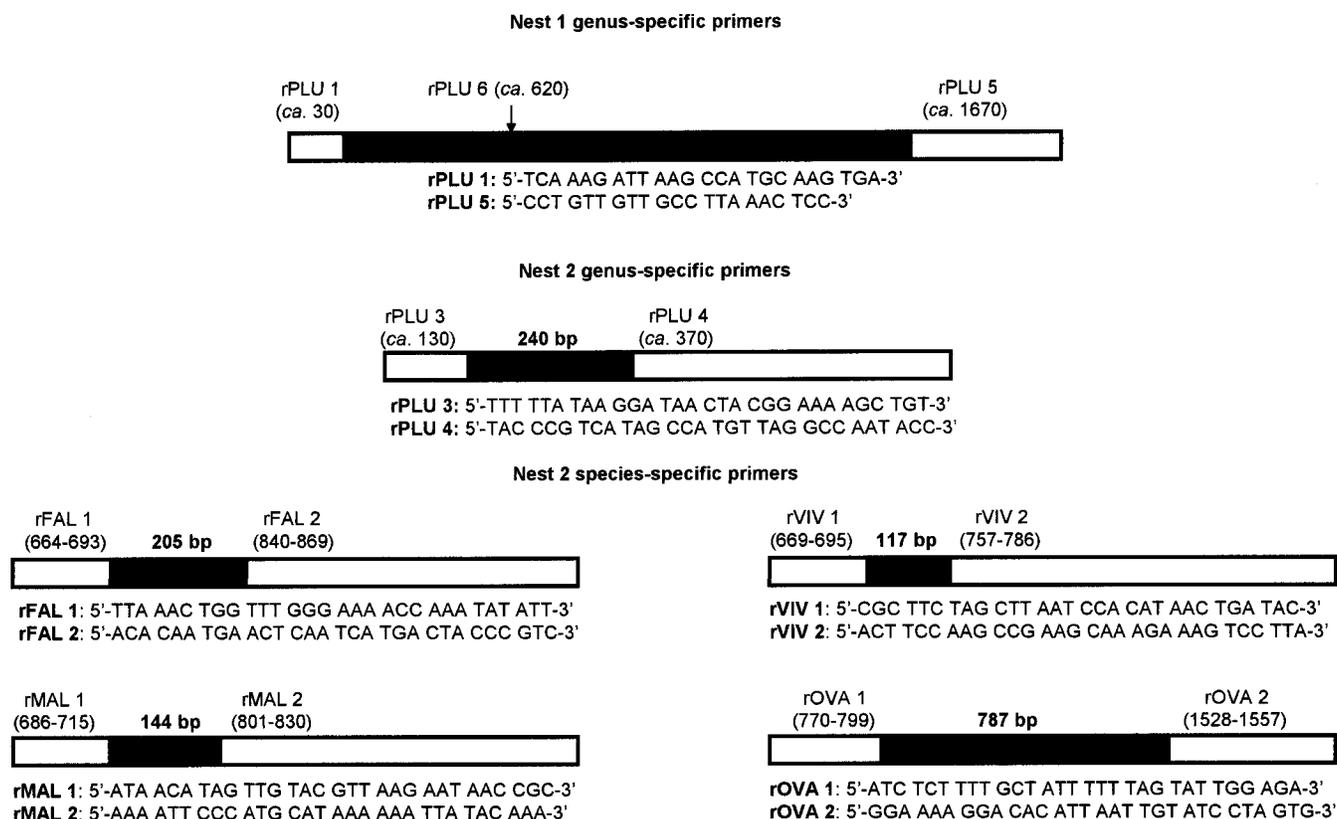


FIGURE 1. Schematic representation of oligonucleotides used in the nested polymerase chain reaction (PCR) assay. The numbers in parentheses indicate the positions of the oligonucleotides on the *Plasmodium* small subunit ribosomal RNA genes.<sup>3,18</sup> The **arrow** indicates the approximate position of oligonucleotide rPLU 6, which was used as nest 1 primer with rPLU 5 in previous nested PCR assays.<sup>3,7</sup> The nest 2 species-specific primers rFAL 1 and 2, rVIV 1 and 2, rMAL 1 and 2, and rOVA 1 and 2 are specific for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*, respectively. The numbers **in bold** indicate the expected sizes of the nest 2 products. ca. = approximately; bp = basepairs.

Program Office. The study was approved by the Universiti Sains Malaysia Research Committee.

**Blood sample collection.** Verbal consent was obtained from all participants before blood samples were obtained. The thick and thin blood films were prepared from each individual together with triplicate fingerprick blood samples, each between 20 and 50  $\mu$ l, that were spotted directly onto filter paper (Whatman 3 MM chromatography paper). Blood spots on filter papers were allowed to air-dry and were placed individually in plastic bags and mailed at room temperature to the Universiti Sains Malaysia, where they were stored at room temperature until processed.

**Microscopic examination.** The thick and thin blood films were stained with Giemsa and examined by microscopists of the Vector-Borne Diseases Control Program Office. The films were then despatched to the Universiti Sains Malaysia where they were examined by an experienced microscopist when there was discordance between PCR and microscopy results. The microscopist had no knowledge of the PCR results or the microscopy results and was instructed to examine the slides carefully for malaria parasites and to determine the parasitemia if the blood films were positive.

**Determination of parasitemia.** To determine the approximate number of parasites per microliter of blood for field samples, the method described by Trape<sup>11</sup> was used in which the number of parasites per white blood cell (WBC) in thick

films was determined and multiplied by 8,000. For *in vitro* cultured blood samples used in sensitivity studies, the number of WBCs per microliter of blood was determined accurately for the initial undiluted blood sample using a T-540 cell counter (Coulter Electronics, Ltd., Luton, United Kingdom), and the parasitemia was determined for thick blood films by counting against 1,000 WBCs.

**Preparation of the DNA template.** The DNA template for the nested PCR assay was prepared from the whole blood spot using the simple Chelex<sup>®</sup> (Bio-Rad Laboratories, Hercules, CA) boiling method.<sup>12</sup> Each spot was boiled in 180  $\mu$ l of Chelex<sup>®</sup> solution and approximately 120  $\mu$ l of DNA template was obtained. Five microliters of this was used in the nested PCR assay, thus corresponding to approximately 0.6–1.4  $\mu$ l of the blood sample.

**Oligonucleotide primers.** Oligonucleotide primers for the nested PCR assay were obtained from Genosys Biotechnologies Inc. (The Woodlands, TX), and were all purified by high-performance liquid chromatography. These primers were designed based on the *Plasmodium* small subunit ribosomal RNA (ssrRNA) genes<sup>13–16</sup> and are shown in Figure 1. In previously reported epidemiologic studies,<sup>7,17</sup> the nest 1 genus-specific primers rPLU 5 and rPLU 6 were used in combination with the four pairs of human malaria species-specific nest 2 primers. In this study, the genus-specific primer rPLU 1 (Jarra W, Snounou G, unpublished data) was

used with rPLU 5 instead of rPLU 6 since this permitted PCR products of nest 1 amplifications to be used as DNA templates with the genus-specific primers rPLU 3 and rPLU 4<sup>18</sup> in nest 2 amplifications. The primers rPLU 3 and 4 have been used in single nest PCR assays to amplify DNA specifically from the four human malaria parasites (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*), from a simian malaria parasite (*P. gonderi*), and from all the rodent malaria parasites (*P. yoelii*, *P. berghei*, *P. chabaudi*, and *P. v. petersi*).<sup>18</sup> These nest 2 genus-specific primers have also been used successfully in nested PCR assays in combination with the nest 1 primers rPLU 1 and rPLU 2 to amplify DNA specifically from rodent, human, and the simian malaria parasites (Jarra W, Snounou G, unpublished data).

**Nested PCR assay.** Each 50- $\mu$ l reaction mixture for nest 1 amplifications contained 5  $\mu$ l of DNA template, 250 nM of each primer (rPLU 1 and rPLU 5), 4 mM MgCl<sub>2</sub>, Boehringer Mannheim (Indianapolis, IN) PCR buffer (50 mM KCl, 10 mM Tris-HCl), 200  $\mu$ M of each deoxynucleoside triphosphate, and 1.25 units of *Taq* DNA polymerase (Boehringer Mannheim). Nest 1 amplification conditions were as follows: step 1, 94°C for 4 min; step 2, denaturation at 94°C for 30 sec; step 3, annealing at 55°C for 1 min; step 4, extension at 72°C for 1 min; repeat steps 2–4 35 times, and step 4 for 4 min. Two microliters of the nest 1 amplification product served as the DNA template for each of the 20- $\mu$ l nest 2 amplifications. The concentration of the nest 2 primers and other constituents were identical to nest 1 amplifications except that 0.5 units of *Taq* DNA polymerase was used. Nest 2 amplification conditions were identical to those of nest 1 except that the annealing temperature in step 3 was 58°C for the species-specific primers (rFAL 1 and 2, rMAL 1 and 2, rVIV 1 and 2, and rOVA 1 and 2) and was 62°C for the genus-specific primers (rPLU 3 and 4). The PCR products of nest 2 amplifications were analyzed by gel electrophoresis and staining with ethidium bromide.

## RESULTS

**Sensitivity of detection.** The sensitivity of detection of the nested PCR assay was determined using DNA templates prepared from 25- $\mu$ l blood spots, each with known numbers of *in vitro* cultured *P. falciparum* parasites. When genus-specific primers for both nest 1 and nest 2 amplifications were used, the nested PCR assay amplified a specific 240-basepair product with DNA template prepared from blood spots of approximately six or more parasites/ $\mu$ l of blood (Figure 2). In experiments to optimize the sensitivity of detection, between six and 31 parasites/ $\mu$ l of blood were detected by nested PCR repeatedly, and on three of 11 occasions a specific product was observed even with DNA template from spots of one parasite/ $\mu$ l of blood. A constant quantity of specific product was observed on agarose gels for positive samples irrespective of the number of parasites present in the samples.

When oligonucleotide primers were used in a single PCR amplification, as opposed to nested reactions, the sensitivity of detection was drastically reduced. Thus, samples with parasitemias < 3,000 parasites/ $\mu$ l of blood, which showed positive results by the nested PCR, were always negative when either the rPLU 1 and rPLU 5, or rPLU 3 and rPLU 4 oli-

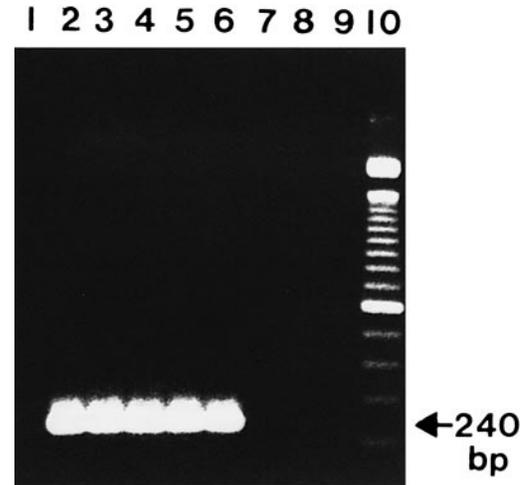


FIGURE 2. Sensitivity of the nested polymerase chain reaction (PCR) assay. Genus-specific primers were used for both nest 1 and 2 amplifications. The PCR products of nest 2 amplifications with DNA templates prepared from 25- $\mu$ l blood spots containing the following numbers of *Plasmodium falciparum* ring-stage parasites/ $\mu$ l of blood: 0 (lane 1), 3,952 (lane 2), 790 (lane 3), 158 (lane 4), 31 (lane 5), 6 (lane 6), 1 (lane 7), 0.2 (lane 8), 0.04 (lane 9). Molecular size markers (100-basepair [bp] ladder) are shown in lane 10.

gonucleotide pairs were used in a single PCR amplification of 35 cycles with *P. falciparum* DNA template prepared by the Chelex<sup>®</sup> method. We also observed a reduction in sensitivity compared with nested PCR amplifications when the four species-specific primer pairs were each used in single PCR amplifications. Furthermore, attempts to include all four species-specific primers in a single nest 2 amplification (multiplex amplification) resulted in a marked decrease in sensitivity of detection and failure to detect the expected specific amplification products from samples containing DNA from the four malaria species. Similar observations of reduced sensitivity for single PCR amplifications and failure of nest 2 multiplex amplifications were noted by Snounou and others with the genus-specific nest 1 primers rPLU 5 and rPLU 6 and the four species-specific primers.<sup>3</sup>

**Evaluation of the nested PCR with field samples.** A total of 129 samples were screened. The malaria prevalence determined by the nested PCR using the genus-specific primers for nest 2 amplifications was 35.6% (46 of 129) compared with 28.7% (37 of 129) by microscopy (Table 1). When the PCR results were compared with the microscopy results, there were 20 discordant results: 11 samples were positive by PCR but were negative by microscopy, one sample was *P. vivax*-positive by the nested PCR but *P. falciparum*-positive by microscopy, six samples were *P. falciparum* and *P. vivax* mixed infections by the PCR but single infections by microscopy, one sample was a triple infection by the PCR but a double infection by microscopy, and one sample was microscopy-positive but PCR-negative (Table 2). The samples that gave discordant results were rescreened blind by microscopy and nested PCR and nine produced concordant results (Table 2). For example, of the 11 PCR-positive but microscopy-negative samples, eight were confirmed as malaria-positive following repeat microscopy (Samples BT 26, 76, 104, 111, 71, 78, 115, and 102) and one sample (BT 12) was negative by both repeat PCR and

TABLE 1  
Comparison of malaria diagnosis by a nested polymerase chain reaction (PCR) with microscopy for 129 field samples

Nested PCR Results	Microscopy results*							Nested PCR Total
	Negative	Pf	Pv	Pm	Po	Pf + Pv	Pf + Pv + Pm	
Negative	85†	1	0	0	0	0	0	86
Pf	4	21	0	0	0	0	0	25
Pv	3	1	1	0	0	0	0	5
Pm	1	0	0	1	0	0	0	2
Po	0	0	0	0	0	0	0	0
Pf + Pv	0	2	4	0	0	4	0	10
Pf + Pv + Pm	0	0	0	0	0	1	0	1
Microscopy total	93	25	5	1	0	5	0	129

\* Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pf + Pv = *P. falciparum* and *P. vivax*; Pf + Pm + Pv = *P. falciparum*, *P. malariae*, and *P. vivax*.

† This figure includes three samples that were malaria positive by PCR using nest 2 genus-specific primers but could not be speciated using nest 2 species-specific primers for the four human malaria species.

microscopy, whereas two samples (BT 104 and 111) remained microscopy-negative but were PCR-positive. For samples that were confirmed as positive by repeat microscopy, parasitemias were very low and ranged from three to 56 parasites/ $\mu$ l of blood. It took 45–90 min to detect parasites and determine parasitemias for each slide. Such prolonged examination of slides by microscopy is clearly not feasible during large-scale epidemiologic studies when microscopists have to process numerous samples in a relatively short time.

Other samples that produced concordant results following re-examination included sample BT 119, which was found to be PCR-positive following repeat nested PCR. The failure to detect malaria in the initial PCR assay is unclear and may be due to operator error. The sample had a parasitemia of 117 parasites/ $\mu$ l of blood and the nested PCR assay was able to consistently detect parasites in other samples with lower parasitemias.

The results of some samples remained discordant after re-examination. For example, despite prolonged re-examination by microscopy of samples that were detected as mixed *P. falciparum* and *P. vivax* infections by PCR but as single infections by microscopy (samples BT 47, 50, 64, 81, 67, and 72), they were still identified as single infections by microscopy. However, repeat nested PCR confirmed all of these samples as mixed *P. falciparum* and *P. vivax* infections. Other examples that gave discordant results included two that were confirmed malaria-positive by microscopy and PCR but could not be speciated by repeat PCR (samples BT 22 and 77), and sample BT 102, which was *P. malariae*-positive by PCR but repeat microscopy found that it had three *P. vivax* parasites/ $\mu$ l of blood. It is difficult to differentiate between young trophozoites of *P. vivax* and *P. malariae* by microscopy. Since the slides were re-examined blind, it is possible that the species was identified incorrectly by microscopy since the repeat PCR confirmed the sample as a *P. malariae* infection.

#### DISCUSSION

In this study, we have described a nested PCR assay that has a sensitivity of detection of at least six parasites/ $\mu$ l of blood using DNA from blood spots with *in vitro* cultured *P. falciparum* on filter paper. This sensitivity, which is greater than that observed using microscopy for malaria detection,

was attained by nested PCR using a simple DNA extraction method for DNA template preparation. A constant quantity of specific product was observed for positive samples irrespective of the number of parasites present. These results confirm previous observations that there is a sharp cut-off point for the nested PCR assay resulting in an all or none detection of parasites.<sup>3</sup> They may explain the irreproducibility of parasite detection in samples with very low parasitemias since there will be variation between the amount of parasite DNA present in each small aliquot taken from a particular sample. For DNA extraction from blood spots on filter papers in this study, the chelating agent Chelex<sup>TM</sup> was used. Recently, we have found that by using InstaGene<sup>TM</sup> (Bio-Rad Laboratories) instead of Chelex<sup>TM</sup>, a larger volume of template can be used in the nested PCR assay, resulting in increased sensitivity of malaria detection without compromising the simplicity of specimen collection or DNA extraction.<sup>19</sup> Therefore, for future epidemiologic studies it is recommended that the new DNA extraction method be used to improve the sensitivity of detection of malaria by the nested PCR.

When evaluated on field samples, the nested PCR assay was found to be more sensitive than microscopy since 11 additional malaria-positive samples were detected by PCR compared with microscopy in the first round of screening. However, two of these *Plasmodium*-positive samples could not be speciated with the species-specific PCR. *Plasmodium*-specific PCR amplification is theoretically more sensitive than species-specific amplification. This is due to the fact that at least two *ssrRNA* genes, all targets for the primers, are present in the plasmodial genome.<sup>13,20,21</sup> The species-specific primers of *P. vivax* and *P. falciparum* are specific for the sexually expressed C-type *ssrRNA* genes of *P. vivax* and *P. falciparum* only.<sup>13,21</sup> Thus, the failure to speciate samples that were *Plasmodium*-positive by PCR might be due to very low parasite levels. Another possibility is that these samples contain *P. ovale* parasites that harbor sequence variations in their *ssrRNA* gene sequences<sup>22</sup> and thus are unable to be amplified with the rOVA 1 and rOVA 2 primer pair. The reason why one sample was identified as *P. falciparum*-positive by microscopy and positive by the genus-specific primers but could not be speciated by the nested PCR is unknown. Variation in the sequence of the *P. falciparum* *ssrRNA* gene is a possibility, but it has not been observed previously.

TABLE 2  
Discordant results obtained using a nested polymerase chain reaction (PCR) assay and microscopy for malaria diagnosis

No.	Sample code	Nested PCR result*	Microscopy result	Repeat microscopy result (parasites/ $\mu$ l of blood)†	Repeat nested PCR result
1	BT 26	Pf	Negative	Pf (9)	Pf
2	BT 76	Pf	Negative	Pf (28)	Pf
3	BT 104	Pf	Negative	Negative	Pf
4	BT 111	Pf	Negative	Negative	Pf
5	BT 71	Pv	Negative	Pv (18)	Pv
6	BT 78	Pv	Negative	Pv (17)	Pv
7	BT 115	Pv	Negative	Pv (4)	Pv
8	BT 102	Pm	Negative	Pv (3)	Pm
9	BT 22	Genus	Negative	Pf (56)	Genus
10	BT 77	Genus	Negative	NR	Genus
11	BT 12	Genus	Negative	Negative	Negative
12	BT 92	Pv	Pf	Pv (69)	Pv
13	BT 47	Pf + Pv	Pv	Pv (384)	Pf + Pv
14	BT 50	Pf + Pv	Pv	Pv (2,160)	Pf + Pv
15	BT 64	Pf + Pv	Pv	Pv (3,280)	Pf + Pv
16	BT 81	Pf + Pv	Pv	Pv (4,480)	Pf + Pv
17	BT 67	Pf + Pv	Pf	Pf (NR)	Pf
18	BT 72	Pf + Pv	Pf	Pf (2,500)	Pf + Pv
19	BT 49	Pf + Pv + Pm	Pf + Pv	Pf (1,200)/Pv (1,866)	Pf + Pv + Pm
20	BT 119	Negative	Pf	Pf (117)	Pf

\* Genus: samples detected as malaria-positive with nest 2 genus-specific primers but could not be speciated with nest 2 species-specific primers. Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*; Pf + Pv = *P. falciparum* and *P. vivax*; Pf + Pm + Pv = *P. falciparum*, *P. malariae*, and *P. vivax*.  
† NR = slides re-examined by microscopy but no result due to poor quality of thick smear and staining.

The greater sensitivity of the PCR assay compared with microscopy was further confirmed when five samples were detected as mixed *P. falciparum* and *P. vivax* infections by PCR on two occasions but identified as single infections by microscopy. The reason why microscopy failed to detect mixed infections could be due to the presence of higher numbers of parasites of one species relative to the other. In four of these samples, high parasitemias between 2,160 and 4,480 parasites/ $\mu$ l of blood were observed. Since the samples were re-examined blind by microscopy, it would be difficult to identify small numbers of the second malaria species when one species was present in high numbers. The dominance of one or more species of malaria over another could also be a reason for the failure to detect *P. malariae* by microscopy in one sample that was confirmed as a triple *P. falciparum*, *P. vivax*, and *P. malariae* infection by repeat PCR, but identified as a double *P. falciparum* and *P. vivax* infection by microscopy. With any PCR assay, there is always the possibility that cross-contamination of samples may lead to false-positive results. However, stringent measures were undertaken to prevent or minimize cross-contamination of samples. These included the use of plugged pipette tips for all procedures including DNA template preparation, the use of four separate areas and four separate sets of pipettes for template preparation, preparation of PCR master mixtures, addition of template to nest 1 and nest 2 amplification reactions and analysis of PCR products, and the storage of solutions used to prepare PCR master mixtures in a room in which amplification products were not stored or analyzed. Furthermore, one uninfected blood spot was included for every 11 blood samples processed and this negative control was always negative by the nested PCR.

The PCR-based assays for the detection of malaria are highly specific and very sensitive but for them to replace microscopy for the routine diagnosis of malaria, they also need to be simple, quantitative, rapid, and inexpensive.

These methods involve multiple steps and are clearly not simple in their present form. In addition, determination of parasite density is vital for disease management and this is difficult to attain with PCR-based assays.<sup>18</sup> Finally, the costs involved in purchasing equipment and assay reagents are very high and the assays are not suitable for prompt diagnosis of single or small numbers of samples. Therefore, it is not proposed that PCR-based assays replace microscopy for routine diagnosis in developing countries where malaria is endemic. Nevertheless, nested PCR assays are extremely valuable tools for obtaining accurate epidemiologic data and this has been demonstrated by several studies in different endemic areas in Southeast Asia<sup>3,7</sup> and Africa.<sup>17,23,24</sup>

The nested PCR assay we have described involves pre-screening the samples for the presence of malaria before speciation of the parasite species. This additional step reduces costs for labor and reagents needed for nested PCR detection of parasites during epidemiologic studies. Cost-effectiveness is achieved even in areas with relatively high endemicity. For example, to screen 100 individuals in an area with a 50% prevalence of malaria, the nested PCR assay described here would involve a total of 400 PCR amplifications: 100 nest 1 and 100 nest 2 amplifications during pre-screening with genus-specific primers, and the expected 50 positive samples can then be speciated with an additional 200 species-specific nest 2 amplifications. Without pre-screening, 500 PCR amplifications (100 nest 1 and 400 nest 2 amplifications) would be required. The assay described would be even more cost-effective in areas of moderate to low endemicity. The use of all four species-specific primers in a single nest 2 multiplex amplification would also drastically improve cost-effectiveness. However, when this was attempted there was a marked decrease in the sensitivity of parasite detection and the ability to detect mixed species infections was substantially reduced.<sup>3</sup>

In conclusion, the higher prevalence of both single and

mixed infections determined by the nested PCR compared with microscopy demonstrates that the nested PCR assay is more specific and sensitive than microscopy for detection of malaria. Compared with the nested PCR assay used in previous studies in which five PCR amplifications were necessary for analyzing each blood sample,<sup>2,7,17</sup> the nested PCR assay described here only requires two PCR amplifications to ascertain whether a sample is positive for *Plasmodium*. Only positive samples are subjected to further speciation and this is therefore a more cost effective nested PCR-based method for screening epidemiologic samples. This nested PCR assay, carried out in a central laboratory, is a sensitive technique for obtaining accurate epidemiologic data. Since it is coupled with a simple blood spot sampling onto filter paper that does not require low temperature storage, it will be particularly valuable for studying communities living in remote regions of the world.

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