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Original article

A novel reverse transcription polymerase chain reaction reveals a high prevalence of *Plasmodium vivax* gametocyte carriage in an endemic area of Thailand

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Background: Gametocytes are precursors of malaria sexual stages that are infective to mosquito vectors and play crucial roles in maintaining cycle of infection. Microscopy cannot determine the status of gametocyte carriage in those who had submicroscopic gametocytemia that may serve as infectious reservoirs in endemic areas. Meanwhile, gametocytes possess stage-specific mRNA that can be detected by molecular methods.

Objective: To develop a sensitive method for detection of *Plasmodium vivax* gametocytes using reverse transcription polymerase chain reaction (RT-PCR) and determine its diagnostic performance in clinical samples.

Materials and Methods: A nested RT-PCR was devised using primers targeting *Pvs25*, a mature gametocyte-specific mRNA transcript of *P. vivax* (nested *Pvs25* RT-PCR). Performance of the assay was evaluated using mRNA extracted from blood samples of 180 febrile patients attending a malaria clinic in Tak Province. Total RNA was extracted from blood samples that were preserved in RNA*later*® and from dried blood on filter papers. Malaria species was determined by microscopy from Giemsa stained blood smears and reaffirmed by nested PCR targeting mitochondrial cytochrome *b* (nested *mtCytb* PCR).

Results: Of 180 blood samples, malaria was diagnosed in 120 patients (69 *P. vivax* and 51 *P. falciparum*) by microscopy and 125 patients by nested *mtCytb* PCR (69 *P. vivax*, 51 *P. falciparum* and 5 coinfections with both these species). Microscopy detected gametocytes in 30 of all 74 (40.5%) *P. vivax* positives by nested *mtCytb* PCR. Meanwhile, 67 of 74 (90.5%) *P. vivax*-positive isolates that were preserved in RNA*later*® gave positive results by nested *Pvs25* RT-PCR. Therefore, nested *Pvs25* RT-PCR identified mature *P. vivax* gametocytes more than twice as frequently as microscopy. The minimum detection threshold for nested *Pvs25* RT-PCR was 10 copies of template DNA whereas no cross-reactivity with other human malaria species was observed. Dried blood collected on filter papers offered comparable results for *Pvs25* mRNA detection with blood stored in RNA preservative with only 2.7% difference in positive rates.

Conclusion: The nested RT-PCR targeting *Pvs25* developed in this study is sensitive and specific for diagnosing mature *P. vivax* gametocytes and can be efficiently applied to both blood samples kept in RNA preservative and dried blood on filter paper.

Keywords: *Plasmodium vivax*, Gametocyte, *Pvs25*, reverse transcription polymerase chain reaction, Thailand

Despite the presence of 5 *Plasmodium* species capable of causing human malaria, *Plasmodium falciparum* and *P. vivax* account for the majority of infections worldwide. Although malaria caused by *P. falciparum* is the most pernicious and contributes the most disease burden of all malaria species on a global scale, *P. vivax* occupies a larger geographical niche and is capable of causing chronic relapsing illness because of the presence of a dormant stage, hypnozoite, in hepatocytes [1, 2]. Currently, malaria caused by *P. vivax* has been estimated to be about 80 million to 300 million clinical cases each year whereas about 2.9 billion people live in disease transmission areas [2, 3]. Monitoring and surveillance of malaria-infected individuals in endemic areas are crucial for understanding the dynamics of disease transmission and assessment of the efficacy of a given control measure [1, 4, 5].

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The presence of asexual stages of malaria in blood smears indicates active replication of parasites in circulation of infected individuals that are responsible for clinical disease, whereas the appearance of gametocytes, the precursors of male and female gametes, in blood samples implies that infected individuals can serve as the infectious reservoirs for disease transmission via onward successive development in mosquito vectors giving rise to infective sporozoites [6]. Although microscopy is considered to be an important and practical tool for malaria diagnosis, its performance relies on various factors such as competency and experience of microscopists, the quality of blood films, and the number of microscopic fields examined [7, 8]. Nevertheless, malaria-infected individuals who harbor parasites below the microscopic detection threshold are not uncommonly encountered [9]. The advent of molecular diagnostic tools using amplification of multicopy target DNA existing in all stages of 5 *Plasmodium,* e.g. *18S rRNA*, mitochondrial cytochrome *b,* has substantially improved malaria diagnosis [10-12]. Under optimal polymerase chain reaction (PCR) conditions, as few as a single parasite can be detected [12]. However, these methods cannot differentiate gametocytes from other stages of *Plasmodium*; therefore detection of malarial mRNA exclusively transcribed during a specific developmental stage is essential [13, 14].

Estimation of gametocyte carriage has important implications for disease transmission because initiation of sexual reproduction and subsequent generation of infective sporozoites ensue after gametocytes are taken up in the blood meal of a vector [13, 14]. Analysis of *P. falciparum* transcriptome has identified about 315 gametocyte-specific mRNAs and some of these are selectively expressed in either male or female gametocytes [15]. Of these, *Pfs25* mRNA transcription occurs when gametocytes become mature (stage V) and are found in both sexes [13, 14]. Homologues of *Pfs25* have been identified in other malaria species including *Pvs25* in *P. vivax* [16]. Analysis of the Pfs25 gene has revealed sequence conservation among field isolates, rendering it an appropriate target for identification of *P. falciparum* gametocytes [9]. Field applications of *P. falciparum* gametocyte detection by reverse transcription polymerase chain reaction targeting *Pfs25* mRNA have revealed substantial numbers of submicroscopic gametocyte carriage. On the other hand, the detection limit of *P. falciparum* and *P. vivax* gametocytes by microscopy is similar to that of asexual blood stages highly variable ranging from 40 to 100 parasites/μL, resulting in much lower gametocyte prevalence than those detected by PCR [9, 17, 18]. Meanwhile, molecular applications for *P. vivax* gametocyte detection have been limited [19, 20], although both *P. falciparum* and *P. vivax* are sympatric in several endemic areas including Thailand [10-12].

The main objective of this study was to develop a nested RT-PCR targeting *Pvs25* (nested *Pvs25* RT-PCR), a homologue of *Pfs25* also exclusively expressed by the time of *P. vivax* gametocyte maturation, for detection of the gametocyte stage of *Plasmodium vivax*. The secondary objective was to compare the efficiency of the nested *Pvs25* RT-PCR method using blood collected onto filter paper (dried blood spot) and in RNA*later*®.

Material and methods

Clinical samples

A total of 180 febrile individuals (120 microscopy positives for any malaria species and 60 microscopy negatives by on-site examination) who visited a malaria clinic in Tak Province, northwestern Thailand, in 2010 were recruited in this study. Inclusion criteria included those who neither had febrile illness nor received drugs with antimalarial effects during the past 2 months before blood sample collection and aged more than 15 years. Exclusion criteria were those having previous antimalarial treatment. Approximately 1 mL of venous blood samples were collected after obtaining informed consent. Fresh blood samples without any preservative (about 200 μL) were spotted onto Whatmann 3MM ChR3 filter paper (Cat. No. 3030917, Maidstone, England), allowed to dry at room temperature, stored in a clean plastic bag and kept at 8–10°C until use. The remaining samples were preserved in EDTA (400 μL) and RNA*later*® (Ambion, Grand Island, NY, USA; 400 μL). The ethical aspects of this study were approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Microscopy

Malaria stages and species were re-examined by microscopy of both thin and thick blood smears stained with Giemsa solution. Samples were considered negative when more 200 fields of 100× objective for thin blood film and >200 white blood cells for thick blood film were examined. Parasite density was

determined from a thick blood film. Estimation of parasite density was performed by assuming a standard count of 7,000 leucocytes/μL as described previously [10, 11].

DNA extraction

Two hundred μL of EDTA-preserved blood samples were used for DNA extraction. The procedures for DNA preparation were performed using a QIAGEN kit following the instruction protocol except for elution with 30 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer.

RNA preparation

Total RNA from each sample was prepared from 200 μL of blood samples that had been preserved in RNA*later*® using a QIAamp RNA blood mini kit (Qiagen, Germany). Generation and amplification of cDNA was performed by using Takara RNA PCR kit (AMV) version 3.0 (Takara, Japan). For samples collected on filter papers, DNA was extracted from the entire blood spot using the same procedure as for blood kept in RNA preservative. Possible genomic DNA contamination during the RNA preparation procedure was excluded by using 2 μL of RNA products of each sample as templates for subsequent RT-PCR assays.

Nested PCR for diagnosing malaria species

Molecular diagnosis of all 5 malaria species causing human infections was performed by nested PCR targeting the mitochondrial cytochrome *b* gene (nested *mtCytb* PCR) of each species. PCR primers and amplification conditions were as described previously [12]. Results were obtained from 2.5% agarose gel electrophoresis, stained with ethidium bromide solution and visualized under UV transillumination.

Nested Pvs25 RT-PCR

The PCR primers were derived from *Pvs25* sequences available in the GenBank database. Primers for primary PCR were PVS25F0 (5′-TAAG TGTATGTGTAACGAAGGG-3′) and PVS25R0 (5′-ATTGACAAGCAGTTTCTCCC-3′). Locations of primers are depicted in **Figure 1**. DNA amplification was carried out in a total volume of 15 μL containing 1 μL of template DNA, 2.5 mM each deoxynucleoside triphosphate, 1.5 μL of 10× PCR buffer, 0.3 μM of each primer and 0.4 unit of ExTaq DNA polymerase (Takara, Seta, Japan). The thermal cycling profile for primary PCR included a preamplification denaturation at 94°C, 1 minute; 35 cycles of denaturation at 94°C, 40 seconds, annealing at 53°C, 30 seconds and extension at 72°C, 30 seconds; and post amplification extension at 72°C, 5 minutes. Secondary PCR was performed using primers PVS25F1 (5′-CTTTCCGAAAATACATG TGAAG-3′) and PVS25R1 (5′-CCAATAGCACAT GAGCAACCT-3′). Secondary PCR was performed using the same thermal cycling profile. All amplification reactions were done in an Applied Biosystem GeneAmp PCR System 9700 thermocycler (PE Biosystems, Foster City, CA). PCR products were analyzed by 2% agarose gel electrophoresis.

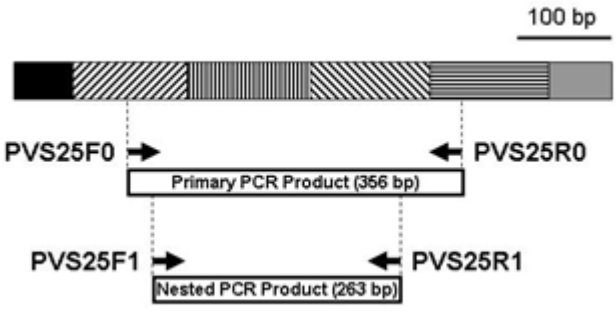

Figure 1. Structure of the Pvs25 gene of *Plasmodium vivax* and locations of PCR primers. Boxes represent sequences encoding the following regions: filled, signal peptide; upward diagonal lines, epidermal growth factor-1 (EGF-1), vertical lines, EGF-2; downward diagonal lines, EGF-3, horizontal lines, EGF-4 and grey, putative glycosylphosphatidyl inositol (GPI) anchored domain. Bar above the diagram denotes 100 bp.

Positive and negative controls

Genomic and cDNA of a clinical isolate (PV800) containing 385 mature *P. vivax* gametocytes/μL were used as positive controls. Sterile water was used as negative control.

Specificity

Genomic DNA of *P. falciparum*, *P. malariae*, *P. ovale* and *P. knowlesi* was included to evaluate specificity of nested *Pvs25* RT-PCR. Five isolates for each malaria species were tested.

Detection limit of nested Pvs25 RT-PCR

The minimum detection limit of the developed nested *Pvs25* RT-PCR method was verify by using known amount of target DNA as a template. Fragment of *Pvs25* spanning 660 bp, designated Pvs25L, that encompassed the target amplification region of the nested *Pvs25* RT-PCR was amplified from *P. vivax* genomic DNA (isolate PV800) using primers PVS25LF, 5′-ATGAACTCCTACTACAGC CTC-3′ and PVS25LR, 5′TTATATGACGTACGA AGGGAC-3′. The thermal cycler profile contained preamplification at 94°C for 1 minute; 35 cycles of 94°C for 40 seconds; 53°C for 30 seconds and 72°C for 30 seconds; and postamplification at 72°C for 5 minutes. Based on the amplified fragment sequence, molecular weight of each copy was equivalent to 6.94×10^{-7} pg; thereby the copy number of each DNA template could be estimated. After the PCR-amplified products were purified from agarose gel electrophoresis, the concentration of each template was determined using the NanoDrop apparatus (Thermo Fisher Scientific, Wilmington, DE, USA). Dilutions at 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2, 10, 1 and 0.1 copy/μL were used as templates to determine the detection limit of the assay. Each dilution of template DNA was tested 4 times under the same amplification conditions.

Data analysis

Molecular detection of malaria parasites based on microscopy is generally superior to microscopy-based diagnosis from blood smears; therefore using results from microscopy as a criterion standard may lead to substantial false positive artifacts by molecular method. Therefore, the sensitivity of gametocyte detection is assessed in terms of (i) detection limit of nested *Pvs25* RT-PCR and (ii) proportion of gametocyte positive rates from nested *Pvs25* RT-PCR, and gametocyte-positive rates from microscopy. Specificity of nested PCR was determined from results of nested *Pvs25* RT-PCR using non-*P. vivax* DNA as templates; thereby, negative results defined specificity of the test.

Results

Specificity of nested Pvs25 RT-PCR

Both genomic DNA and cDNA of a *P. vivax* isolates used as positive controls generated single-band PCR products with the nested *Pvs25* RT-PCR. PCR products from primary and secondary PCR contained 356 bp and 263 bp, respectively. No amplification could be observed when genomic DNA of unmatched species of *Plasmodium* were used as templates, indicating the specificity of the developed method (**Figure 2**).

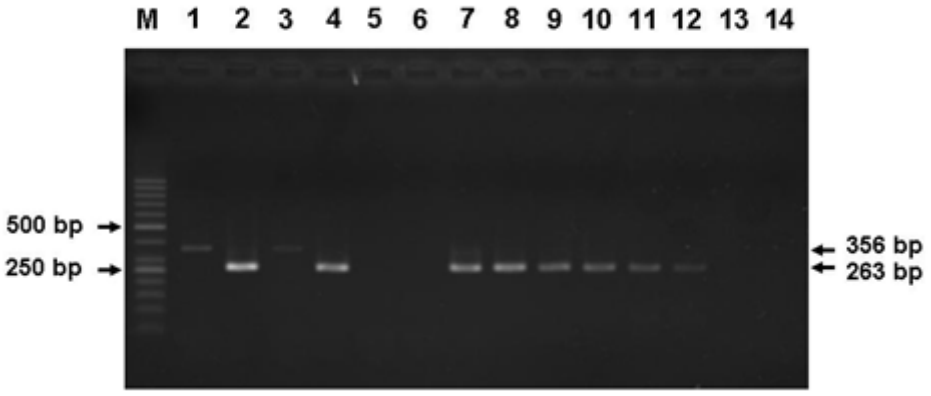

Figure 2. Representative 2% agarose gel electrophoresis of amplified products from nested RT-PCR targeting *Pvs25*. Lanes are M, 50-bp ladder marker; (1), primary PCR of *P. vivax* genomic DNA; (2), secondary PCR of *P. vivax* genomic DNA; (3) primary PCR of *P. vivax* cDNA; (4), secondary PCR of *P. vivax* cDNA; (5) water as negative control; (6) secondary PCR of *P. falciparum* genomic DNA; and lanes 7–14, secondary PCR using *Pvs25L* products as templates containing 10^6, 10^5, 10^4, 10^3, 10^2, 10, 1, and 0.1 copy, respectively. Molecular size is shown beside the gel.

Detection limit of nested Pvs25 RT-PCR

When known copy numbers of the Pvs25L fragment were analyzed in the nested *Pvs25* RT-PCR assay, reproducible amplification occurred with 10 or more copies of template DNA. Further dilution to 5 copies of template gave 2 positive results out of 5 repeated assays whereas no products were visible under agarose gel electrophoresis with amplification of 1 copy of DNA template. Therefore, the minimum detection limit of the nested *Pvs25* RT-PCR developed herein was 10 copies of template DNA (**Figure 2**).

Microscopy and nested mtCytb PCR

Of 180 field isolates, microscopy diagnosed 69 patients having *P. vivax* infections whereas 51 patients were infected with *P. falciparum*. The parasite densities for isolates containing *P. vivax* ranged from 40 to 33,000 parasites/μL (median, 8,520 parasites/μL). For *P. falciparum*, higher parasite densities were observed (range 7,840–164,800 parasites/μL, median 32,570 parasites/μL). Analysis by nested *Cytb* PCR revealed that 114 malaria-positive samples by microscopy showed concordant results (**Table 1**). Nested *Cytb* PCR detected 5 isolates having coinfections with both species (3 *P. vivax*- and 2 *P. falciparum*-positives by microscopy). One isolate containing only the ring stage with a parasite density of 1,480 parasites/μL diagnosed as *P. falciparum* by microscopy shown to be positive only with primers specific for *P. vivax*. Furthermore, five of 60 samples that were negative by microscopy contained *P. falciparum* (n = 3) and *P. vivax* (n = 2) as diagnosed by nested *mtCytb* PCR. In total, the ratios of positive detection of *P. falciparum* and *P. vivax* by nested *mtCytb* PCR to microscopy were 1.1 and 1.07, respectively. No other *Plasmodium* species were detected in this study population (**Table 1**).

Gametocyte detection by microscopy and nested Pvs25 RT-PCR

Characteristic *P. vivax* gametocytes were observed in 30 isolates by microscopy (40.5% of all *P. vivax* positives by nested *mtCytb* PCR), whereas 5 *P. falciparum*-positive isolates contained gametocytes on blood smears (8.9% of all *P. falciparum* positives by nested *mtCytb* PCR). By contrast, nested RT-PCR could detect *Pvs25* mRNA in 67 of 74 (90.5%) *P. vivax*-positive isolates. None of *P. falciparum*-positive samples gave positive tests for nested *Pvs25* RT-PCR. However, nested *Pvs25* RT-PCR gave negative results in isolates with cryptic *P. vivax* infections (2 microscopy-negative, but nested *Cytb* PCR-positive samples for *P. vivax*) (**Table 2**). Nested *Pvs25* RT-PCR was 2.23 times more sensitive than microscopy for detecting *P. vivax* gametocytes.

Evaluation of blood spotted on filter paper for Pvs25 mRNA detection

Total mRNA extracted from blood spotted onto filter papers of all 180 isolates before reverse transcriptase treatment did not generate visible products by nested *Pvs25* RT-PCR. On the other hand, cDNA from 65 of 74 (87.8%) *P. vivax* positives by nested *Cytb* PCR yielded positive results in nested *Pvs25* RT-PCR. All *P. falciparum* positive samples and 2 *P. vivax* positive samples by nested *Cytb* PCR gave negative results (**Table 2**). Therefore, *P. vivax* gametocyte detection by nested *Pvs25* RT-PCR using mRNA extracted from filter paper conferred positive results 2.17 times more than that by microscopy.

Table 1. Microscopy and nested *Cytb* PCR detection of *Plasmodium* infections in 180 febrile individuals

Nested *Cytb* PCR	Microscopy			Total
	P. vivax	*P. falciparum*	Negative	
P. vivax	66	1	2	69
P. falciparum	0	48	3	51
P. vivax and *P. falciparum*	3	2	0	5
Negative	0	0	55	55
Total	69	51	60	180

Table 2. Detection of malarial gametocytes by microscopy and nested *Pvs25* RT-PCR from blood preserved in RNA*later*® and dried blood on filter papers of 180 febrile individuals

Category	Microscopy	Nested *Pvs25* RT-PCR (RNA*later*®)		Nested *Pvs25* RT-PCR (Filter paper)	
		Positive	Negative	Positive	Negative
P. vivax					
asexual blood stages	69	NA	NA	NA	NA
gametocytes	30 (40.5%)*	67 (90.5%)*	2	65 (87.8%)*	4
P. falciparum					
asexual blood stages	51	NA	NA	NA	NA
gametocytes	5 (8.9%)*	0	51	0	51
Negative	60	0	60	0	60

NA = not applicable, *Percentage of gametocyte positive isolates per total *P. falciparum* positives (n = 56) or *P. vivax* positives (n = 74) by nested *mtCytb* PCR.

Discussion

Current intervention aiming at abrogation or reduction of malaria transmission among those harboring malarial gametocytes in circulation in Thailand is by administration of primaquine, an antigametocytocidal agent [21]. However, assessment of drug efficacy has been hampered by the low sensitivity of microscopy to diagnose gametocytes existing at densities lower than the microscopy detection threshold [7]. Although previous studies have applied different molecular methods to detect *Pvs25* mRNA, the sensitivity of detection has not been assessed in a study using real-time PCR assay [22], whereas another study reported a minimum detection limit of 100 copies of cDNA/μL based on real-time quantitative nucleic acid sequence-based amplification [23]. Herein, we devised a sensitive *Pvs25* mRNA detection using a nested RT-PCR method that could reproducibly detect as low as 10 copies of cDNA templates. The method did not confer cross-amplification with malaria species other than *P. vivax*, indicating the specificity of the test.

Despite that malaria transmission requires the presence of mature gametocytes in the peripheral circulation of infected hosts, both microscopically patent gametocytemic patients and those who had submicroscopic levels of gametocytemia can contribute to malaria transmission [17, 18]. The frequency of mosquito infection seems not to rely on the presence or absence of *P. vivax* gametocytes in the peripheral blood of infected Thai patients based on microscopic detection [24]. By contrast, a study in the Peruvian Amazon revealed that human-derived *P. vivax* isolates that contained gametocytes above the microscopy detection threshold were more infectious to the local malaria main vector species, *Anopheles darlingi*, than those with submicroscopic gametocytemia [22]. Discrepancies could arise from various factors such as differences in vector species that might influence oocyst development, differences in the numbers of mature gametocytes in clinical samples and differences in the competency of microscopists. These factors could be identified if both studies deployed the same molecular method for gametocyte detection. It is noteworthy that *Pvs25* mRNA is transcribed exclusively during maturation of gametocytes; therefore, positive detection indicates both species and stage of the parasite [16]. Although it has been suggested that most immature gametocytes of *Plasmodium* undergo sequestration, mainly in the bone marrow and probably in the spleen of infected hosts [9], it is not uncommon to observe immature gametocytes in the peripheral blood of malaria patients [25]. Accurate differentiation between near mature gametocyte (stage IV) and fully mature crescent-shape gametocytes (stage V) of *P. falciparum* by microscopy may not be ambiguous because of the discernible pointed ends of the former and rounded ends of the latter. However, such distinctive features observed under microscopy cannot be applied to *P. vivax* gametocytes because both stages are round to oval shaped, with only size difference [9]. Therefore, the nested RT-PCR developed in this study could circumvent limitation of microscopy in terms of sensitivity of gametocyte detection and accurate assignment of a mature gametocyte stage.

The prevalence of *P. vivax* gametocytes by microscopy detection has shown a significantly higher

rate among isolates collected during the dry season than during the rainy season in an endemic area of Thailand [26]. However, our study has revealed that substantial number of *P. vivax*-infected patients (90.5%) harbored gametocytemia by the time of sample collection through both seasons. Therefore, seasonal trends might not be observed when a more sensitive gametocyte detection method is used. Importantly, all *P. vivax*-infected patients in this study had febrile onset 1 to 2 days prior to blood sampling, suggesting that complete gametocytogenesis could occur early during the course of clinical symptoms. Similarly, Nakazawa and colleagues reported that microscopy detected gametocytes in a small subset (16.2%) of *P. falciparum*-infected patients in Tak province, whereas all the patients had *Pfs25* mRNA transcripts in their circulation, indicating the high prevalence of mature gametocytes in the study population [27]. It could be that early appearance of gametocytes seems to be common to both *P. falciparum* and *P. vivax*, albeit the majority having a level below that of microscopy detection. Presentation of gametocytes at the beginning of the illness could ensure transmission of malaria parasites to other hosts before being eradicated from the host's circulation by antimalarial drugs or being destroyed by host immunity [20]. It is important to note that recruitment of patients in our study is based on history taking, including those without febrile illness and taking antimalarial drugs during the 2 months before blood sample collection. The high prevalence of *P. vivax* gametocyte-positive samples in this study strongly suggests that clinical history was reliably obtained.

Collection of blood samples, particularly those that require RNA preservation could be compromised in some circumstances in malaria endemic areas with limited resource. Blood spotted onto filter papers has reportedly preserved the integrity of both host and *Plasmodium* RNA as demonstrated by successful detection of various cytokine mRNAs in malaria-infected mice [28] and *P. falciparum* specific mRNA, respectively [29]. In this study, nested RT-PCR could detect *Pvs25* mRNA from dried blood on filter papers obtained from 64 of 74 *P. vivax*-infected patients, whereas the same blood samples preserved in RNA*later*® offered 2 more positive results (2.9% difference in gametocyte-positive rates). Therefore, our study provides a supportive evidence that dried blood on filter papers offers satisfactory results, comparable those from with blood samples properly kept in RNA preservative for detection of *P. vivax* mRNA.

Conclusion

We have devised a sensitive and specific nested RT-PCR for detection of mature *P. vivax* gametocytes by amplification of *Pvs25* cDNA. Field application of the method revealed that substantial numbers of *P. vivax*-infected patients had mature gametocytes in circulation early in the course of infection. The method can be efficiently applied to both blood samples kept in RNA preservative and dried blood on filter paper.

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