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Hexaplex PCR Detection System for Identification of Five Human *Plasmodium* Species with an Internal Control

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Malaria remains one of the major killers of humankind and persists to threaten the lives of more than one-third of the world's population. Given that human malaria can now be caused by five species of *Plasmodium*, i.e., *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and the recently included *Plasmodium knowlesi*, there is a critical need not only to augment global health efforts in malaria control but also, more importantly, to develop a rapid, accurate, species-sensitive/species-specific, and economically effective diagnostic method for malaria caused by these five species. Therefore, in the present study, a straightforward single-step hexaplex PCR system targeting five human *Plasmodium* 18S small-subunit rRNAs (ssu rRNAs) was designed, and the system successfully detected all five human malaria parasites. In addition, this system enables the differentiation of single infection as well as mixed infections up to the two-species level. This assay was validated with 50 randomly blinded test and 184 clinical samples suspected to indicate malaria. This hexaplex PCR system is not only an ideal alternative for routine malaria diagnosis in laboratories with conventional PCR machines but also adds value to diagnoses when there is a lack of an experienced microscopist or/and when the parasite morphology is confusing. Indeed, this system will definitely enhance the accuracy and accelerate the speed in the diagnosis of malaria, as well as improve the efficacy of malaria treatment and control, in addition to providing reliable data from epidemiological surveillance studies.

The global impact of malaria, a parasitic disease transmitted through the bites of an infected female *Anopheles* mosquito, is persistent and very critical. The latest World Malaria Report stated that malaria affects approximately half of the world's population, with an estimated 216 million cases and 655,000 deaths worldwide in the year 2010. The main focal regions of this disease include Africa, Southeast Asia, and Eastern Mediterranean regions (37). Currently, malaria ranks as the fifth cause of death from infectious diseases, after respiratory infections, HIV/AIDS, diarrheal diseases, and tuberculosis (2).

The known causative agents of human malaria include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and the recently included *Plasmodium knowlesi*, which is still recognized as a zoonotic species. Generally, *P. vivax* infections have the widest global distribution, and this species is prevalent in subtropical and temperate regions, with 2.5 billion people at risk and 80 to 300 million cases reported annually (19). Although *P. falciparum* is the second most prevalent species, it is the most lethal species, as it is responsible for more than 90% of malaria mortality annually (12). The cytoadherence properties of *P. falciparum*-infected red blood cells (RBCs) to the host epithelium and sequestration into the peripheral vasculature are the main pathogenic factors for potential death from *P. falciparum* infection (38). *Plasmodium falciparum* infections are concentrated in the tropical and subtropical regions of Africa, Central and Southeast Asia, and America (12). Infections caused by other species are relatively sparse, with *P. malariae* being limited to subtropical areas (4) and *P. ovale* and *P. knowlesi* restricted to sub-Saharan Africa and forested areas in Southeast Asia, respectively (3, 6).

In addition to *P. falciparum* infection, infection with the newly recognized human pathogen *P. knowlesi* is also potentially fatal, especially when the parasite burden is high (hyperparasitemia) (28). Previously, *P. knowlesi* was considered a simian parasite. It

has been reported that human infection with *P. knowlesi* is not new but was misdiagnosed until the availability of molecular tools, such as PCR, enabled differentiation of *P. knowlesi* from *P. falciparum* and *P. malariae* (28). Morphologically, *P. knowlesi* resembles *P. falciparum* in the early blood ring stages and *P. malariae* in other stages (6). Currently, reports from an increasing number of epidemiology studies based on *knowlesi* malaria in Malaysian Borneo are being published, with improved information on disease severity and clinical complications (7–9, 35). The shortest erythrocytic replication period (24 h) and high parasite loads have been identified as the two key features that lead to severe malaria and malaria death in *P. knowlesi* infections. Thus far, seven malarial deaths caused by *P. knowlesi* infection have been reported in Sarawak state (7–9), and six have been reported in Sabah state (35). Although *knowlesi* malaria cases have been largely concentrated in the forested areas of Southeast Asian countries (6, 8, 28), traveler's cases of *P. knowlesi* have also been reported in Europe (1, 13). Hence, with increased global transboundary traveling, there is a critical need to include *P. knowlesi* infection in a malaria diagnosis. Only with an effective diagnostic tool to discriminate the five malaria species can efforts to heighten the awareness among key stakeholders (e.g., clinicians, public health officers, and patients) on *P. knowlesi* clinical manifestations and treatment management be achieved with greater success.

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In general, financial resources, an uncomplicated diagnostic procedure, and personnel training, as well as rapid availability of test results, are the major considerations in malaria diagnosis (18). To date, conventional light microscopy examination of Giemsa-stained thin and/or thick smear films still remains the gold standard for routine laboratory diagnosis, and this can be attributed to its rapidity, low cost, and simplicity, as well as its reliability in most settings, even though in many cases this detection approach has been shown to have low sensitivity and specificity, especially in low-level parasitemia cases, as well as in mixed infections (16, 18, 38). The microscopy approach is only accurate when the minimum parasite titer in a blood sample is 50 parasites/ μ l; however, it is able to identify and further quantify parasite numbers in the patient samples, both of which are significant in assessing the disease severity and the prescription of adequate therapy. Overall, the accuracy of results varies depending on the experience and skill of laboratory technicians (18, 38). Confusion due to morphological similarities of blood stages of some *Plasmodium* species with others is one of the major challenges in malaria diagnosis (5, 18). Misdiagnosis of the *Plasmodium* species, especially in overlooking lethal *Plasmodium* species, will have a significant negative impact on the effectiveness of treatment, control measures, and the prognoses for patients (16, 18). Essentially, such misdiagnosis will definitely stifle global malaria eradication efforts.

Alternatively, antigen detection based on immunochromatographic dipstick methods, which require only 15 to 20 min to obtain results and need less technical interpretation, are applied in most diagnostic laboratories. Nonetheless, factors such as variable detection thresholds and field stability, low sensitivity (100 to 200 parasites/ μ l), and most importantly the inability to distinguish the parasites at the species level limit the usage of available commercial kits in some cases (16, 18).

The advent of molecular tools such as PCR has provided more specific, sensitive, and reliable molecular techniques for the diagnosis of malaria (5, 16, 18). Genus-specific and species-specific sequences of the asexual stage-expressed 18S small-subunit (ssu) rRNA, circumsporozoite surface protein (csp), a nuclear gene encoding a cysteine protease, and the cytochrome *b* (*cytb*) gene have comprised several common targets in molecular studies of malaria (5, 18). PCR is able to detect parasites at a low titer of 5 parasites/ μ l of blood (18), and the most recent study attained a detection limit of 0.5 parasites/ μ l (16) in the latent or recrudescence stage of malaria disease. Although PCR assays have improved the sensitivity and specificity for the diagnosis of malaria over microscopic or rapid diagnostic tests (RDTs), in many cases the time duration required to obtain results is much longer, with at least 3 h to 1 day required for sample processing (16, 18).

PCR-based amplification assays, including nested PCR (27–29), seminested PCR (26), multiplex PCR (14, 20, 22), real-time or quantitative PCR (qPCR) (23, 25), and more recently, loop-mediated isothermal amplification (LAMP) (11), have been developed to identify malaria parasites to the species level. In general, nested PCR (27–29) has been considered the molecular gold standard for malaria detection. However, this assay is time-consuming and labor-intensive, because it requires a large number of reagents and disposable consumables, as at least six separate PCRs are needed to test for the five human malaria infection species. On the other hand, real-time PCR or qPCR is a rapid assay, and the result is obtained in a more straightforward manner based on completion of amplification without any downstream analysis through

gel electrophoresis. The qPCR assay also allows parasite quantification, which cannot be achieved by other conventional PCR approaches; however, the costs of reagents and equipment are much higher than for any conventional PCR assays. A more recent approach, LAMP, is a simple and less technically demanding technique that involves isothermal amplification of targeted DNA for identification of each *Plasmodium* species. LAMP results can be detected either by agarose gel electrophoresis or turbidity assessment by the naked eye or with a turbidimeter (11). However, the results obtained with LAMP are qualitative, and there is no specific indicator to determine whether it is a true-positive or false-positive result, due to potential contamination. Furthermore, large-scale screening efforts using LAMP are still under assessment. The development of a multiplex LAMP that includes many primers would also be challenging.

Therefore, to overcome these limitations and challenges, a straightforward single-step multiplex PCR system for simultaneous identification of five human *Plasmodium* species was developed. To date, the published descriptions of seminested and single-step multiplex PCR assays could only differentiate up to two species (mainly focused on *P. falciparum* and *P. vivax*) to four species (excluding *P. knowlesi*), although the PCR approach has very high specificity and sensitivity (14, 20, 22, 26). With the WHO malaria eradication programs back on the global health agenda, it is of paramount significance that a rapid, accurate, species-sensitive/species-specific, and economically efficient system be made available for the institution of effective treatment management and control. Essentially, with a highly effective diagnosis system, the ultimate aim is to increase the chances for an infected patient's recovery and survival, hence reducing morbidity and eliminating mortality due to malaria.

MATERIALS AND METHODS

Clinical sample collection. A total of 184 whole-blood samples were collected from patients suspected to have malaria and admitted to the University of Malaya Medical Center (UMMC), Kuala Lumpur, Malaysia, and the Telupid Health Clinic, Sabah, Malaysia, from 2008 to 2010. An aliquot of each sample was used for routine biological diagnosis in the laboratories, and the remainder of sample was extracted to obtain the total genomic DNA. Another 20 whole-blood samples were collected from healthy individuals and used as negative controls for the present study. Written informed consent was obtained from each patient and healthy donor before the blood sample was taken. The study was approved by the Ethics Committee reviewer board of the UMMC, Malaysia (MEC reference number 709.2).

Microscopic examination. For patients with suspected malaria, blood films were prepared at the time of admission prior to antimalaria treatment. Microscopic analysis was performed by experienced microscopists at each hospital, and the level of parasitemia was estimated. Thin and thick blood smears from whole-blood samples were stained with either 3% or 10% (vol/vol) Giemsa stain in phosphate-buffered saline (PBS; pH 7.2) and examined under a microscope for the presence of malaria parasites at a magnification of $\times 1,000$ with immersion oil. The parasite counts were estimated with a system using scores that ranged from + to + + + +, whereby + indicated 4 to 40 parasites/ μ l, ++ indicated 41 to 400 parasites/ μ l, +++ indicated 401 to 4,000 parasites/ μ l, and + + + + indicated $>4,000$ parasites/ μ l of blood (35). Blood films were defined as negative if no parasite was observed in 300 microscopic fields. The microscopy data (species and parasite counts) were retained for subsequent comparative analysis.

In the PCR sensitivity test, the total numbers of RBCs and parasitized RBCs were counted in at least 25 microscopic fields on thin blood smears.

TABLE 1 *Plasmodium* species and GenBank accession numbers for the 18S ssu rRNA sequences

<i>Plasmodium</i> species	Accession no.	No. of bp
<i>P. vivax</i>	X13926 U03079	~215
<i>P. ovale curtisi</i>	AB182489 AB182490 AF145337 L48987	~304
<i>P. ovale wallikeri</i>	AB182491 AB182492	
<i>P. cf. malariae</i> ^a	AF487999 AF488000	~341
<i>P. malariae</i>	AB489196 AB489195 AF145336	
<i>P. knowlesi</i>	L07560 AY327556 DQ350261 DQ350269 DQ641524	~284
<i>P. falciparum</i>	M19172	~453

^a *P. cf. malariae* refers to a species similar to *P. malariae*.

Parasitemia was expressed as the number of RBCs infected with asexual parasites divided by the total number of RBCs scanned in 25 microscopic fields, multiplied by the total RBC count/ μ l for the patient (36).

DNA template preparation. DNA was extracted from 200 μ l of each blood sample by using a QIAamp DNA minikit (Qiagen, Germany), according to the manufacturer's instructions, and then eluted into 100 μ l of sterile double-distilled water, thus producing a corresponding concentration of approximately 2 μ l of blood per μ l of DNA. The purity and concentration of DNA samples were determined using a NanoPhotometer apparatus (Implen, Germany). DNA was stored at -20°C , and 1.5- μ l aliquots were used for each PCR.

Design of hexaplex primers. The primers for the hexaplex PCR were designed to specifically amplify the region encompassing the multicopy, stable, and highly conserved 18S ssu rRNA gene of the five human *Plasmodium* spp., based on multiple alignments of sequences available in GenBank (Table 1). Basically, nucleotide sequences for all five *Plasmodium* species were aligned and analyzed for differences and conserved regions. The primer pairs were then designed and checked through a BLAST search (<http://www.ncbi.nih.gov>) to ensure no nonspecific amplification from nontarget loci. Finally, a single reverse genus-specific and five forward species-specific primers of human malaria parasites were designed for this multiplex PCR system. A pair of human housekeeping β -hemoglobin primers was added to this multiplex system, and they served as an internal control for the confirmation of a successful DNA extraction procedure and PCR.

Sensitivity and specificity analyses. The parasitemia levels of the five human *Plasmodium* spp., i.e., *P. vivax*, *P. falciparum*, *P. knowlesi*, *P. malariae*, and *P. ovale*, obtained from clinical samples were estimated, and each of the DNA samples was extracted. The extracted DNA of each species was then 10-fold serially diluted (10^{-1} to 10^{-7}) with a human DNA solution (10 ng/ μ l), and 1 μ l of the DNA mixture was used in each PCR in the sensitivity validation assay. The sensitivity and specificity of the present hexaplex system to five human *Plasmodium* spp. were compared using nested PCR as the molecular gold standard, whereas microscopy exami-

nation results were used as a reference for the presence of *Plasmodium* species. In addition, the specificity of the hexaplex PCR was also tested on non-*Plasmodium* organisms, i.e., *Toxoplasma* spp., *Giardia* spp., *Aeromonas* spp., *Serratia* spp., and *Vibrio* spp.

Hexaplex PCR amplification. Fifteen microliters of PCR reagent mixture containing 20 mM Tris-HCl, 20 mM KCl, 5 mM $(\text{NH}_4)_2\text{SO}_4$, 3.0 mM MgCl_2 , 0.2 μ M each deoxynucleoside triphosphate (dNTP), empirically determined concentrations of primers (0.6 μ M F_{453} , M_{341} , and O_{304} primers, 0.5 μ M K_{284} primer, 0.4 μ M V_{215} primer [all species-specific forward primers], 0.6 μ M single genus-specific reverse primer, and 0.25 μ M each β -hemoglobin primer [forward and reverse]), 1 U of Maxima Hot Start *Taq* DNA polymerase (Fermentas), and 1.5 μ l (~ 10 ng) of template DNA was used in the detection study. The reactions mixtures were subjected to an initial denaturation at 95°C for 5 min, 35 amplification cycles at 95°C for 30 s, 56°C for 30 s, and 65°C for 40 s, followed by a final extension at 65°C for 10 min in a thermal cycler (TaKaRa Bio, Japan). The amplified products were visualized on 3.0% (wt/vol) agarose gel (Promega, Madison, WI) stained with ethidium bromide.

Nested PCR amplification. The first nested PCR was performed in a final volume of 15 μ l that consisted of $1\times$ optimized Dream *Taq* buffer, 0.3 mM each dNTP, 0.3 μ M each of the rPLU1 and rPLU5 primers (27), 2.0 mM MgCl_2 , 1.5 μ l of DNA template (~ 10 ng), 1.0 U Dream *Taq* DNA polymerase (Fermentas). PCR amplifications were carried out with an initial denaturation step at 95°C for 3 min; 30 repeated cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min 30 s; and an extension step at 72°C for 7 min.

The second nested PCR mixture contained 10 mM Tris-HCl, 50 mM KCl, 0.08 (vol/vol) Nonidet P-40, 1.5 mM MgCl_2 , 0.2 mM each dNTP, 0.3 μ M each pair of primers, i.e., rFAL1/2, rVIV1/2, rMAL1/2, rOVA1/2, and Pmk8/Pmk9 (27, 28), and 0.5 U of *Taq* DNA polymerase (Fermentas), as well as 0.5 μ l of amplicons from the first nested PCR, used as the DNA template, in a final volume of 15 μ l. An alternative primer pair targeting both *P. ovale curtisi* and *P. ovale wallikeri* from the multiplex PCR was added to the second nested PCR (20). The PCR thermal cycling was the same as for the first nested PCR, with the exception that the temperature was changed to 72°C for 30 s in the 30 repeated cycles. Both PCR amplifications were performed in a thermal cycler (TaKaRa Bio). The first and second nested PCR products were visualized on 1% and 2% (wt/vol) agarose gels (Promega), respectively, stained with ethidium bromide.

Plasmodium plasmid constructions and DNA sequencing. Five human *Plasmodium* spp. clones encompassing whole 18S ssu rRNA genes were constructed. Approximately 1.6 to 1.7 kb of the ssu rRNA gene fragment of each species sample was amplified using the rPLU1 and rPLU5 primers (27) as described for the nested PCR amplification sessions. The PCR products were further gel purified using the QIAquick gel extraction kit (Qiagen, Germany) prior to cloning into the pCRII-Topo TA cloning system (Invitrogen Life Technologies) and transformed into Top 10 competent *Escherichia coli* cells. The successful constructs were selected by colony PCR based on the boiling approach (31) using T7 and SP6 primers and then sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems) for clone confirmation. Finally, the five *Plasmodium* clones developed in this study served as positive controls in the subsequent PCR amplifications.

Random blind test and clinical screening. To validate the sensitivity and specificity of the present hexaplex system for species determination, a random blind test on 50 DNA samples (randomly drawn from 171 microscopy-confirmed clinical samples) and a detection study on 184 clinical samples were carried out together with nested PCR for comparison purposes.

RESULTS

Microscopy examination. A total of 171 malaria samples used in the present study were confirmed by microscopy examination, with 154 samples diagnosed as single infections (67 cases of *P. vivax*, 51 cases of *P. falciparum*, 33 cases of *P. malariae*, and 3 cases

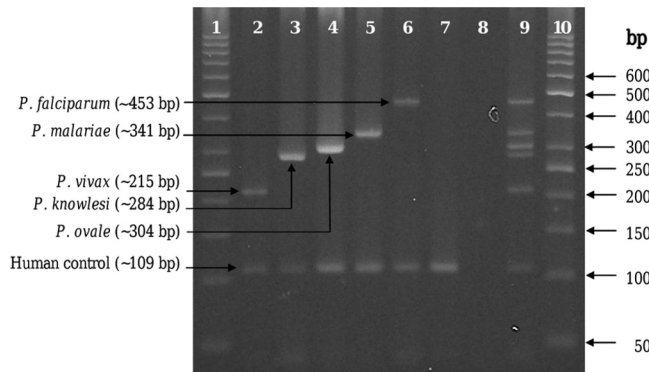


FIG 1 Hexaplex PCR results. All *Plasmodium* spp. primers target the 18S ssu rRNA genes of *Plasmodium* species. Lanes 1 and 10, 50-bp DNA ladder (Fermentas); lanes 2 to 6, DNA samples from malaria patients; lane 7, healthy control sample; lane 8, DNA blank; lane 9, hexaplex DNA ladder.

of *P. knowlesi*), 11 cases were either *P. malariae* or *P. knowlesi* infection, and 6 cases were mixed infections (two were *P. falciparum* mixed with *P. vivax*, two were *P. falciparum* mixed with *P. malariae*, and two were *P. vivax* mixed with *P. malariae*). Of those samples, 42% (72/171) were scored in the + category, 9% (15/171) were ++, 18% (31/171) were +++, and 31% (53/171) were +++. The other 13 blood samples from patients with malaria clinical symptoms, as well as samples from 20 healthy individuals recruited for the present study, served as negative controls, as the samples were negative by microscopy.

Primer design and hexaplex PCR optimization. Given that the ssu rRNA sequence normally tracks the phylogeny of a biological species accurately (5), alignment of the 18S ssu rRNA gene sequences from five human *Plasmodium* spp. was performed using the CLUSTAL W program (33) and BioEdit version 7.0.9 software (10) to obtain the consensus sequences for primer design. The primers were designed and tested for secondary structures, i.e., hairpin and dimer structures, possible false priming, and cross-dimers by using the Primer Premier software (Premier Biosoft International, CA). The primers with the secondary structure were redesigned with slight modifications. The selected primers were then tested through a BLAST search and *in silico* PCR (<http://insilico.ehu.es/PCR/>) as described previously for the prediction of amplicons (32, 34). PCR conditions for each of the primer pairs were optimized individually and then mixed together to perform a multiplex PCR for the detection of five species of human malaria parasites. Overall, the lengths of the PCR products generated from

this PCR assay were ~453 bp for *P. falciparum*, ~341 bp for *P. malariae*, ~304 bp for *P. ovale*, ~284 bp for *P. knowlesi*, and ~215 bp for *P. vivax* (Fig. 1). The human internal positive-control primer pair generated an ~109-bp PCR fragment (Fig. 1). This patented detection system (reference number PI 2010 003802) was produced by Reszon Diagnostics International.

Sensitivity analysis. The starting parasitemia levels of the blood samples were 25,000 parasites/ μ l of blood for *P. vivax*, 25,250 parasites/ μ l for *P. knowlesi*, 26,500 parasites/ μ l for *P. ovale*, 26,980 parasites/ μ l for *P. malariae*, and 15,000 parasites/ μ l for *P. falciparum*. The final detection limits of the hexaplex PCR can be lowered to 0.025 parasites/ μ l (10^{-6}) for *P. vivax*, 0.25 parasites/ μ l (10^{-5}) for *P. knowlesi*, 0.027 parasites/ μ l (10^{-6}) for *P. ovale*, 0.27 parasites/ μ l (10^{-5}) for *P. malariae*, and 0.15 parasites/ μ l (10^{-5}) for *P. falciparum* (Fig. 2).

The ratios between two or more parasite species can vary substantially in clinical samples with mixed infections (5, 38). Therefore, DNA and primer competition effects in mixed infection samples were also tested. Granted that there is a lack of natural acquired mixed infections, three experimental mixtures, i.e., 100 pg/ μ l of *P. vivax* clone DNA and 1, 10, or 100 pg/ μ l of *P. falciparum* clone DNA, were experimentally created to determine the sensitivity of this hexaplex PCR. The serial dilutions of the DNA mixtures were performed using a DNA solution containing 10 ng/ μ l of human DNA. This was taken into consideration due to the presence of a large amount of human DNA in the actual clinical samples; in addition, a pair of human housekeeping primers was included as internal positive controls in the present detection system. The inclusion of human DNA to simulate mixed clone DNA also aimed to validate the possibility of detection constraints for the hexaplex PCR.

Subsequently, a sensitivity test with 12 different mixtures of DNA using two of the five human *Plasmodium* spp. for each set, together with another set of mixtures with all five *Plasmodium* spp., resulted in successful detection in the simulated mixtures (a total of 60 mixtures were tested) (data not shown). A total of 10 possible combinations of DNA to create two-species-simulated mixed infections with equal clone DNA concentrations (100 pg/ μ l) is shown in Fig. 3. In addition, to test whether these primers could improve the detection of clinical mixed infections, a total of 30 experimental random mixed infections (2 to 3 *Plasmodium* species) were created using clinical samples, and these were screened using the present hexaplex PCR and a nested PCR as the molecular gold standard for identifying mixed infections. All

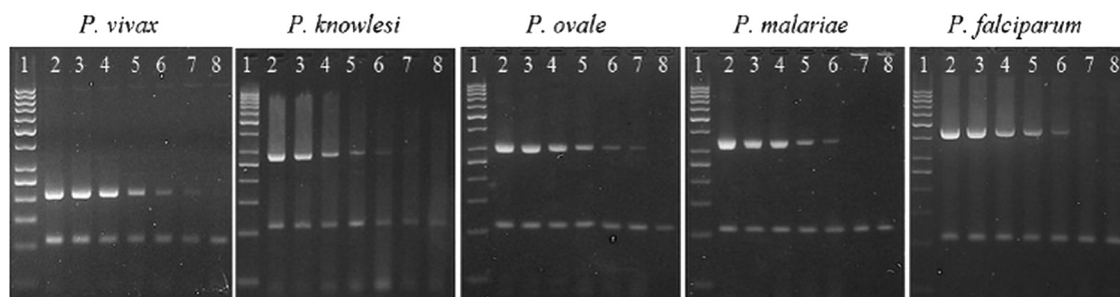


FIG 2 Detection limits of the hexaplex PCR system to five human *Plasmodium* species. Lane 1, 50-bp DNA ladder (Fermentas); lanes 2 to 8, 10-fold serial dilutions of all five *Plasmodium* spp. DNA samples from malaria patients, using 10 ng/ μ l of human DNA. Dilutions of *Plasmodium* DNA samples: lane 2, 10^{-1} ; lane 3, 10^{-2} ; lane 4, 10^{-3} ; lane 5, 10^{-4} ; lane 6, 10^{-5} ; lane 7, 10^{-6} ; lane 8, 10^{-7} .

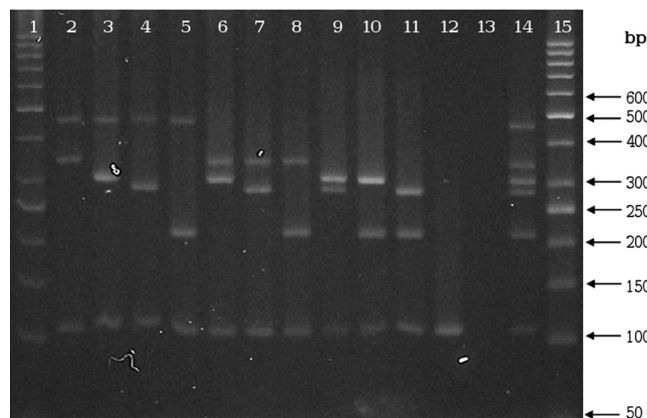


FIG 3 Hexaplex PCR results based on simulated mixed cloned DNA. Lanes 1 and 15, 50-bp DNA ladder (Fermentas); lane 2, *P. falciparum* and *P. malariae*; lane 3, *P. falciparum* and *P. ovale*; lane 4, *P. falciparum* and *P. knowlesi*; lane 5, *P. falciparum* and *P. vivax*; lane 6, *P. malariae* and *P. ovale*; lane 7, *P. malariae* and *P. knowlesi*; lane 8, *P. malariae* and *P. vivax*; lane 9, *P. ovale* and *P. knowlesi*; lane 10, *P. ovale* and *P. vivax*; lane 11, *P. knowlesi* and *P. vivax*; lane 12, human healthy control DNA; lane 13, DNA blank; lane 14, hexaplex DNA ladder.

mixed *Plasmodium* spp. infections were successfully detected in the simulated mixtures of clinical samples (Fig. 4).

Specificity analysis. To estimate the analytical specificity of the *Plasmodium* hexaplex assay, DNA samples obtained from 2 isolates of non-*Plasmodium* organisms, i.e., *Toxoplasma gondii*, *Giardia duodenalis*, *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas caviae*, *Aeromonas aquariorum*, *Serratia* sp., *Vibrio harveyi*, and *Vibrio parahaemolyticus*, were screened. DNA from 20 healthy individuals was also tested for the specificity and possibility of nonspecific fragments. None of the *Plasmodium* PCR product was detected by hexaplex PCR when the DNA was obtained from all non-*Plasmodium* samples (data not shown). Only an approximately 109-bp band that represented the human β -hemoglobin gene in human samples was observed on the gel.

Random blind testing and clinical screening. The present hexaplex system demonstrated the specificity of the PCR primers for their target species. In addition, it also ensured the sensitivity and specificity for at least two-species detection based on experimentally created mixed infections without any significant diagnostic limitations. Both the hexaplex and nested PCR gave concordant results for species determinations in all 50 samples in the random blind test as well as in the 184 clinical samples. Among 171 microscopy-confirmed malaria samples, PCR assays indicated that there was only one case of mixed infection (i.e., *P. falciparum* and *P. vivax*), with the rest of the 170 malaria samples showing single infection. PCR multiplex-based species identification showed 39.8% (68/171) of samples were *P. vivax*, 29.8% (51/171) were *P. falciparum*, 28.1% (48/171) were *P. knowlesi*, and 1.2% (2/171) were *P. malariae*, as well as an imported *P. ovale* case (0.6%; 1/171), which was misdiagnosed as *P. vivax* by microscopy (15). Among 13 microscopy-negative clinical samples, 5 samples were for single infections (i.e., 1 by *P. falciparum*, 1 by *P. vivax*, and 3 by *P. knowlesi* infections). The results were further confirmed by nested PCR, and similar results were obtained.

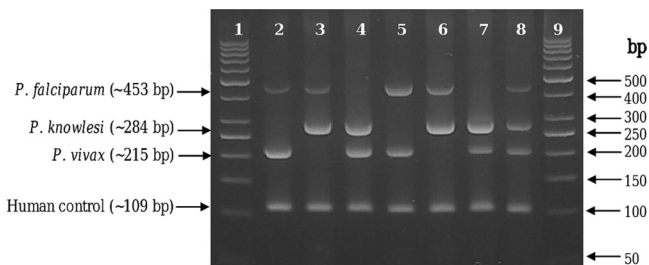


FIG 4 Hexaplex PCR results based on simulated clinical mixed infections. Lanes 1 and 9, 50-bp DNA ladder (Fermentas); lanes 2 to 7, random mixed infections of two *Plasmodium* species; lane 8, random mixed infections of three *Plasmodium* species.

DISCUSSION

Malaria is a serious global health challenge, and the disease can become severe, leading to morbidity if left untreated, especially for infections by two fatal species, i.e., *P. falciparum* and *P. knowlesi*. Thus far, effective malaria vaccines are still unavailable (17, 24); therefore a rapid, accurate, species-sensitive/species-specific, and economically efficient diagnostic tool is crucial to increase the effectiveness of treatment and control management.

Giemsa-stained blood smears are the most widely used approach for malaria diagnosis. However, this method is often challenging for *Plasmodium* species identification, especially when parasitemia drops below 40 parasites/ μ l of blood, as well as in cases of mixed infections (18, 38). The detection limit can vary among microscopists, with the level routinely in excess of 50 parasites/ μ l, and down to approximately 5 to 10 parasites/ μ l for an expert (38). Conventional PCR assays, such as nested PCR (27–29), seminested PCR (26), and multiplex PCR (20), have shown in many cases to improve the sensitivity and specificity over microscopic evaluations or RDTs, as they can detect parasitemia levels of <5 parasites/ μ l and probably as low as 0.004 parasites/ μ l for *P. ovale* (20).

Multiplex PCR is one of the research priorities for malaria diagnosis in the future (16). Generally, multiplex PCR systems face difficulties in primer design and the cumbersome optimization steps that are needed to establish a highly sensitive and specific assay. To the best of our knowledge, two multiplex PCR assays based on conventional PCR technology (i.e., a seminested PCR [26] and a single-tube multiplex PCR [20]) that can detect up to four species, excluding *P. knowlesi*, have been described. In light of the emergence of potentially fatal human infections with *P. knowlesi*, which was previously considered a zoonotic parasite, a primer targeting the ssu rRNA of *P. knowlesi* was designed and included in the present hexaplex diagnostic system.

In 2010, Sutherland and coworkers reported that there were two nonrecombining sympatric forms of *P. ovale* with dimorphism of genetic haplotypes, i.e., *P. ovale curtisi* (classic type) and *P. ovale wallikeri* (variant type), and only *P. ovale curtisi* can be identified by nested PCR based on the primers targeting the ssu rRNA gene (30). For this reason, an alternative PCR primer pair specific to both *P. ovale* subspecies (20) was also included in the second nested PCR. In our present study, we took this into consideration by designing a degenerate *P. ovale* primer that covered both the classic and variant types of *P. ovale*. However, due to sample limitation, the detection of both *P. ovale* variants could only be tested through *in silico* PCR. In this study, the *P. ovale*

clone developed was confirmed as *P. ovale curtisi* through sequencing. As there were only one *P. ovale* and two *P. malariae* infection cases detected during the study period, more samples must be obtained in the future from other countries (especially African countries) to carry out further sensitivity and specificity validation tests, especially for the case of *P. ovale wallikeri* infection.

In our development of this hexaplex PCR assay, all the optimization steps as well as sensitivity and specificity tests were performed using five *Plasmodium* spp. from clinical samples with known parasitemia. The sensitivity and specificity of the assay were also ensured by random blind testing ($n = 50$) and clinical screening ($n = 184$), as well as experimental mixed infections based on clinical samples ($n = 30$) and cloned DNA ($n = 60$), and they showed concordance with results using nested PCR. The present hexaplex PCR revealed a high sensitivity, with the final detection limits stated above in Results. All five human *Plasmodium* parasites were detectable below 0.5 parasites/ μ l of blood sample in the sensitivity validation.

Overall, optimized nested PCR in our laboratory was able to detect *Plasmodium* species at a low titer, with an average of 0.01 parasites/ μ l, which is slightly more sensitive than our hexaplex PCR system. However, our present single-round hexaplex PCR results showed 100% concordance with the results obtained through nested PCR. Furthermore, five missed malaria infections were successfully detected from 13 microscopy-negative clinical samples. This may have been due to the fact that our tested samples had a parasitemia level above the detection limits of both approaches. The only limitation of the present study was that we were unable to further evaluate the sensitivity and specificity for *P. malariae* and *P. ovale*, due to limited samples in this study period; however, the current hexaplex PCR has been validated on sufficient numbers of clinical malaria samples, especially for the three most prevalent malaria agents in our local area, i.e., *P. vivax*, *P. falciparum*, and *P. knowlesi*, which have multiple ranges of parasitemia counts; most significantly, the system successfully and consistently picked up the two most potential fatal infectious agents, i.e., *P. falciparum* and *P. knowlesi*.

In mixed infections, primers and DNA competition effects can contribute to inconsistencies of results in most of multiplex PCR assays; however, the multiplex PCR assay developed and described here did not present this problem. The only limitation in evaluation of the present hexaplex PCR is the lack of naturally acquired mixed infections. However, in the mixtures of two parasite species in the 60 experimentally simulated mixed infection tests using clones DNA with different DNA ratios and 30 clinical samples, we successfully identified the species in all the samples, thereby confirming that this hexaplex PCR system is robust and capable of detecting mixed infections at least up to the two-species level without any diagnostic constraints.

In addition, the hexaplex primer set used here was specific to all five human *Plasmodium* spp., with no nonspecific band observed in all-non-*Plasmodium* samples or in samples from 20 human healthy controls. As the internal positive-control primer pair used in the hexaplex PCR system targeted the human β -hemoglobin gene, an approximately 109-bp fragment was present in all of the human DNA samples. A random blind test based on 50 DNA samples was conducted, and no cross-reactions among the five species-specific primers were observed. This assay was further validated with 184 clinical samples, and the results demonstrated

100% concordance with the results obtained through nested PCR, the current molecular gold standard. There was only one case of *P. vivax*-*P. falciparum* mixed infection detected among 171 microscopy-confirmed samples in the present study. The rest of the samples were single-species infections, including another five samples identified by microscopy as mixed infections: one *P. falciparum*-*P. vivax* infection as well as two *P. malariae*-*P. vivax* mixed infections (as identified by microscopy) were actually single infections of *P. vivax*, while another two cases of *P. falciparum*-*P. knowlesi* mixed infection were single infections of *P. knowlesi*. Interestingly, only two samples PCR confirmed as *P. malariae* infection (4.5%) among the 44 samples identified with microscopy as either *P. malariae* or *P. malariae*/*P. knowlesi* infection. This finding was similar to that in the study done by Singh and colleagues in 2004, indicating that most microscopy-diagnosed *P. malariae* infections turn out to be *P. knowlesi* infections based on a nested PCR assay (28). Both findings further support the sensitivity and specificity of PCR assays over microscopic examination for the diagnosis of malaria, especially for *P. knowlesi* cases.

Some of the main advantages of the present novel hexaplex PCR system over available seminested (26) and nested (27–29) PCR assays include the hexaplex system being less labor-intensive and faster (reduced hands-on time) due to its single-step PCR, compared to at least two and/or six PCRs conducted for the seminested and nested PCR assays, respectively, in the identification of all five *Plasmodium* spp. The fewer steps reduce the usage of PCR reagents and disposable consumables. The time required for this single-step hexaplex PCR is only 3 h, versus approximately 6 h for seminested PCR and about 20 h for nested PCR, including time for PCR preparation, conducting the PCR, and agarose gel electrophoresis; thus, the current hexaplex PCR significantly shortens the time for diagnosis. In addition, a single-tube reaction is also recommended, as it avoids the risk of carryover and possible external contamination during transfer of PCR amplicons from primary to secondary PCR mixtures and agarose gel electrophoresis for the detection of PCR products. However, this contamination can be avoided if good laboratory practices for molecular amplification techniques are followed.

Currently, multiplex PCR is widely applied for the rapid diagnosis of many infectious diseases (20, 31, 32, 34). With well-designed primers used under fully optimized conditions, most of the multiplex systems have shown high sensitivity and specificity. For instance, the results from our present hexaplex PCR and nested PCR assays were in agreement. Multiplex PCR is an ideal and affordable method in most of laboratories or field settings, especially in developing countries or countries with lower economic resources, since the conventional PCR thermal cycler and agarose gel electrophoresis system remain the cheapest molecular assay compared with the real-time PCR assay, which utilizes expensive reagents (probes), consumables, and equipment. In addition, multiplex PCR assays also allow large-scale screening of samples within a short period, especially in high-malaria-burdened regions where there are limited clinical personnel and experienced microscopists. The workflow for microscopic examination can be very burdensome and time-consuming when huge sample sizes must be handled.

The lack of consistency in the current standard operating procedures and diagnostic approaches among different laboratories is the major constraint for malaria epidemiology studies. This problem has made it difficult for results from distinct

diagnostic approaches to be compared. To reconcile differences between the management and the diagnosis of malaria, it is important to develop a diagnostic standard or parameter that facilitates interlaboratory comparisons of results. A good example is the development of the first WHO International Standard for *P. falciparum* DNA nucleic acid amplification technique (NAT)-based assay. This assay was calibrated in an international collaborative study involving 14 laboratories from 10 different countries and can be used to consistently access *P. falciparum* detection methods (21). It is hoped that similar standards for other *Plasmodium* species will be developed in the near future to accommodate implementation of consistency for multiplex assays among laboratories.

Overall, the hexaplex PCR system highlighted in this paper provides a set of primers that are sensitive and specific for simultaneous detection of single infections as well as mixed infections. Screening of *Plasmodium* spp. with this assay has proven to be rapid, beneficial, and could significantly improve detection procedures. The availability of this system may indeed facilitate efforts in the global malaria eradication programs. With a rapid and highly effective diagnostic system such as this, evaluation of malaria morbidity and mortality will be more accurate. In addition, this tool can be used for future global molecular epidemiological studies to produce consistent and reliable data regarding malaria distribution, including sub-clinical cases.

Conclusions. A hexaplex PCR method targeting 18S ssu rRNA genes and that accurately detects all five human malaria parasites in single infections as well as two-species mixed infections was successfully developed in this study. Significantly, the rapidity of this straightforward single-step procedure offers a more practical and clinically acceptable alternative for accurate and effective diagnosis of patients presenting with symptoms indicative of malaria disease. This hexaplex PCR assay is currently available commercially from Reszon Diagnostics International.

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