

Evaluation of new multiplex PCR primers for the identification of *Plasmodium* species found in Sabah, Malaysia

Cheronie Shely STANIS¹, Beng Kah SONG², Tock Hing CHUA^{1,*}, Yee Ling LAU³, Jenarun JELIP⁴

¹Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Kota Kinabalu, Sabah, Malaysia

²School of Science, Monash University, Bandar Sunway, Malaysia

³Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

⁴Jabatan Kesihatan Negeri Sabah, Malaysia

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Background/aim: Malaria is a major public health problem, especially in the Southeast Asia region, caused by 5 species of *Plasmodium* (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*). The aim of this study was to compare parasite species identification methods using the new multiplex polymerase chain reaction (PCR) against nested PCR and microscopy.

Materials and methods: Blood samples on filter papers were subject to conventional PCR methods using primers designed by us in multiplex PCR and previously designed primers of nested PCR. Both sets of results were compared with microscopic identification.

Results: Of the 129 samples identified as malaria-positive by microscopy, 15 samples were positive for *P. falciparum*, 14 for *P. vivax*, 6 for *P. knowlesi*, 72 for *P. malariae*, and 2 for mixed infection of *P. falciparum*/*P. malariae*. Both multiplex and nested PCR identified 12 *P. falciparum* single infections. For *P. vivax*, 9 were identified by multiplex and 12 by nested PCR. For 72 *P. malariae* cases, multiplex PCR identified 58 as *P. knowlesi* and 10 as *P. malariae* compared to nested PCR, which identified 59 as *P. knowlesi* and 7 as *P. malariae*.

Conclusion: Multiplex PCR could be used as alternative molecular diagnosis for the identification of all *Plasmodium* species as it requires a shorter time to screen a large number of samples.

Key words: Multiplex polymerase chain reaction, malaria, microscopy, *Plasmodium*

1. Introduction

The World Health Organization (WHO) reported that in 2012 malaria caused 207,000,000 clinical cases and about 627,000 deaths worldwide. The report also stated that, 3,400,000,000 people still live in areas that are at risk of malaria transmission in 106 countries (<http://www.cdc.gov/malaria/about/facts.html>). The African region has the highest number of deaths with 562,000 people dying, half of which are children, and this is followed by the Southeast Asia region with 42,000 deaths annually (1).

Malaria is a major public health problem in tropical and subtropical areas, especially in the Southeast Asia region. Until recently, there were 4 recognized species of *Plasmodium* (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) that cause human malaria. *P. knowlesi*, found in many parts of Southeast Asia (2–4), previously misidentified as *P. malariae* and originating from Old World monkeys, is now recognized as the fifth malarial species and has also been recorded outside of Asia (5). Human malaria parasites are highly specific with humans

as the host and with female *Anopheles* mosquitoes (e.g., *An. balabacensis*) as the vectors (6).

Conventional light microscopy using Giemsa-stained blood films is a simple method and considered as the 'gold standard' to detect malaria parasites. This method is still being used to diagnose malaria as it can determine parasite morphologies and differentiate stages among *Plasmodium* species. However, it is time-consuming, it requires a well-trained microscopist, and the accuracy varies depending on the expertise of the microscopist. Misdiagnoses may occur when there is mixed infection and low levels of parasitemia (7).

Even though the rapid diagnostic test (RDT) performs a fast detection of *Plasmodium* species, it has limitations in identifying all of the 5 human malaria species, with only *P. falciparum* and *P. vivax* being identified without problems (8,9). Furthermore, the RDT also may not be suitable for mixed infections, as it only reports the absence or presence of *Plasmodium* sp. (8) and performs poorly in *P. knowlesi* cases (10).

* Correspondence: chuath.ums@gmail.com

Polymerase chain reaction (PCR) offers a good alternative to microscopy in terms of sensitivity and specificity and could be used as a confirmatory tool for microscopy identification of malaria parasites (11,12). Molecular methods, such as single-step PCR (13), loop-mediated isothermal amplification (LAMP) assay (14), nested PCR (15,16), multiplex PCR (17,18), multiplex real-time PCR (19), and real time PCR (20–23) basically use conventional PCR techniques to detect and identify malaria parasites. Even though PCR assay initially requires expensive equipment (a thermal cycler and gel electrophoresis tools and documentation), it is considered reasonably cheap in the long run for large-scale malaria detection and speciation, and it is highly sensitive in terms of detection (17), especially in regions where malaria is endemic.

The aim of this study was to design a set of primers based on different genes of *Plasmodium* and test these new primers in multiplex PCR against nested PCR and microscopy on blood samples collected from the Kudat Division of Sabah, Malaysia. It is hoped that this new set of highly sensitive primers could lead to more efficient detection and speciation of malaria parasites in patients.

2. Materials and methods

2.1. Study sites and sample collection

Blood samples in the form of spots on Whatman filter paper were collected from patients by hospital personnel in the Kudat Division, Sabah, where the malaria incidence is high, at 3 locations: Hospital Kudat, Hospital Kota Marudu, and Hospital Pitas (Figure 1). Sample collection

was done from March 2012 to December 2013. This study was approved by the National Medical Ethics Committee, Sabah, Malaysia (NMRR-11-453-9471).

For *P. ovale*, SSU rRNA nest 1 PCR plasmid clone 54 (MRA-180, BEI Resources/MR4, ATCC Manassas, VA, USA), contributed by PA Zimmerman, was included in the multiplex PCR. This was used as the positive control for *P. ovale*. The plasmid vector preparation was as described in a previous study (24).

2.2. Microscopy

Microscopic identification of malaria parasites was performed by technicians using microscopy at magnification of 100× with immersion oil in each hospital following the method as described by Trape (25). The parasitemia determination for field sample (number of parasites per microliter of blood) was performed using the formula of number of parasites per white blood cell (WBC) in thick blood films multiplied by 8000.

2.3. Extraction of parasite DNA

The DNA extraction from blood filter paper samples was performed using a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions with minor modifications (elution time was extended to 5–10 min to yield more DNA and 60 µL of DNA was eluted for the first elution to get more concentrated DNA) and samples were stored at –20 °C until used as the PCR template.

2.4. Primer designing

Four genes were amplified, namely the cytochrome b gene (*cytb*), the dihydrofolate reductase gene (*dhfr*), the small subunit ribosomal RNA gene (*SSU rRNA*), and the merozoites surface antigen gene (*Msp-1*) (Table 1). The gene

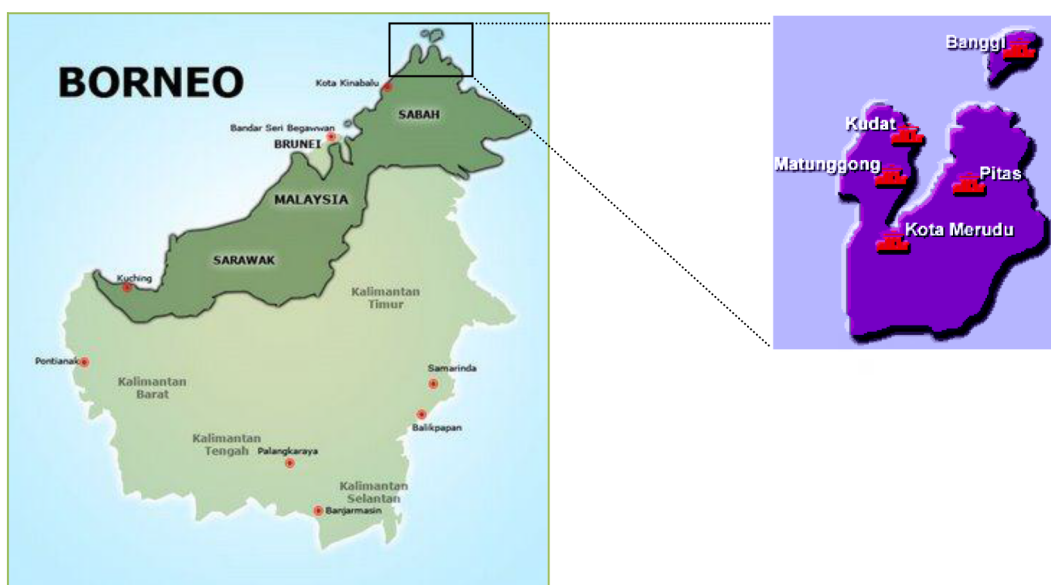


Figure 1. Map of Borneo Island showing the administrative regions in the northern part of Sabah.

Table 1. Details of primers for the selected gene sequences of various *Plasmodium* species. Primer for *P. ovale* was taken from Singh et al. (26).

Species	Target gene	Primer code	Melting temp, T _m (°C)	GC content (%)	Expected size (bp)	GenBank accession no.
Human	Beta-actin gene	ACTB_F2 ACTB_R2	60.8 57.1	55.0 55.6	208	M10277
<i>P. falciparum</i>	Cytochrome b gene	T6-PFCYF2 T7-PFCYR2	63.2 63.5	45.0 47.6	456	JX893153
<i>P. vivax</i>	Merozoite surface protein gene	T19-PVMF1 T20-PVMR1	62.9 60.6	50.0 50.0	320	GQ890970
<i>P. knowlesi</i>	Dihydrofolate reductase gene	T8-PKDF1 T30-PKDR3	65.2 61.7	52.2 45.0	111	JQ409299
<i>P. malariae</i>	Merozoite surface protein gene	T22-PMMF2 T23-PMMR1A	60.1 60.2	45.0 40.0	539	FJ824669
<i>P. ovale</i>	Small subunit ribosomal RNA gene	rOVA 1 rOVA 2	53.5 55.7	26.7 36.7	787	AF145337

sequences of all 4 human *Plasmodium* parasites occurring in Sabah (namely *P. falciparum*, *P. malariae*, *P. vivax*, and *P. knowlesi*) were taken from the GenBank website (www.ncbi.nlm.nih.gov/nucleotide). The sequences were then aligned using Clustal2W Software (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and analyzed manually for differences to generate new forward and reverse primers for multiplex reaction. The newly designed primers were checked using the BLAST program and analyzed individually to check for hairpin formations using OligoAnalyzer 3.1 Software (<http://eu.idtdna.com/calc/analyzer>). Finally, the primer for *P. ovale* (26) and a pair of human housekeeping β -actin gene primers (to serve as an internal control) (27) were added in the multiplex reaction.

2.5. Sensitivity and specificity analyses

For sensitivity testing, the parasite samples used were F29 for *P. knowlesi* (42,067 parasites/ μ L of blood), K60 for *P. falciparum* (6760 parasites/ μ L of blood), P4 for *P. vivax* (10,450 parasites/ μ L of blood), and F46 for *P. malariae* (11,047 parasites/ μ L of blood). The parasite DNA of each of these samples was mixed with human DNA solution and serially diluted 10-fold (from 10^{-1} to 10^{-6}) to establish the minimum number of parasites that could be detected. For specificity testing, each of the primers was tested against all human *Plasmodium* species and simian *Plasmodium* species (*P. inui* and *P. coatneyi*).

2.6. Multiplex polymerase chain reaction amplification

A volume of 20 μ L of reaction mixture, which had 2 μ L of DNA template, was added to a PCR master mix containing 2.5 mM MgCl₂, 200 μ M deoxynucleoside triphosphates (dNTPs), various predetermined concentrations of primers (0.25 pmol Pf, Pk, Po; 0.45 pmol Pv, Pm; 0.15

pmol ACTB), and 1.0 U of GoTaq Flexi DNA polymerase (Promega, USA) in the PCR buffer. The final volume of master mixture was adjusted with nuclease-free water. The amplification involved a single round of PCR by using PCR thermal cycler ESCO Swift MaxPro that started with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, ending with a final extension at 72 °C for 10 min. Between 8 to 10 μ L of PCR product was loaded onto a 3% agarose gel (Promega, Madison, WI, USA), which was the recommended gel percentage (28), for 30 min at 100 V using 1X TAE buffer. The gels were stained with ethidium bromide for 15 min and were visualized under an ultraviolet transilluminator.

For the samples found to be negative by multiplex PCR, they were subject to singleplex PCR for reidentification.

2.7. Nested PCR amplification

Nested PCR was carried out according to the method described previously (26,29) with a slight modification (using 25 μ L of master mixture for nest 1 instead of 50 μ L). Nest 2 amplification conditions were identical to all species-specific primers (rFAL 1 and 2, rMAL 1 and 2, rVIV 1, and Pmk8 and Pmkr9) for which the annealing temperature was 58 °C. Other conditions remained the same and all PCR reactions were carried out using a thermal cycler (ESCO Swift MaxPro).

2.8. Cloning and construction of *Plasmodium* plasmids

The selected PCR amplicons of each *Plasmodium* sp. were sent to First BASE (First BASE Laboratories Sdn. Bhd., Selangor, Malaysia) for cloning in glycerol stock to be used as a positive control for the multiplex PCR and stored at -80 °C in a freezer.

2.9. Multiplex PCR on artificial mixed infections and random blind samples

Artificial mixed infections were obtained by mixing 2 µL of clone DNA of each *Plasmodium* species and tested with multiplex PCR using 2 µL of the mixture. In addition, a total of 10 blind blood samples were randomly chosen for further validation of the multiplex PCR protocol.

2.10. Clinical sample screening and analysis of percentage sensitivity and specificity

The identification of *Plasmodium* species in 129 samples determined by microscopy and evaluated by multiplex PCR and Singh's protocol were: *P. falciparum* (n = 15), *P. vivax* (n = 14), *P. knowlesi* (n = 6), *P. malariae* (n = 72), mixed *P. falciparum/P. malariae* (n = 2), and healthy samples (n = 20). The multiplex PCR results were compared with the nested PCR of Singh and microscopy methods. The results were put into a 2 × 2 table (Table 2) and the sensitivity and specificity percentages were calculated based on the formulas according to Wong and Lim (30) in Table 2.

3. Results

According to the microscopic examination the malaria species of 72 out of 129 samples were identified as *P. malariae*, 15 as *P. falciparum*, 14 as *P. vivax*, 6 as *P. knowlesi*, and 2 as mixed infection of *P. falciparum/P. malariae*, while another 20 samples were negative.

For the 72 *P. malariae* cases, multiplex PCR identified 58 as *P. knowlesi*, but 10 as *P. malariae* and 2 as negative (F31 and F44), whereas nested PCR identified 59 as *P. knowlesi*, 7 as *P. malariae*, and 4 negative (F31, K38, K41, and K33). Both multiplex PCR and nested PCR identified 12 out of 15 of *P. falciparum* single infections, 2 cases of *P.*

knowlesi, and 1 negative sample (Table 3). For *P. vivax*, only 9 were identified by multiplex and 12 by nested PCR, while 1 case was identified as *P. knowlesi* by both PCR methods. In addition, 4 *P. vivax* cases were shown to be negative by multiplex, while 1 was shown as negative by nested PCR. Both nested and multiplex PCR identified the mixed infection of *P. falciparum/P. malariae* as *P. falciparum* monoinfection. The 20 noninfected samples taken from healthy persons were used as the negative control and only human DNA was amplified by multiplex PCR. An example of the result of multiplex PCR assay is shown in Figure 2.

The negative results produced by multiplex PCR were reevaluated using the same primer pairs of multiplex PCR but each primer was singly run using singleplex PCR. When the multiplex PCR primers were used in a singleplex, it worked well. For example, the singleplex PCR successfully amplified the *P. knowlesi* sample (F44) collected from Hospital Kudat, which was not detected using multiplex PCR (lane 3, Figure 3). Singleplex PCR also produced similar results to multiplex PCR where a *P. malariae* sample (K33) was successfully amplified (lane 7, Figure 3), which was negative by nested PCR.

For random blind testing, the multiplex PCR successfully amplified the targeted genes of the 10 blood samples each containing a different *Plasmodium* sp. and produced the expected band in the electrophoresis gel. Three of the blood samples were found to be infected with *P. knowlesi* (S1, S6, and S7), 4 with *P. falciparum* (S2, S3, S4, and S9), 2 with *P. malariae* (S5 and S10), and 1 with *P. vivax* (S8) (Figure 4).

The parasitemia levels used for sensitivity analysis determined using microscopic examination were

Table 2. Results of a comparative diagnostic test presented as a 2 × 2 table.

Results of diagnostic test to be compared with "standard"	Results of "standard" diagnostic test	
	Species present	Species absent
Test positive for the species	True positive (a)	False positive (b)
Test negative for the species	False negative (c)	True negative (d)
$\% \text{sensitivity} = \frac{\text{Number of true positives}}{(\text{Number of true positives} + \text{Number of false negatives})} \times 100$ $= \frac{a}{(a + c)} \times 100$ (1)		
$\% \text{specificity} = \frac{\text{Number of true negatives}}{(\text{Number of false positives} + \text{Number of true negatives})} \times 100$ $= \frac{d}{(b + d)} \times 100$ (2)		

Table 3. Results of identification of malaria species using microscopy and PCR methods. Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pk = *P. knowlesi*; Pm = *P. malariae*; Neg = Negative results (uninfected).

<i>Plasmodium</i> species (identified by microscopy)	Pf	Pv	Pk	Pm	Pf + Pm	Neg	Total of species identified by PCR
No. of samples	15	14	6	72	2	20	
Multiplex PCR							
Pf	12	-	-	2	2	-	16
Pv	-	9	-	-	-	-	9
Pk	2	1	6	58	-	-	67
Pm	-	-	-	10	-	-	10
Neg	1	4	-	2	-	20	27
Summary	12 Pf	9 Pv	6 Pk	2 Pf	2 Pf	20 Neg	129
	2 Pk	1 Pk		58 Pk			
	1 Neg	4 Neg		10 Pm			
				2 Neg			
Nested PCR							
Pf	12	-	-	2	2	-	16
Pv	-	12	-	-	-	-	12
Pk	2	1	6	59	-	-	68
Pm	-	-	-	7	-	-	7
Neg	1	1	-	4	-	20	26
Summary	12 Pf	12 Pv	6 Pk	2 Pf	2 Pf	20 Neg	129
	2 Pk	1 Pk		59 Pk			
	1 Neg	1 Neg		7 Pm			
				4 Neg			

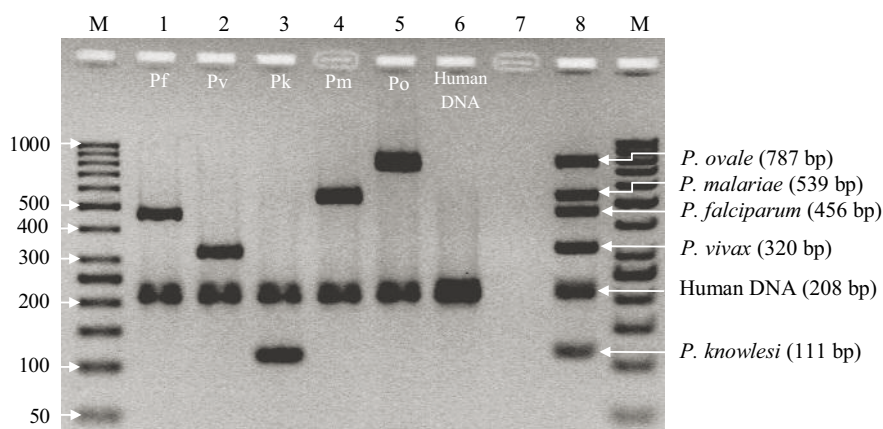


Figure 2. Results of multiplex PCR assay of malaria samples. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder, Lane 1: *P. falciparum*, Lane 2: *P. vivax*, Lane 3: *P. knowlesi*, Lane 4: *P. malariae*, Lane 5: *P. ovale*, Lane 6: healthy sample human DNA, Lane 7: negative control (DNA blank), Lane 8: positive control for all 6 *Plasmodium* species and human DNA.

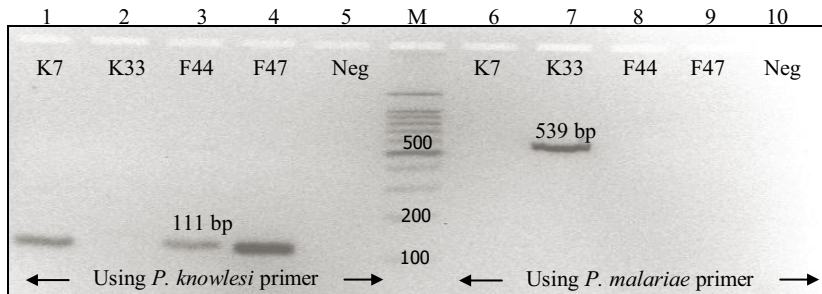


Figure 3. An example of results from singleplex PCR assay using our primers on blood samples containing *P. malariae* as identified by microscopy. Samples K7, F44, and F47 were identified as *P. malariae* while K33 was identified as *P. knowlesi*. Lane M: 100 bp DNA ladder (Promega), Lanes 1 and 6: blood sample K7, Lanes 2 and 7: blood sample K33, Lanes 3 and 8: blood sample F44, Lanes 4 and 9: blood sample F47, Lanes 5 and 10: negative control (DNA blank).

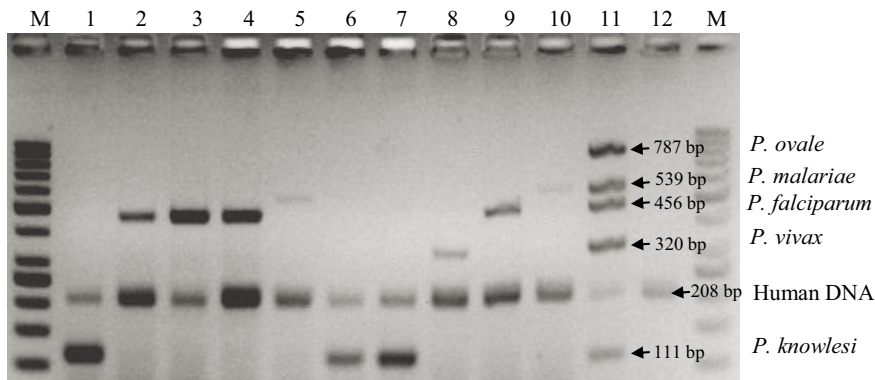


Figure 4. Results of multiplex PCR assay of samples containing *Plasmodium* species in blind testing. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder; Lanes 1, 6, and 7: blood samples (S1, S6, and S7) from patients infected with *P. knowlesi*; Lanes 2, 3, 4, and 9: blood samples (S2, S3, S4, and S9) from patients infected with *P. falciparum*; Lanes 5 and 10: blood samples (S5 and S10) from patients infected with *P. malariae*; Lane 8: blood sample (S8) from patient infected with *P. vivax*; Lane 11: positive control for all 6 species; Lane 12: negative control (DNA blank).

42,067 parasites/ μL of blood for *P. knowlesi*, 6760 for *P. falciparum*, 10,450 for *P. vivax*, and 11,047 for *P. malariae* for samples F29, K60, P4, and F46 respectively. The parasitemia of *P. ovale* was unknown as the samples were provided by a researcher from the United Kingdom. As the parasitemia estimate cannot be assumed to be accurate and the volume of the blood spot was not known, it is not possible to calculate accurately the detection level of the malaria species in terms of parasites/ μL . Nevertheless, based on the PCR results of the above mentioned samples, the malaria species were identifiable up to the detection of 10^{-3} dilution for *P. knowlesi*, 10^{-5} for *P. falciparum*, 10^{-3} for *P. vivax*, 10^{-4} for *P. malariae* primers, and 10^{-2} for *P. ovale* primers (Figure 5). This is equivalent to estimates of parasitemia of 42 parasites/ μL of blood for *P. knowlesi*, 0.06 for *P. falciparum*, 10 for *P. vivax*, and 1 for *P. malariae*.

For specificity analysis, the results showed that each of the multiplex primers is only specific to a particular human *Plasmodium* species in PCR of blood samples containing only 1 of the 5 *Plasmodium* species and no nonspecific fragments were observed (Figure 6). When simian *Plasmodium* (*P. inui* and *P. coatneyi*) from macaques were tested for specificity, we found that there was no amplification in the multiplex PCR. Only the human internal control beta-actin gene and the positive control for both simian *Plasmodium* species were observed on the gel (Figure 7).

In multiplex PCR the positive control of *P. ovale* was created by mixing the sample with a human DNA sample for the detection of both the amplified gene from *P. ovale* and human DNA. For the simulated mixed cloned DNA of the 5 *Plasmodium* species, the expected band for each

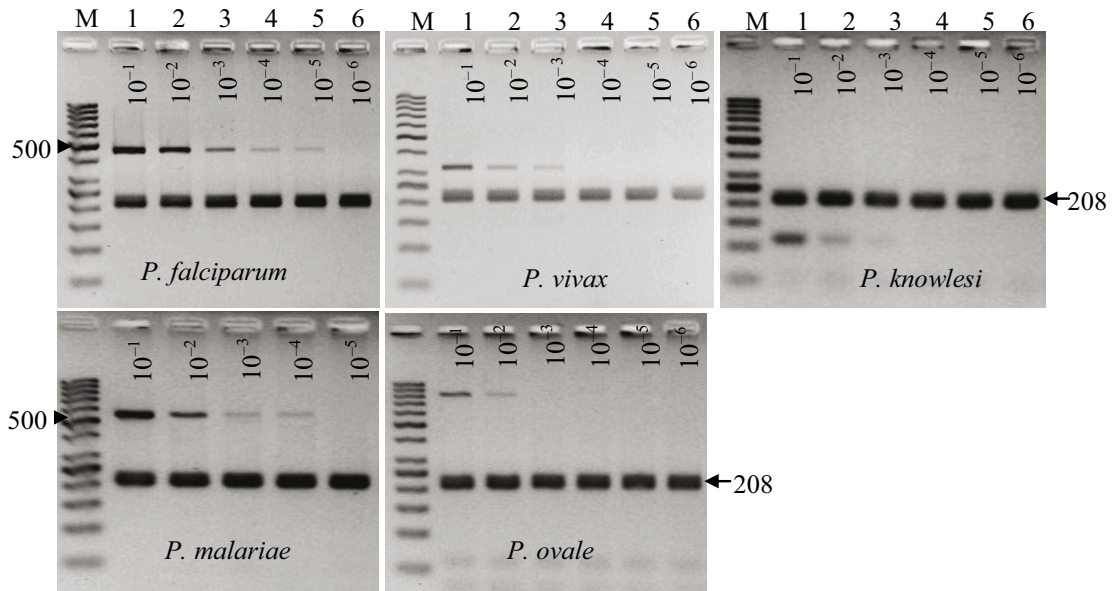


Figure 5. Sensitivity tests of primers for each of the *Plasmodium* species using blood samples. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder; Lane 1: 10^{-1} dilution, Lane 2: 10^{-2} dilution, Lane 3: 10^{-3} dilution, Lane 4: 10^{-4} dilution, Lane 5: 10^{-5} dilution, Lane 6: 10^{-6} dilution.

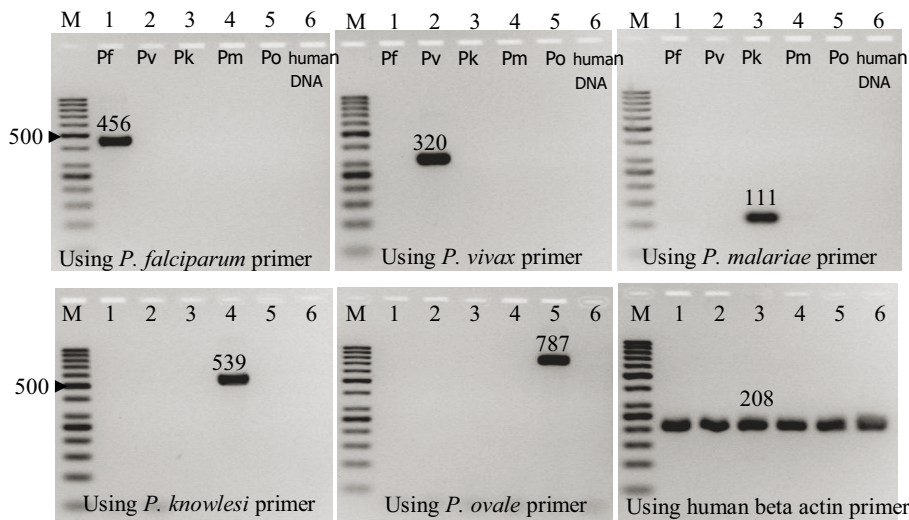


Figure 6. Specificity PCR tests of each of the new primers against all 5 human *Plasmodium* spp. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder; Lane 1: blood sample containing *P. falciparum*, Lane 2: *P. vivax*, Lane 3: *P. knowlesi*, Lane 4: *P. malariae*, Lane 5: *P. ovale*, Lane 6: human DNA.

Plasmodium species was clearly amplified identifying the species (Figure 8), even for the simulated sample containing terms of sensitivity analysis using Eq. (1) in Table 2, for *P. falciparum*, microscopy was only 75% sensitive compared to both nested PCR and multiplex PCR (Table 4), while nested PCR achieved 75% sensitivity when compared to multiplex PCR. On the other hand, both microscopy and nested PCR achieved 100% sensitivity for *P. vivax* and *P.*

malariae when compared to the multiplex PCR method, respectively. For *P. knowlesi*, microscopy showed only 8.8% sensitivity as compared to nested PCR and 8.9% sensitivity as compared to multiplex PCR, whereas nested PCR showed 86.6% sensitivity compared to multiplex PCR.

As for specificity analysis using Eq. (2) in Table 2 for *P. knowlesi*, microscopic identification was as good as molecular identification with 100% specificity. However,

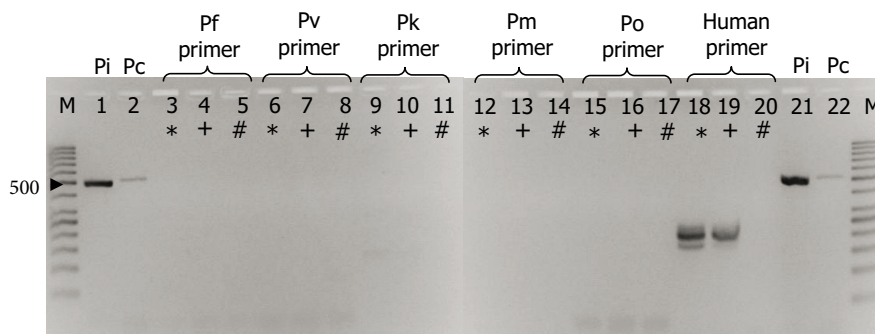


Figure 7. Specificity PCR test of all 6 primers against simian *Plasmodium* spp. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder; Lanes 1 and 21: positive control of *Plasmodium inui* (479 bp) (Pi); Lanes 2 and 22: positive control of *Plasmodium coatneyi* (504 bp) (Pc); (*)Lanes 3, 6, 9, 12, 15, and 18: DNA template containing *P. inui*; (+)Lanes 4, 7, 10, 13, 16, and 19: DNA template containing *P. coatneyi*; (#)Lanes 5, 8, 11, 14, 17, and 20: negative control (dH₂O).

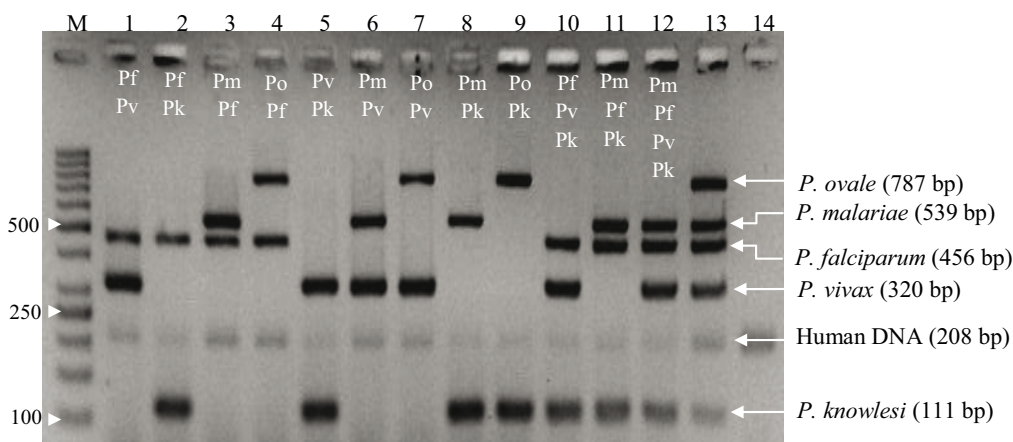


Figure 8. Results of multiplex PCR assay using simulated mixed cloned DNA. Lane M: Thermo Scientific GeneRuler 50 bp DNA ladder; Lane 1: *P. falciparum* and *P. vivax*; Lane 2: *P. falciparum* and *P. knowlesi*; Lane 3: *P. falciparum* and *P. malariae*; Lane 4: *P. falciparum* and *P. ovale*; Lane 5: *P. vivax* and *P. knowlesi*; Lane 6: *P. vivax* and *P. malariae*; Lane 7: *P. vivax* and *P. ovale*; Lane 8: *P. knowlesi* and *P. malariae*; Lane 9: *P. knowlesi* and *P. ovale*; Lane 10: *P. falciparum*, *P. vivax*, and *P. knowlesi*; Lane 11: *P. falciparum*, *P. malariae*, and *P. knowlesi*; Lane 12: *P. falciparum*, *P. vivax*, *P. knowlesi*, and *P. malariae*; Lane 13: positive control for all species; Lane 14: negative control (DNA blank).

when nested PCR was compared with multiplex PCR, the specificity for nested PCR was only 66.7%.

For *P. vivax*, the specificity test results for microscopy versus nested PCR and multiplex PCR were 91% and 80%, respectively. Only 87% specificity was shown by nested PCR when compared to multiplex PCR.

Similarly, for *P. falciparum*, only 87% specificity was achieved by microscopy when compared to both nested and multiplex PCR. However, nested PCR only showed 83.3% specificity for *P. falciparum* when compared to multiplex PCR.

The lowest specificity was about 23% by the microscopy method for *P. malariae* as compared to both the molecular

methods. Comparing the nested PCR method against multiplex PCR, the specificity for nested PCR was 100% (Table 4).

4. Discussion

In this multiplex system the mitochondrial cytochrome b gene for *P. falciparum* was targeted because it has a highly conserved region. The high degree of conservation might be due to structural constraints on the genome and the fact that their genome size and gene arrangement remain unchanged for a long period of time (31). Therefore, with improved PCR technique, the cytochrome b gene can also be targeted (32). The dihydrofolate reductase gene

Table 4. Comparison of sensitivity and specificity of the 3 diagnostic tests, i.e. microscopic identification, molecular identification using nested PCR, and multiplex PCR primers.

Species	Percentage (%)	Comparison of identification methods		
		Microscopy Nested PCR	Microscopy Multiplex PCR	Nested PCR Multiplex PCR
<i>P. falciparum</i>	Sensitivity	75	75	75
	Specificity	87	87	83.3
<i>P. vivax</i>	Sensitivity	100	100	100
	Specificity	91	80	87
<i>P. knowlesi</i>	Sensitivity	8.8	8.9	86.6
	Specificity	100	100	66.7
<i>P. malariae</i>	Sensitivity	100	100	100
	Specificity	23.6	24.4	100

was targeted in identifying *P. knowlesi* in this study as it provides good sensitivity for PCR assay (33). Although previously this gene was only studied because of its own unique characteristics, especially due to the point mutation that causes drug resistance (34), this gene is important in multiplex PCR as the system provides faster identification and detection, and hence effective treatment or drugs for *P. knowlesi* infection could be implemented immediately. *MSP-1* was chosen in this study as the target gene for *P. vivax* and *P. malariae* because high polymorphisms are detected in this gene, which are often used as markers to detect parasites from the DNA of malaria patients (35).

The combination of newly designed primers for all 4 human malaria parasites with the existing primers of *P. ovale* (26) has provide a good multiplex PCR system. Besides, the target gene of this species is highly conserved and, according to a comparable study (36), the *P. ovale* primers of nested PCR are able to detect levels as low as 0.4 parasites/ μ L of blood as compared to multiplex PCR (18) and seminested multiplex PCR (37), thus ensuring the effectiveness of *P. ovale* identification in our multiplex PCR.

Of the 129 blood samples, 72 of them identified by microscopy were diagnosed to be infected with *P. malariae*. However, both multiplex and nested PCR identified only 10 and 7 samples, respectively, actually as *P. malariae*, and most of them were identified as *P. knowlesi*, which were evidently misdiagnosed due to the similar morphology between these species. Most of the *P. malariae* positive samples were collected from Hospital Kota Marudu and only a few from Hospital Kudat and Hospital Pitas.

The 2 cases of mixed infection of *P. falciparum*/*P. malariae* identified by microscopy were actually *P. falciparum* monoinfection, as determined by both PCR

methods. The reason why microscopy misdiagnosed the films is probably that the level of parasitemia was high (3,494,400 parasites/ μ L), leading to too much overlapping of parasites. False positives could occur due to the poor blood film preparations (8), but this can be overcome by improving the slide preparation technique (38).

We chose to use blood spots on Whatman filter paper for sample collection as it has been shown (39) that blood spot filter paper does not result in signal loss when the DNA extraction is not performed immediately, as compared to a fresh whole blood sample. It was reported that the success of the PCR technique relies on the quality of DNA, which could be affected by factors such as inappropriate blood sample collection and sample storage (15). Therefore, Cox-Singh et al. (40) suggested that coupling the blood sampling, for example dried blood on filter paper and the molecular extraction method, might increase the sensitivity of parasite detection and identification.

The ability to detect *P. knowlesi* by singleplex and not multiplex, for example as in F44 (lane 3, Figure 3), might be due to factors like primer and DNA template competition, which may complicate the multiplex reaction since all primers that are specific to each of the *Plasmodium* species are combined together. It has been demonstrated that optimization of primer concentration plays an important role since the concentration of primer for the "weak" loci region must be increased while that of the "strong" loci region must be decreased (28).

Microscopy can be considered as the cheapest method for identifying *Plasmodium* species with the shortest hands-on time in handling a single sample. Unfortunately, comparison among microscopy and molecular methods (41) revealed that microscopy lacks sensitivity and specificity even though it is cost-effective. In addition,

this method is not suitable to screening large numbers of samples in a short period of time since it requires experienced microscopists to handle a large number of samples. If the level of parasitemia is low in a sample, it will take 45–90 min to examine the parasites, excluding the slide preparation (27).

Although nested PCR provides a reliable method for identifying *Plasmodium* species (42) based on one target gene (SSU rRNA) that is highly conserved, it is not as rapid as the multiplex PCR proposed here. For every unknown sample the set of whole nested primers has to be used in order to identify the *Plasmodium* species. This method may not be suitable for screening large numbers of samples in a short period of time as it requires 2 successive PCR amplifications, and it is costly as more reagents are needed (26,29). The time taken for nested PCR to be completed from DNA extraction until gel band analysis is approximately 6 h per sample.

In contrast to nested PCR, our multiplex primers target multiple genes (Table 1) and could be considered as a faster and more cost-effective molecular method for screening a large number of samples within a short period of time. One advantage of this single-step multiplex PCR is that it has relatively fewer steps and hence uses less PCR reagents and disposable consumables (17,18,43). Overall, the system has shortened the time needed to identify *Plasmodium* species to approximately 3 h including DNA extraction per sample, which is half the time needed for nested PCR. Others also found that single-round multiplex PCR can be completed within 3 h for all *Plasmodium* species upon the arrival of a specimen (18,44). More importantly, a single tube reaction is also preferred in order to avoid the risk of contamination during transfer of materials from one PCR tube to a second tube.

In 2013, prevalences of the 4 *Plasmodium* species in Sabah as given by the Sabah State Health Department

were 35% for *P. knowlesi*, 29% for *P. falciparum*, 30% for *P. vivax*, and 6% for *P. malariae*. Therefore, it is important to examine *P. knowlesi* accurately as the erythrocytic replication period is the shortest (24 h) among the species, which may lead to death of the patient. It has been reported that *P. knowlesi* was the commonest severe infection occurring in the main hospital in Kota Kinabalu (Hospital Queen Elizabeth), Sabah (10,45). As such, this study suggests that our multiplex primers could be of great potential as an alternative method as they can distinguish *P. knowlesi* from other malarias.

The comparative study highlighted in this paper provides information about which method is faster and more accurate in identifying the *Plasmodium* species so that patients can be treated quickly and effectively. Screening of *Plasmodium* species using the PCR assays has proven to be rapid and may be useful in monitoring the global molecular epidemiological studies and also in providing reliable data regarding malaria distribution.

In conclusion, multiplex PCR assay could be used as an alternative molecular diagnosis for the detection of all 5 *Plasmodium* species and it can shorten the time required to screen a large number of samples. Moreover, this assay performed especially well for identifying *P. knowlesi*, which was the most common species infecting patients in Sabah, Malaysia.

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