Comparison of Three Molecular Methods for the Detection and Speciation of Five Human Plasmodium Species

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Abstract. In this study, three molecular assays (real-time multiplex polymerase chain reaction [PCR], merozoite surface antigen gene [MSP]-multiplex PCR, and the PlasmoNex Multiplex PCR Kit) have been developed for diagnosis of Plasmodium species. In total, 52 microscopy-positive and 20 malaria-negative samples were used in this study. We found that real-time multiplex PCR was the most sensitive for detecting P. falciparum and P. knowlesi. The MSP-multiplex PCR assay and the PlasmoNex Multiplex PCR Kit were equally sensitive for diagnosing P. knowlesi infection, whereas the PlasmoNex Multiplex PCR Kit and real-time multiplex PCR showed similar sensitivity for detecting P. vivax. The three molecular assays displayed 100% specificity for detecting malaria samples. We observed no significant differences between MSP-multiplex PCR and the PlasmoNex multiplex PCR kit (McNemar’s test: P = 0.1489). However, significant differences were observed comparing real-time multiplex PCR with the PlasmoNex Multiplex PCR Kit (McNemar’s test: P = 0.0044) or real-time multiplex PCR with MSP-multiplex PCR (McNemar’s test: P = 0.0012).

INTRODUCTION

Malaria is a devastating disease that affects people in many tropical and subtropical regions. The World Health Organization has estimated that 627,000 people died of malaria in 2012. Five Plasmodium species are known to cause malaria in humans: P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi. Among these species, P. falciparum causes the greatest morbidity and mortality in humans.2 Also, the simian malaria infecting humans, P. knowlesi, is known to produce rapid hyperparasitemia because of its ability to replicate every 24 hours, which can result in life-threatening complications and death.3

In endemic regions, individuals have been found to display simultaneous infections involving more than one human malaria species.4 These mixed infections, which are often unrecognized and underestimated, produce non-specific clinical manifestations of malaria that contribute to presumptive diagnosis and treatment of patients.5 For this reason, laboratory tools that are capable of accurately diagnosing all five human malaria species are essential for estimating clinical prognosis and monitoring therapeutic responses, particularly within geographical areas that harbor drug-resistant parasites.

Existing methods of malaria diagnosis include microscopy, rapid diagnostic tests (RDTs), and molecular tools.6 However, for more than a century, microscopy has been the gold standard for malaria diagnosis in endemic areas, because it is relatively inexpensive and allows for quantification of parasitemia. Nevertheless, this method requires highly experienced personnel to accurately diagnose and quantify malaria parasites. Indeed, two- to threefold discrepancies in parasite quantification can be observed between individuals. In fact, even experienced microbiologists have found to misdiagnose cases involving low parasitemia or mixed infections.7–9 Moreover, morphological similarities between P. knowlesi and P. malariae often result in misdiagnosis.10 Although RDTs are generally specific for detecting P. falciparum and P. vivax infections, they are non-specific for other malaria species.11 Additionally, RDTs are known to produce false-positive results because of residual antigen, which can persist for weeks after treatment and parasite clearance.12

Molecular methods, such as nested polymerase chain reaction (PCR) and real-time PCR, allow accurate species identification and are valuable for distinguishing species in cases of mixed malaria infection.13 In this regard, Snounou and others14 developed a nested PCR method, which targets the 18S ribosomal DNA (rDNA) gene and allows for the discrimination between distinct malaria species based on differentially sized PCR products. Although this method is widely used, it is expensive and laborious, because it requires separate PCR reactions for each human malaria species. Therefore, efforts have been made to develop single-reaction assays for the rapid and specific identification of all five human malaria species. In this regard, protocols for seminested multiplex15 and single-round multiplex PCR16 were recently established based on targeting a region of the 18S gene. Notably, in a recent report published by Mixson-Hayden and others,17 the seminested multiplex assay was found to display similar sensitivity to the nested assay for the detection of P. falciparum and P. vivax but more sensitivity for identifying P. malariae (0.04 parasites [p]/μL). Its detection threshold for P. ovale was found to be 4 p/μL. However, the detection limits for the multiplex single-round protocol were found to be 4 p/μL for P. ovale and 40 p/μL for P. malariae and P. vivax. In addition, a high-throughput multiplex S′-nuclease quantitative PCR (qPCR) assay was recently developed by Reller and others18 for clinical diagnosis of human malaria species. This method was reported to be highly sensitive for all five Plasmodium species, showing a detection limit of 1–6 p/μL blood. Furthermore, the PlasmoNex Multiplex PCR Kit, which was commercially introduced in 2012, claims to detect all five human malaria species and requires a DNA concentration of at least 35 ng/μL. Nevertheless, preliminary results obtained in our laboratory have indicated that this kit shows low sensitivity for detecting P. knowlesi infection. In this study, we have evaluated the sensitivity and specificity of three molecular methods (the PlasmoNex Multiplex PCR Kit, in-house merozoite surface antigen gene (MSP) -multiplex PCR, and real-time multiplex PCR) using microscopy and nested PCR as gold standards.

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9 INTRODUCTION

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MATERIALS AND METHODS

Clinical samples. Fifty-two malaria-positive blood samples were collected from the University Malaya Medical Center (UMMC) in Malaysia. These samples were evaluated using microscopy and nested PCR assay. In addition, 20 blood samples were collected from healthy donors. Ethical approval for this study was obtained from the Medical Ethics Committee of the UMMC (reference number 817.18).

Microscopic examination. Thick and thin blood films were prepared and examined by skilled personnel who had extensive experience in identifying malaria parasites. Parasitemia was assessed per 1,000 erythrocytes in thin films and per 200 white blood cells (WBCs) in thick films. Films were considered as negative if no parasites were observed after counting 500 leukocytes.

Rapid diagnostic kit. The BinaxNOW Malaria Kit (Innverness Medical International, United Kingdom) was used to analyze microscopy-confirmed cases. The manufacturer’s protocol was, however, modified to use a drop of ethylenediaminetetraacetic acid (EDTA)-treated whole blood. Test results were analyzed after 15 minutes.

DNA extraction from whole blood. For nested PCR and multiplex PCR, DNA was extracted from the blood samples of malaria-infected and non-infected patients using the DNAeasy Blood and Tissue Kit based on the manufacturer’s protocol (Qiagen, Hilden, Germany). Briefly, proteinase K (20 μg/mL) was added to EDTA-treated whole blood (100 μL). Buffer AL (200 μL) and ethanol (200 μL) were subsequently added. The sample was transferred to a DNAeasy spin column and centrifuged. The flow-through was discarded, and buffer AW1 (500 μL) was added. The column was centrifuged again, and the flow-through was discarded. This step was repeated using buffer AW2. The column was placed into a fresh tube and the DNA was eluted using buffer AE (50 μL). Finally, the purified DNA was stored at −20°C until further use.

Nested PCR assay. Species identification for the malaria-infected samples was achieved through nested PCR, which targets the Plasmodium small subunit ribosomal RNA (ssrRNA) gene. The primers used for the assay have been previously described.10,18 The reaction mixture for the first PCR step included 4 μL DNA template, 250 mM/L each primer (Primer for 18S rDNA), 1 μL diluted Taq polymerase (supplied with the kit) was added to each PCR tube, which contained PlasmoNex PCR mix. DNA was extracted from the blood sample (1.5 μL) and added to the PCR mix. PCR amplification was initiated at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 30 seconds, and final extension at 72°C for 2 minutes. Final extension was performed at 72°C for 5 minutes, after which the PCR products were analyzed by electrophoresis using 3% (wt/vol) agarose gels.

Primer design. Based on GenBank sequence alignments, multiplex PCR primers were designed to specifically amplify a distinct region of the MSP for each of the human Plasmodium species (Table 1).

MSP-Multiplex PCR. Initially, a monoplex PCR was carried out for each primer set to determine specificity, which was followed by rigorous optimization to develop our multiplex PCR detection assay. Briefly, multiplex PCR was performed in a 25 μL reaction mixture that contained 4 μL template DNA, 1× buffer, 0.4 μL each P. knowlesi, P. ovale, P. vivax, and P. falciparum primers, 0.8 μM P. malariae primers, 200 μM dNTP mix, 1.5 mM MgCl2, and 1 U Taq polymerase (Promega, Madison, WI). PCR amplification was initiated at 95°C for 5 minutes, which was followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and elongation at 72°C for 2 minutes. Final extension was performed at 72°C for 5 minutes. The amplified PCR products were resolved by agarose gel electrophoresis, and the amplified fragment lengths were determined by ethidium bromide staining and gel documentation.

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5' to 3')</th>
<th>Amplicon (bp)</th>
</tr>
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<tbody>
<tr>
<td>P. knowlesi forward</td>
<td>GCACCATAGTACGAAAGCA</td>
<td>1,069</td>
</tr>
<tr>
<td>P. knowlesi reverse</td>
<td>TCCGAGGTCTTGTGGTG</td>
<td></td>
</tr>
<tr>
<td>P. ovale forward</td>
<td>ACTGCCAGGAGCACA</td>
<td>850</td>
</tr>
<tr>
<td>P. ovale reverse</td>
<td>GGAACTACATCTTGTAGACG</td>
<td></td>
</tr>
<tr>
<td>P. vivax forward</td>
<td>GAAGCATAATTTGCGCA</td>
<td>677</td>
</tr>
<tr>
<td>P. vivax reverse</td>
<td>GTTTCTACTATATTGTCC</td>
<td></td>
</tr>
<tr>
<td>P. falciparum forward</td>
<td>AGGTGCAAGTGCTCAAAG</td>
<td>508</td>
</tr>
<tr>
<td>P. falciparum reverse</td>
<td>CGTCTAATTCTTTGACCG</td>
<td></td>
</tr>
<tr>
<td>P. malariae forward</td>
<td>GGTTCAGATGCAAGAGAT</td>
<td>291</td>
</tr>
<tr>
<td>P. malariae reverse</td>
<td>AGGGCAAGTTCTCTAC</td>
<td></td>
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</table>
products were viewed on 1% (wt/vol) agarose gels, which were stained with SYBR Safe DNA Gel Stain (Invitrogen) and visualized with the Gel Doc system (Bio-Rad).

**Analytical sensitivity of MSP-multiplex PCR.** The DNeasy Blood and Tissue Kit (Qiagen) was used to extract DNA from infected blood samples (100 μL), which were obtained from patients with 0.04% parasitemia (2 × 10^3 p/μL) for each species. The DNA was eluted in 50 μL sterile Tris-EDTA (TE) buffer. Therefore, 1 μL sample contained DNA equivalent to 4,000 parasites. The stock DNA was aliquoted and stored at −20°C, from which six 10-fold serial dilutions were prepared to achieve a final concentration equivalent to 0.004 p/μL. Assay detection limits were established for samples corresponding to individual *Plasmodium* species as well as mixed samples (i.e., DNA cocktails of multiple species), which were done in triplicate. Ultimately, 1 μL each sample was analyzed by real-time PCR, MSP-multiplex PCR, and the PlasmoNex Multiplex PCR Kit.

**Analytical specificity of MSP-multiplex PCR.** Specificity of all three assays was tested using gDNAs of all five species of human malaria.

**Clinical sensitivity and specificity.** The clinical sensitivity and specificity of three assays was calculated using 72 whole-blood samples with microscopy and nested PCR as the reference method. Sensitivity was calculated as (number of true positives)/(number of true positives + number of false negatives), and specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives).

**Statistical analysis.** Results from the comparisons between MSP-multiplex PCR and the PlasmoNex Multiplex PCR Kit, between the PlasmoNex Multiplex PCR Kit and real-time PCR, and between MSP-multiplex PCR and real-time PCR assays are shown in a 2 × 2 table. Agreement of results between the two methods was assessed using Cohen’s κ-test\(^{19}\) for concordance and McNemar’s test for discordance.

**RESULTS**

Our analysis revealed that the real-time multiplex PCR was more sensitive (81%) for detecting *P. vivax*, *P. falciparum*, and *P. knowlesi* infections compared with the PlasmoNex Multiplex PCR Kit (62%) and MSP-multiplex PCR (50%). Indeed, of 52 malaria samples that tested positive by both microscopy and nested PCR, 42 samples were correctly identified by real-time multiplex PCR. In contrast, the PlasmoNex Multiplex PCR Kit and MSP-multiplex PCR were able to accurately detect 32 and 25 samples, respectively.

Although the detection limit of the MSP-multiplex PCR assay was more sensitive than that of the PlasmoNex Multiplex PCR Kit for detecting *P. knowlesi* and other *Plasmodium* species (Figure 1A), it was only capable of detecting down to 400 and 4,000 p/μL, respectively. In contrast, the nested PCR assay displayed a detection threshold of 4 p/μL for all *Plasmodium* species, constituting an approximately 1,000-fold difference in sensitivity (Figures 1F–J). However, the real-time multiplex PCR assay could be used to detect 1–6 p/μL. Because we relied on microscopic parasite counts for determining our initial species concentrations (in parasites per microliter), it is possible that these values did not directly correspond to known DNA quantities because of the presence of schizonts.

![Figure 1](image-url) Detection limits for the three multiplex assays based on 10-fold serial dilutions of five *Plasmodium* species from 4,000 p/μL stocks. Lane 1 = 4,000 p/μL; lane 2 = 400 p/μL; lane 3 = 40 p/μL; lane 4 = 4 p/μL; lane 5 = 0.4 p/μL; lane 6 = 0.04 p/μL; lane 7 = 0.004 p/μL. One microliter of each dilution was amplified using the various methods. Representative results are shown for (A–E) MSP-multiplex PCR assay, (F–J) nested PCR method, and (K and L) the PlasmoNex Multiplex PCR Kit. The nested PCR method amplified up to 4 p/μL for each *Plasmodium* species. The MSP-multiplex PCR assay amplified up to 400 p/μL for *P. knowlesi* and 4,000 p/μL for *P. ovale*, *P. falciparum*, *P. vivax*, and *P. malariae*. The PlasmoNex Multiplex PCR Kit amplified up to 4,000 p/μL for both *P. knowlesi* and *P. ovale*, whereas no amplification was observed for other *Plasmodium* species. The positive process control (109 bp) was used to validate the results. (A, F, and K) *P. knowlesi*. (B, G, and L) *P. ovale*. (C and H) *P. vivax*. (D and I) *P. falciparum*. (E and J) *P. malariae*. M = GeneRuler Express DNA Ladder (Thermo Scientific, Waltham, MA). N = negative control (distilled water).
Thus, this potential discrepancy might have affected our ability to replicate previously reported sensitivity results. Notably, all three assays showed 100% specificity, because we observed no amplification of other gDNA.

Furthermore, we found that each of the multiplex assays was incapable of simultaneously amplifying all five _Plasmodium_ species from mixed samples (4,000 p/µL each species). Therefore, these molecular tools were more effective when analyzing monoinfected samples, suggesting that primer competition might contribute to their diminished performance in heterogeneous samples. Therefore, in cases of suspected mixed infection, our in-house _MSP_-multiplex method could be performed using individual species-specific primers to enhance specificity. In terms of clinical sensitivity and specificity, real-time PCR detected all of the _P. knowlesi_-positive samples, whereas the PlasmoNex Multiplex PCR Kit and _MSP_-multiplex PCR only identified 9 of 15 specimens each. In addition, the _MSP_-multiplex PCR assay performed slightly better than real-time multiplex PCR and the PlasmoNex Multiplex PCR Kit with regard to _P. ovale_, with a detection limit of 5,000 p/µL. However, among 18 _P. vivax_-positive samples, _MSP_-multiplex PCR only detected 9 samples, whereas real-time PCR and the PlasmoNex Multiplex PCR Kit were each capable of identifying 14 samples. Furthermore, both the PlasmoNex Multiplex PCR Kit and _MSP_-multiplex PCR assay performed poorly in the detection of _P. falciparum_, identifying 9 and 7 of 17 total samples, respectively. In contrast, real-time multiplex PCR distinguished 13 of 17 _P. falciparum_ samples. Nevertheless, all three methods were found to be 100% specific for detecting malaria infection (Table 2). Results from our comparison between the PlasmoNex Multiplex PCR Kit and _MSP_-multiplex PCR assay are presented in Table 3. Cohen’s _κ_-value of 0.656 indicated good agreement between the two methods. Also, McNemar’s test confirmed that there was no significant difference between the two methods (_P_ = 0.1489). However, when the PlasmoNex Multiplex PCR Kit was compared with the real-time multiplex PCR assay (Table 4), a significant difference was observed (McNemar’s test value: _P_ = 0.0044), despite the fact that Cohen’s _κ_-value (0.727) indicated good agreement. Comparing real-time PCR with _MSP_-multiplex PCR (Table 5), Cohen’s _κ_-value revealed moderate agreement (Cohen’s _κ_-value: 0.568), and the difference was statistically significant (_P_ value = 0.0012).

In addition, the cost of each assay, including the price of reagents and plastic consumables, was calculated based on a single sample. In this regard, the real-time multiplex PCR was found to be the most expensive ($14.80 US per sample) followed by the PlasmoNex Multiplex PCR Kit ($9.00 US per sample), nested PCR ($1.00 US per sample), and _MSP_-multiplex assay ($0.50 US per sample). Finally, we considered the time required for each of the assays based on PCR cycles: real-time multiplex PCR (60 minutes), the PlasmoNex Multiplex PCR Kit (70 minutes), nested PCR (6 hours), and _MSP_-multiplex PCR (101 minutes).

**DISCUSSION**

Although nested PCR represents the most consistent and accurate method for detecting low levels of malaria parasites, it is tedious and time-consuming. Therefore, the generation of rapid, highly sensitive/specific, cheap, and widely accessible detection assays is essential for effectively combating malaria transmission. In this regard, comparative studies evaluating available diagnostic assays are required to determine the most efficacious methods. Therefore, in this study, we have assessed three distinct molecular methods for _Plasmodium_ detection, including an in-house multiplex PCR assay.
Notably, although alternative molecular methods using loop-mediated isothermal amplification (LAMP) have been reported,\textsuperscript{25-27} this technology has not been sufficiently evaluated in field conditions. Also, to date, multiplex LAMP assays remain to be developed. Thus, advancement of this technique could yield novel clinical diagnostic tools for field-based detection of malaria.

In conclusion, the development of sensitive, simple, and accessible molecular diagnostic tools, such as multiplex assays, is critical for combating malaria. Our comparative study has shown that MSP-multiplex PCR assay and the PlasmoNex Multiplex PCR Kit display poor sensitivity compared with real-time multiplex PCR. Therefore, these two assays should not be used in diagnostic settings requiring more than 80% sensitivity. Future studies involving larger sample sizes may be needed to verify these findings. Nevertheless, our results suggest that additional optimization of real-time PCR sensitivity could enhance on-time and point-of-care diagnosis of malaria patients in endemic regions.

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REFERENCES


### Table 5

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<th></th>
<th>MSP-multiplex PCR</th>
<th>Real-time multiplex PCR</th>
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<tbody>
<tr>
<td>Positive</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>26</td>
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Number of microscopy-positive samples = 52. Cohen’s k = 0.508; McNemar’s test: P = 0.0012 (difference between the two methods is considered to be very statistically significant).


