

Multiplex 5' Nuclease Quantitative Real-Time PCR for Clinical Diagnosis of Malaria and Species-Level Identification and Epidemiologic Evaluation of Malaria-Causing Parasites, Including *Plasmodium knowlesi*

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Molecular diagnosis of malaria offers many potential advantages over microscopy, including identification of malaria to the species level in an era with few experienced microscopists. We developed high-throughput multiplex 5' nuclease quantitative PCR (qPCR) assays, with the potential to support large studies, to specifically identify *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. We compared qPCR to microscopy and confirmed discordant results with an alternative target PCR assay. The assays specifically detected 1 to 6 parasites/ μ l of blood. The clinical sensitivities (95% confidence intervals [CIs]) of the 4-plex assay to detect microscopically confirmed malaria were 95.8% (88.3 to 99.1%) for *P. falciparum*, 89.5% (75.2 to 97.1%) for *P. vivax*, 94.1% (71.3 to 99.9%) for *P. ovale*, and 100% (66.4 to 100%) for *P. malariae*. The specificities (95% CIs) were 98.6% (92.4 to 100%) for *P. falciparum*, 99% (84.8 to 100%) for *P. vivax*, 98.4% (94.4 to 99.8%) for *P. ovale*, and 99.3% (95.9 to 100%) for *P. malariae*. The clinical specificity for samples without malaria was 100%. The clinical sensitivity of the 5-plex assay for confirmed *P. knowlesi* malaria was 100% (95% CI, 69.2 to 100%), and the clinical specificity was 100% (95% CI, 87.2 to 100%). Coded retesting and testing with an alternative target PCR assay showed improved sensitivity and specificity of multiplex qPCR versus microscopy. Additionally, 91.7% (11/12) of the samples with uncertain species by microscopy were identified to the species level identically by both our multiplex qPCR assay and the alternative target PCR assay, including 9 *P. falciparum* infections. Multiplex qPCR can rapidly and simultaneously identify all 5 *Plasmodium* species known to cause malaria in humans, and it offers an alternative or adjunct to microscopy for clinical diagnosis as well as a needed high-throughput tool for research.

Malaria is an important cause of morbidity and mortality worldwide, with up to half of the world's population at risk (1). More than 1 million deaths occur each year, of which 90% are in sub-Saharan Africa (1); however, many cases go unreported. Commercial rapid diagnostic tests (RDTs) for malaria yield only qualitative results and vary widely in diagnostic sensitivity and specificity (2). Microscopy has been the historic diagnostic standard but depends on the skill of the microscopist. Further, Bayesian latent-class models suggest that microscopy can be insensitive (with likely sensitivity ranges of 50 to 90%) for low-level parasitemia (3). Under optimal conditions, the analytical sensitivity of thick-film microscopy is 10 to 30 parasites per μ l of blood (4). Moreover, the differentiation of species can be difficult even for the most experienced microscopists. Simian *Plasmodium knowlesi* was found to cause potentially fatal infections in humans only after molecular tools enabled its distinction from the morphological mimics *Plasmodium falciparum* and *Plasmodium malariae* (5, 6). Finally, large epidemiologic and clinical studies require not only detection of low-level parasitemia and accurate identification to the species level but also high throughput to be practical. Therefore, we designed high-throughput multiplex 5' nuclease quantitative PCR (qPCR) assays to simultaneously identify, quantitate, and distinguish the 5 species of *Plasmodium* now recognized to cause human malaria.

MATERIALS AND METHODS

Cohort of patients with clinical malaria and control subjects. The study cohort included patients with clinical malaria after travel to or residence in regions in which malaria is endemic (most frequently sub-Saharan

Africa or Southeast Asia), from whom blood samples were submitted between 2004 and 2012 for clinical diagnosis of malaria by thin- and thick-blood-smear microscopy at the Johns Hopkins University and Duke University clinical microbiology laboratories or a university-based reference laboratory. To assess clinical sensitivity, discarded deidentified EDTA-anticoagulated blood samples from the aforementioned cohort that had been archived and stored at -20 to -80°C were used. To assess clinical specificity, discarded deidentified EDTA-anticoagulated blood samples from patients who presented to Johns Hopkins Hospital with no known bloodstream infection or with microscopy-confirmed babesiosis (a clinical mimic of malaria) were used.

Ethics. The study was reviewed by the ethics committee of the Johns Hopkins University School of Medicine. Institutional review board approval to use archived discarded deidentified samples was granted, since obtaining consent was deemed both impractical and unnecessary (Johns Hopkins Medicine protocol NA_00021376).

Blood samples from patients. For evaluation of a 4-color 384-well platform designed to detect and to distinguish *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* malaria, 148 samples from the aforementioned cohort of patients with microscopic diagnoses of clinical malaria (72 *P. falciparum* samples, 38 *P. vivax* samples, 17 *P. ovale* samples, 9 *P. malariae* samples, and 12 *Plasmodium* sp. samples) were available, including DNA

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TABLE 1 Primers and probes for detection and identification of *Plasmodium* species

Species	Primer or probe (amplicon length [bp]) ^a	Sequence ^b
<i>P. falciparum</i>	Primer, forward	CCACATCTAAGGAAGGCAGCAG
	Primer, reverse	CCTCCAATTGTTACTCTGGGAAGG
	Probe (145)	Cy5-CCCACCATTCCAATTACAA-BHQ1
<i>P. vivax</i>	Primer, forward	ACGCCAAGTTCGGATTATGG
	Primer, reverse	CCGTCATTTCTTCTTCATACTGAG
	Probe (157)	TET-TTGATCTGAGGCACTCGCTCCG-BHQ1
<i>P. ovale</i>	Primer, forward	TATCCTCGGAGCACCATTATGAG
	Primer, reverse	ACGGCAAACCGACCCTCTC
	Probe (81)	FAM-ACTTCTCCGTTTTCG-BHQ1
<i>P. malariae</i>	Primer, forward	CCAACAATACATACACATTAGAACC
	Primer, reverse	GTAGGATATAAAGCATACACAAAGTG
	Probe (145)	Texas Red-ATCTAGTAATGGCTCC-BHQ1
<i>P. knowlesi</i>	Primer, forward	TAACATGGTAATCATACTAAGG
	Primer, reverse	TAAGGAAATGCCAACTCTTG
	Probe (117)	TAMRA-TCAGCCAACAACACTTACAG-BHQ1

^a PCR amplification targets were 18S (small-subunit) rRNA (*P. falciparum*) (8), *AMA1* (*P. vivax*), plasmepsin 4 (*P. ovale*) [GenBank accession no. AF001209] and *P. malariae* [GenBank accession no. AF001210]), and strain H chromosome 13 plasmepsin (*P. knowlesi*) [GenBank accession no. XM_002260879.1].

^b TET, tetrachloro-6-carboxyfluorescein; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

previously extracted from blood samples from 17 patients with malaria (11 *P. ovale* samples, 5 *P. malariae* samples, and 1 indeterminate *Plasmodium* sp. sample). Since 12 samples were not identified microscopically to the species level, 136 samples obtained from 100 patients with malaria (50 *P. falciparum* cases, 24 *P. vivax* cases, 17 *P. ovale* cases, and 9 *P. malariae* cases) were used for assessment of clinical sensitivity and specificity. The non-*Plasmodium* panel used for assessment of specificity included 11 discarded deidentified blood samples, i.e., 1 sample spiked with *Trypanosoma brucei rhodesiense*, 4 samples from patients with no known bloodstream infection, 2 samples from patients with microscopy-confirmed babesiosis, and 4 blood smear-negative samples obtained during monitoring of parasitemia in 2 patients with recent *P. falciparum* malaria.

For extension of the 4-plex assay to a 5-plex 96-well platform that also detects and distinguishes *P. knowlesi*, 10 EDTA-treated blood samples were obtained from 10 Malaysian patients with *P. knowlesi*, as confirmed by microscopy and nested PCR assays (5). A subset of available samples was used to assess the sensitivity and specificity of the 5-plex assay and included 33 blood samples from patients with microscopy-confirmed clinical malaria (7 *P. falciparum* samples, 7 *P. vivax* samples, 5 *P. ovale* samples, 4 *P. malariae* samples, and 10 *P. knowlesi* samples) and 4 samples from patients without malaria (2 with bacteremia and 2 with no known bloodstream infection).

Preparation of DNA. DNA was prepared from archived EDTA-anticoagulated blood samples with the automated QIASymphony SP system (Qiagen Inc., Valencia, CA). Blood samples (800 μ l to 1 ml) were brought to 1 ml with 1 \times phosphate-buffered saline before extraction with a QIASymphony DNA Midi kit; the final elution volume was 200 μ l. Blood samples of insufficient volume were extracted with spin columns and a DNeasy Blood & Tissue kit (Qiagen); the elution volume was 200 μ l, which then was concentrated to 100 μ l.

DNA was prepared from 152 blood samples (141 *Plasmodium* samples and 11 non-*Plasmodium* samples), 121 by the QIASymphony system and 31 by the Qiagen DNeasy Blood & Tissue kit. The MagNA Pure System or MagNA Pure Compact System was used for the 17 previously extracted samples.

Development of multiplex 5' nuclease (TaqMan) quantitative real-time PCR assays. We initially selected amplification targets for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* based on prior publications (7

and available gene sequences in GenBank. We verified specific 18S rRNA gene primers and probes only for *P. falciparum* (8); hence, we targeted the apical membrane antigen 1 (*AMA1*) gene in *P. vivax* and the plasmepsin genes in *P. ovale* (GenBank accession no. AF001209), *P. malariae* (GenBank accession no. AF001210), and *P. knowlesi* (GenBank accession no. XM_002260879.1). We used the human beta-actin (*ACTB*) gene as an internal amplification control. We optimized multiplex reactions using uniform conditions with AlleleID version 6.1 software (Premier Biosoft International, Palo Alto, CA).

The primers (forward and reverse) and probes targeting 18S rRNA (*P. falciparum*) (8), *AMA1* (*P. vivax*), and plasmepsin (*P. malariae*, and *P. knowlesi*) are shown in Table 1. The forward and reverse primers for *ACTB* were GTGCTCAGGGCTTCTTGTC and CCATGTCGTCCCAGTTGGT, respectively, and the probe was Texas Red- or hexachloro-6-carboxyfluorescein (HEX)-ACCATGCCACCATCACGCC C-black hole quencher 1 (BHQ1). Amplification using primers for *P. ovale* based on plasmepsin 4 and using SYBR green allowed detection of all 9 samples initially tested; however, only 4 could be detected using a probe based on this sequence. To clarify potential genetic heterogeneity, 1,000-bp sections of the *P. ovale* plasmepsin gene were amplified from these 4 blood samples by qPCR; these sections were cloned, sequenced, and aligned with reference sequences to identify conserved and variable regions and to guide query and design of new primers/probes using AlleleID.

Multiplex qPCR was first performed using a C1000 thermal cycler with a CFX384 real-time PCR (4-color) system (Bio-Rad, Hercules, CA) with 384-well plates. Initial evaluations of primers were conducted with reaction mixtures containing 1 μ l DNA template, 200 nM each primer, and iQ SYBR green supermix (Bio-Rad). Reaction mixtures that contained probes were prepared with 1 μ l blood DNA, 200 nM each primer and probe, and 2 \times iQ Multiplex Powermix (Bio-Rad), in a final total volume of 10 μ l. Each qPCR run included a human blood DNA positive-control sample for each of the 4 major *Plasmodium* species (courtesy of David Sullivan), 2 to 4 human blood DNA negative-control samples, 2 to 4 control samples without template (water only), plasmid standards for quantitation of each *Plasmodium* sp. (diluted to provide 10⁵, 10³, 10¹, and 10⁰ copies/ μ l DNA input), and validation or unknown samples in duplicate. Quantitative PCR assays for *ACTB* were run separately, since the

CFX384 real-time PCR system is a 4-color system. Standard two-step qPCR was performed, with initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 55°C for 30 s. All samples were run in duplicate wells, and most assays were reproduced at least twice during the course of assay development and optimization.

To establish a consistent baseline and accurate cycle threshold (C_T) and end relative fluorescence unit (RFU) values, we applied the baseline with curve fit algorithm to the data for each sample, using Bio-Rad CFX384 Manager software. Curves generated using this algorithm were manually inspected and corrected if the logarithmic features expected with DNA amplification were not observed or if any systematic technical errors were detected. Endpoint analysis was conducted with Bio-Rad CFX384 system software. Different tolerances, i.e., percentages of the difference between the maximum RFU value and the average RFU value for 2 to 4 negative-control samples for each fluor on the plate over the last 5 qPCR cycles, were calculated. Each tolerance value was added to the average negative-control RFU value over the last 5 cycles to establish a potential cutoff value above which a sample would be considered positive. Various cutoffs were examined, and sensitivity and specificity were maximized using receiver operating characteristic curves.

To extend the multiplex assay to include *P. knowlesi*, a probe and a primer pair were designed to recognize a specific portion of the *P. knowlesi* strain H chromosome 13 plasmepsin gene (GenBank accession no. XM_002260879.1) (Table 1). After initial testing in a singleplex assay, *P. knowlesi* identity was confirmed by melting curve analysis. The analytical sensitivity and linearity of *P. knowlesi* DNA standards were determined using a plasmid containing the region of the *P. knowlesi* plasmepsin gene targeted by the assay. The *P. knowlesi* assay was then integrated into the 4-plex assay for *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae*, which required adaptation of the previously optimized 4-plex assay to a 5-plex assay using the Bio-Rad iCycler iQ5 multicolor real-time PCR detection system for 96-well plates. For the iQ5 system, each reaction mixture included 2.5 μ l of DNA, 12.5 μ l of iQ Multiplex Powermix (Bio-Rad), and primers and probes at final concentrations of 200 nM each. Runs included duplicates of template, plasmid standard, DNA positive- and negative-control, and no-template control samples. Standard two-step qPCR was again performed, and analysis was similarly accomplished using Bio-Rad iQ5 software.

Correlation of multiplex PCR and microscopy findings. Multiplex qPCR testing was performed with masking (blinding) with respect to the microscopy (smear) results. As the primary analysis of the performance of our qPCR assay, we considered microscopy the gold standard. Samples for which qPCR results did not agree with smear results were considered discordant. Discordant samples were retested with at least one additional PCR method, i.e., our multiplex qPCR assay, an alternative target PCR assay used by the Mayo Clinic Reference Laboratory (9), and 2 singleplex assays for *P. falciparum* (targeting the 18S [1/4 targets in our multiplex assay] and cytochrome B [CYTB] [8] genes), and melting curve analysis (the CYTB target for *P. falciparum* melts at 76°C and *P. vivax* AMA1 at 82.5°C). As a secondary analysis, we considered samples with identical results with two different DNA targets as true-positive PCR results, since assigning a species is not always possible with microscopy. In a final analysis, we assessed the performance of clinical microscopy versus composite PCR testing for accurate identification of clinical malaria to the species level.

Quantitation of parasitemia. Comparisons of quantitation of parasitemia by microscopy versus qPCR testing were performed for samples for which parasitemia had been determined by microscopy. Microscopic parasitemia values of <1% and <0.5% were considered 0.5% and 0.25%, respectively, in tests of correlations between microscopic and molecular parasitemia results.

RESULTS

Analytical sensitivity and specificity. The 4-plex assay detected 10^5 to 10^0 copies of *Plasmodium* spp.; *P. falciparum* contains four

to six 18S rRNA gene copies per parasite, and each of the other species contains one gene (AMA1 or the specific plasmepsin gene target) copy or more per parasite. Detection was linear ($R^2 \geq 0.99$) and efficient (>85% to <115%). The assay did not detect the targets in blood spiked with *Trypanosoma brucei rhodesiense*. The analytical limit of sensitivity for each *Plasmodium* species component of the 5-plex assay was 10 to 100 copies/ μ l, similar to that of the 4-plex qPCR, and detection was linear from 10^1 to 10^5 copies.

Clinical sensitivity. Among blood samples from patients with microscopically identified *P. falciparum* malaria that were tested with the 4-plex assay, 95.8% (69/72 samples) were qPCR positive for *P. falciparum* (discordant qPCR results: *P. malariae*, 1 sample; negative, 2 samples). One discordant sample was confirmed as *P. malariae* on retesting and alternative target PCR testing (results of singleplex assays and melting curve analyses for *P. falciparum* also were negative). Of the two initially negative samples by multiplex qPCR, one was confirmed as *P. falciparum* by multiplex qPCR retesting, alternative target PCR testing, and our singleplex *P. falciparum* assays. The other remained negative for *P. falciparum* on repeat multiplex qPCR testing but was confirmed as *P. falciparum* by alternative target PCR assays and our singleplex *P. falciparum* assays.

Among blood samples from patients with microscopically identified *P. vivax* malaria that were tested with the 4-plex assay, 89.5% (34/38 samples) were qPCR positive for *P. vivax* (discordant qPCR results: *P. ovale*, 2 samples; *P. falciparum*, 1 sample; negative, 1 sample). Of the two samples that were initially identified as *P. ovale* by multiplex qPCR, one was confirmed as *P. ovale* by both multiplex qPCR retesting and alternative target PCR testing and was negative for *P. falciparum* by singleplex PCR assays. The other sample was confirmed as *P. ovale* by multiplex qPCR retesting, could not be identified to the species level by alternative target PCR testing, and was *P. falciparum* negative by singleplex assays. The sample initially identified as *P. falciparum* by multiplex qPCR testing was confirmed to be *P. vivax* by multiplex qPCR retesting, by alternative target PCR testing, and by singleplex PCR testing with melting curve analysis. The sample that was initially negative by multiplex qPCR testing remained negative by multiplex qPCR retesting and by alternative target PCR testing.

Of blood samples from 17 patients with *P. ovale* malaria by microscopy, 94.1% (16/17 samples) were positive for *P. ovale* by the 4-plex qPCR assay (discordant result: negative, 1 sample). This sample was confirmed as *P. ovale* by alternative target PCR testing. Of blood samples from 9 patients with *P. malariae* malaria by microscopy, 100% (9/9 samples) were positive for *P. malariae* by the 4-plex qPCR assay.

All samples that were tested in duplicate amplified human *ACTB*. If the analyses were restricted to 1 sample per patient, then the sensitivity of multiplex qPCR for microscopic *P. falciparum* was 94.0% (47/50 samples) and that for *P. vivax* was 91.7% (22/24 samples) (*P. ovale* and *P. malariae* values were unchanged, since only 1 sample was available for each patient). The clinical sensitivity of the 5-plex assay for both *P. falciparum* and *P. vivax* microscopy-confirmed malaria was 100% (7/7 samples), that for *P. ovale* was 100% (5/5 samples), that for *P. malariae* was 100% (4/4 samples), and that for *P. knowlesi* was 100% (10/10 samples).

Clinical specificity. The clinical specificities of the 4-plex qPCR assay for detection of *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* in blood samples from patients with microscopy-confirmed malaria were 98.6% (70/71 samples), 99.0% (104/105 sam-

TABLE 2 Initial performance of multiplex PCR versus microscopy for detection of microscopy confirmed clinical malaria^a

Microscopy result (n)	PCR performance (% [95% CI])			
	Sensitivity	Specificity	Positive predictive value	Negative predictive value
<i>P. falciparum</i> (72)	95.8 (88.3–99.1)	98.6 (92.4–100)	98.6 (92.3–100)	95.9 (88.5–99.1)
<i>P. vivax</i> (38)	89.5 (75.2–97.1)	99 (84.8–100)	97.1 (85.1–99.9)	96.3 (90.8–99)
<i>P. ovale</i> (17)	94.1 (71.3–99.9)	98.4 (94.4–99.8)	88.9 (65.3–98.6)	99.2 (95.6–100)
<i>P. malariae</i> (9)	100 (66.4–100)	99.3 (95.9–100)	90 (55.5–99.7)	100 (97.3–100)
<i>P. knowlesi</i> (10)	100 (69.2–100)	100 (87.2–100)	100 (69.2–100)	100 (87.2–100)

^a Results for species other than *P. knowlesi* were from testing of 143 samples with a 4-plex 384-well assay. Results for *P. knowlesi* were based on testing of a 37-sample subset with a 5-plex 96-well assay. CI, confidence interval.

ples), 98.4% (124/126 samples), and 99.3% (133/134 samples), respectively. One *P. vivax*-smear-positive sample was initially detected as *P. falciparum* by our multiplex assay; repeat testing with the multiplex assay, alternative-target PCR testing, singleplex assays, and melting curve analyses all indicated *P. vivax* (i.e., the multiplex qPCR *P. falciparum* result was a false-positive finding). One *P. falciparum*-smear-positive sample that was initially positive for *P. falciparum* and *P. vivax* by our multiplex qPCR assay was positive only for *P. falciparum* with repeat testing with the multiplex assay, alternative target PCR testing, singleplex assays, and melting curve analyses (i.e., the multiplex *P. vivax* result was a false-positive finding). The two *P. vivax*-smear-positive samples identified as *P. ovale* by the multiplex assay were confirmed on retesting; further, although one sample could not be identified to the species level by alternative target PCR testing, the other was identified as *P. ovale* (both likely true *P. ovale* cases). Finally, the *P. falciparum*-smear-positive sample identified as *P. malariae* by the multiplex qPCR assay was confirmed as *P. malariae* on retesting and by alternative target PCR testing (likely a true *P. malariae* case).

No *Plasmodium* DNA was detected in blood samples from patients with no known bloodstream infection or patients with confirmed babesiosis (clinical specificity of 100% for patients without malaria). The persistence of *P. falciparum* DNA was detected in one smear-negative blood sample from a patient who had been smear positive for *P. falciparum* 48 h earlier. The clinical specificity of the 5-plex assay for both *P. falciparum* and *P. vivax* microscopy-confirmed malaria was 100% (30/30 samples), that for *P. ovale* was 100% (32/32 samples), that for *P. malariae* was 100% (33/33 samples), and that for *P. knowlesi* was 100% (27/27 samples).

Performance of multiplex qPCR versus microscopy for the diagnosis of microscopy-confirmed clinical malaria. Table 2 summarizes the performance data for our multiplex qPCR assay, relative to microscopy, for the diagnosis of malaria. In primary

data analysis, the sensitivities of the 4-plex qPCR assay for microscopically identified *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* malaria were 95.8% for *P. falciparum*, 89.5% for *P. vivax*, 94.1% for *P. ovale*, and 100% for *P. malariae*, and the specificities were 98.6% for *P. falciparum*, 99.0% for *P. vivax*, and 98.4% for *P. ovale*, and 99.3% for *P. malariae*. Using the 5-plex assay, the sensitivity and specificity for *P. knowlesi* were both 100%.

Performance of multiplex qPCR versus microscopy after resolution of qPCR-microscopy discrepancies. In secondary data analysis (Table 3), the sensitivities of the 4-plex qPCR assay for microscopically identified *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* malaria were at least 97.2% (69/71 samples) for *P. falciparum*, 97.1% (34/35 samples) for *P. vivax*, 94.7% (18/19 samples) for *P. ovale*, and 100% (10/10 samples) for *P. malariae*, and the specificities were 98.6% (71/72 samples) for *P. falciparum*, 99.1% (107/108 samples) for *P. vivax*, 100% (124/124 samples) for *P. ovale*, and 100% (133/133 samples) for *P. malariae*.

Additionally, 91.7% of samples (11/12 samples) of uncertain species by microscopy were definitively identified to the species level by multiplex qPCR, including 9 *P. falciparum* samples (confirmed by multiplex qPCR testing twice, alternative target PCR testing, and our singleplex assays), 1 *P. ovale* sample, and 1 *P. vivax* sample. The remaining sample of uncertain species might have been a coinfection. It was initially qPCR positive for *P. vivax* and *P. ovale*; repeat multiplex qPCR testing, singleplex assays, and melting curve analyses suggested *P. falciparum*/*P. vivax* coinfection, but alternative target PCR testing showed only *P. vivax*.

Performance of clinical microscopy versus composite PCR (≥ 2 assays) for identifying microscopy-confirmed clinical malaria to the species level. Finally, with the benchmark standard of PCR confirmed by ≥ 2 assays, the sensitivity of microscopy for identifying *P. falciparum* to the species level would be at best 88.8% (71/80 samples), since 9 *P. falciparum* infections were reported as indeterminate species and 1 was reported as *P. malariae*

TABLE 3 Final performance of multiplex PCR versus microscopy for detection of microscopy-confirmed clinical malaria after resolution of discrepancies^a

Microscopy result (n)	PCR performance (% [95% CI])			
	Sensitivity	Specificity	Positive predictive value	Negative predictive value
<i>P. falciparum</i> (71)	97.2 (90.2–99.7)	98.6 (92.5–100)	98.6 (92.3–100)	97.3 (90.5–99.7)
<i>P. vivax</i> (35)	97.1 (85.1–99.9)	99.1 (94.9–100)	97.1 (85.1–99.9)	99.1 (94.9–100)
<i>P. ovale</i> (19)	94.7 (74.0–99.9)	100 (97.1–100)	100 (81.5–100)	99.2 (95.6–100)
<i>P. malariae</i> (10)	100 (69.2–100)	100 (97.3–100)	100 (69.2–100)	100 (97.3–100)
<i>P. knowlesi</i> (10)	100 (69.2–100)	100 (87.2–100)	100 (69.2–100)	100 (87.2–100)

^a Results for species other than *P. knowlesi* were from testing of 143 samples with a 4-plex 384-well assay. Results for *P. knowlesi* were based on testing of a 37-sample subset with a 5-plex 96-well assay.

TABLE 4 Performance of clinical microscopy versus composite (≥ 1 target and retesting) PCR for species-level identification of clinical malaria^a

PCR result (n)	Microscopy performance (% [95% CI])			
	Sensitivity	Specificity	Positive predictive value	Negative predictive value
<i>P. falciparum</i> (80)	88.8 (79.7–94.7)	95.9 (88.6–99.2)	95.9 (88.6–99.2)	88.8 (79.7–94.7)
<i>P. vivax</i> (37)	97.3 (85.8–99.9)	89.7 (82.8–94.6)	75.0 (60.4–86.4)	99.1 (94.9–100)
<i>P. ovale</i> (20)	85.0 (62.1–96.8)	92.5 (86.7–96.4)	63.0 (42.4–80.6)	97.6 (93.3–99.5)
<i>P. malariae</i> (10)	90.0 (55.5–99.7)	92.4 (86.7–96.1)	45.0 (23.1–68.5)	99.3 (95.9–100)
<i>P. knowlesi</i> (10)	100 (69.2–100)	100 (87.2–100)	100 (69.2–100)	100 (87.2–100)

^a Results for species other than *P. knowlesi* were from testing of 143 samples with a 4-plex 384-well assay. Results for *P. knowlesi* were based on testing of a 37-sample subset with a 5-plex 96-well assay.

(Table 4). Similarly, the clinical sensitivities for identifying *P. vivax* and *P. ovale* to the species level would be $\leq 97.2\%$ (35/36 samples) and $\leq 85.0\%$ (17/20 samples), respectively, since 1 *P. vivax* sample and 1 *P. ovale* sample were reported as indeterminate species and 2 *P. ovale* samples were erroneously reported as *P. vivax*. Finally, the sensitivity of microscopy for identifying *P. malariae* to the species level would be $\leq 90.0\%$ (9/10 samples), since 1 *P. malariae* sample was reported as *P. falciparum*. The specificities of microscopy for accurately identifying *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* to the species level (95.9% [71/74 samples], 89.0% [105/118 samples], 92.5% [124/134 samples], and 92.4% [133/144 samples], respectively) were similarly worse than the specificities of our multiplex assay, particularly for *P. vivax*.

Quantitation of parasitemia. Quantitation of parasitemia by microscopy and qPCR was performed for 52 confirmed *P. falciparum* samples and 19 confirmed *P. vivax* samples. The median microscopic parasitemia value (and interquartile range [IQR]) for *P. falciparum* was 0.5% (IQR, 0.25% to 2.0%), and that for *P. vivax* was 0.5% (0.25% to 0.5%). The median molecular parasitemia value for *P. falciparum* was 1.2×10^5 copies/ μL (IQR, 5.1×10^4 to 4.1×10^5 copies/ μL), and that for *P. vivax* was 1.3×10^5 copies/ μL (IQR, 3.2×10^4 to 2.4×10^5 copies/ μL). In contrast to the median and IQR values, the minimum and maximum values differed for both microscopic (*P. falciparum*, 0.5% to 50%; *P. vivax*, 0.25% to

1%) and molecular (*P. falciparum*, 2.0×10^3 to 1.1×10^7 copies/ μL ; *P. vivax*, 3.5×10^2 to 4.9×10^5 copies/ μL) parasitemia findings. The correlation between microscopic and molecular parasitemia findings (Fig. 1) was statistically significant for *P. falciparum* (Spearman correlation, $P < 0.001$) but not for *P. vivax* ($P = 0.155$).

DISCUSSION

The gold standard for the diagnosis of clinical malaria has been microscopy. However, microscopy is subjective and laborious, requires expertise gained by rigorous training and experience, and cannot effectively support large studies. RDTs based on immunochromatographic antigen-detection systems have been implemented in some diagnostic laboratories as a complement to microscopy. Although they are rapid, simple, and easy to interpret, RDTs target proteins specific to *P. falciparum* or *P. vivax* or those common to all *Plasmodium* species (2) and cannot specifically identify *P. malariae*, *P. ovale*, and *P. knowlesi* (2, 10). Further, commercially available RDTs vary widely in diagnostic sensitivity (e.g., lacking sensitivity for some strains of *P. falciparum* and up to 50% of *P. knowlesi*) (11) and specificity (e.g., *P. vivax* in patients with coinfections and high *P. falciparum* parasitemia levels). Lastly, RDTs cannot be used to monitor therapy, because they are qualitative (2).

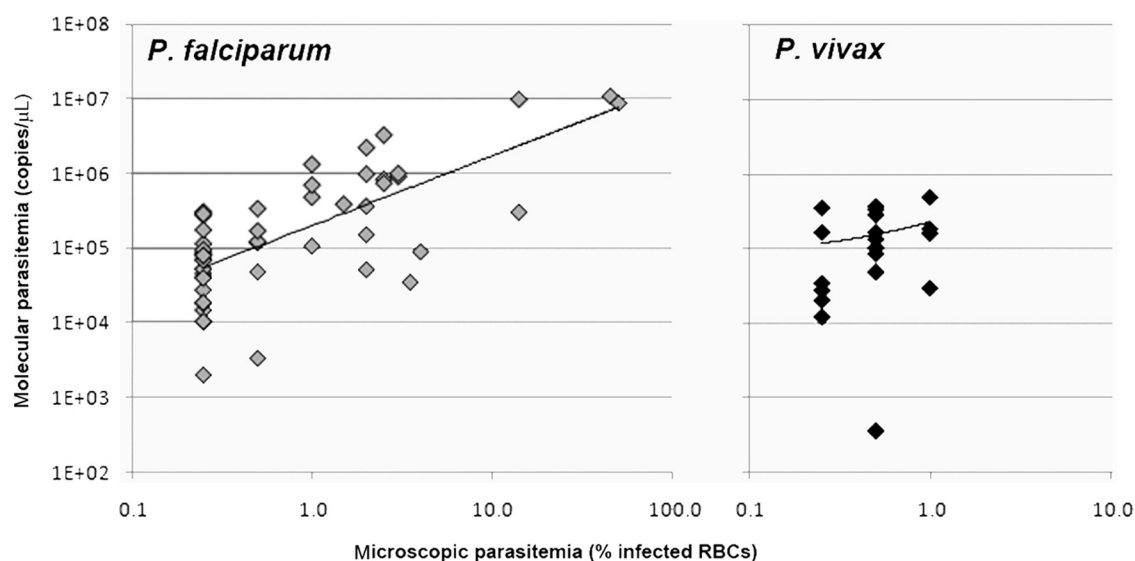


FIG 1 Clinical microscopic (lower limit, 0.5% or 1%) versus molecular parasitemia for *P. falciparum* (Spearman rank correlation, $P < 0.0001$) and *P. vivax* (Spearman rank correlation, $P = 0.155$). RBCs, red blood cells.

Molecular assays offer an attractive alternative approach. Conventional PCR assays are time-consuming, and nested conventional PCR assays enable identification to the species level but with a high risk of cross-contamination. A few real-time PCR assays have been described (10). However, most target one (most commonly *P. falciparum*) or two of the four major *Plasmodium* species, with similarly limited evaluation of specimens from patients with clinical malaria. For example, several assays only distinguish *P. falciparum* from other *Plasmodium* species (12, 13). One assay cannot detect *P. malariae* (14) and additionally could miss variants of *P. ovale* (15, 16). Clinical specificity is not ensured, since blood samples from patients with *P. ovale* and *P. malariae* are difficult to obtain and infrequently tested. Recently, a multiplex PCR system targeting 18S small-subunit rRNA genes and capable of detecting all 5 known species of malaria in humans was reported, using nested PCR as the molecular gold standard (17). However, these results have yet to be corroborated outside Malaysia; further, the group tested only 2 clinical samples from patients with *P. malariae* and 1 from a patient with *P. ovale* malaria.

We describe multiplex quantitative real-time PCR assays for malaria that are sensitive and specific for detection of the 4 major *Plasmodium* species as well as the recently recognized species *P. knowlesi*. Detection and definitive species-level identification of *P. falciparum* and *P. knowlesi* are important, since both species are likely to be clinically urgent (18) and misdiagnosis of *P. knowlesi* as *P. malariae* has been associated with excess mortality due to delayed treatment with intravenous artesunate therapy (19). Furthermore, relapses have occurred from failure to administer antihypnozoite treatment to patients with *P. vivax* parasites that were microscopically misidentified as *P. knowlesi* (20). Multiplex quantitative real-time PCR assays can be conducted rapidly (<4.5 h from DNA preparation to PCR amplification), and the 384-well (4 species) and 96-well (5 species) formats both enable high throughput, as required for large clinical and epidemiologic studies. The assays are quantitative over a wide range of parasitemia levels and can detect as few as 1 to 6 parasites/ μ l of blood, well below the limit of detection of microscopy.

Since increased blood volume improves sensitivity and automated extraction supports high throughput, we extracted DNA from a large volume (usually 1 ml) of blood with an automated platform. Further, we chose multiplex real-time qPCR, since this automated, quantitative, closed-system format supports high throughput and decreases contamination (increases specificity). We also obtained and tested a large number of blood samples from patients with clinically suspected and clinical microbiology laboratory-confirmed microscopic malaria, and we enriched our specimen repository with additional samples from patients with rare *P. ovale*, *P. malariae*, and *P. knowlesi* infections. We compared qPCR to the existing standard of microscopy as our primary analysis but we also completed extensive additional testing to adjudicate discordant qPCR and microscopy results, since microscopy can be insensitive, relative to other methods (3), and identification to the species level is not always possible.

Resolution of discordant results and testing by qPCR of specimens from patients with malaria caused by indeterminate *Plasmodium* species (secondary analysis) showed improved sensitivity and specificity for all 4 *Plasmodium* targets in the 4-plex qPCR assay versus microscopy. However, this is likely a significant underestimate, since we did not test samples from

patients who had epidemiologically and clinically suspected malaria but had negative blood smears, due to effective prophylaxis or treatment. Of paramount clinical importance, both the sensitivity and specificity of detecting *P. falciparum* were higher with our qPCR assay (97.2% and 98.6%, respectively) than with microscopy (88.8% and 95.9%, respectively). The multiplex qPCR assay was able to identify additional infections with *P. falciparum* that had not been identified to the species level by microscopy, which could improve clinical care by identifying infections more likely to be severe. We also found that infections with less-common species of malaria may be reported as more-common, morphologically similar species when there is doubt, such as reporting of *P. ovale* as *P. vivax* or *P. malariae* as *P. falciparum*. Although epidemiologic histories could suggest the species (e.g., *P. ovale* is much more likely than *P. vivax* in a patient from West Africa) (21), clinical and reference laboratories rarely are provided such important information about pretest probability. Furthermore, epidemiologic histories also may be misleading, since recent work has shown that *P. vivax* merozoites do not absolutely require Duffy surface antigens to invade erythrocytes (22) and clinical *P. vivax* malaria can occur in some Duffy-negative individuals (23). These findings highlight the importance of developing and applying species-specific molecular assays to support rigorous scientific enquiry in addition to clinical care.

Improved sensitivity and specificity of multiplex qPCR versus microscopy for the detection of clinical and subclinical malaria are plausible. In a study in which nested PCR was considered the gold standard, multiplex qPCR was comparable to microscopy for 30 symptomatic patients (24); additionally, 2 *P. ovale* infections that were microscopically misdiagnosed as *P. vivax* were correctly identified by multiplex qPCR. Although assessment of the clinical sensitivity and specificity for *P. malariae* was not possible (no patient had *P. malariae* infection), the assay is now used routinely for identification of malaria to the species level in Alberta, Canada (24). In addition, a multiplex assay based on similar primers (7) was found to aid proper microscopic identification in a study of 89 patients with predominantly *P. falciparum* malaria (only 6 *P. vivax* cases, 3 *P. ovale* cases, and 1 *P. malariae* case) evaluated by a large, university-based, clinical microbiology laboratory in Switzerland (25). In that study, the multiplex qPCR assay correctly identified to the species level *P. falciparum* initially identified as *P. malariae* and *P. ovale* initially classified as *P. vivax* and detected mixed infections with low levels of parasitemia.

Because our study population included predominantly symptomatic returning travelers (individuals lacking preexisting protective immunity) presenting for routine clinical care, microscopic parasitemia was reported most often for *P. falciparum* malaria and levels were relatively low. Further, quantitation of microscopic parasitemia was performed in accordance with clinical practice (no quantitation for findings of <0.5% or <1%). Consequently, data that would allow correlation of microscopic and molecular parasitemia were limited. Nevertheless, since the average normal erythrocyte count for adults is $>4.0 \times 10^6$ cells/ μ l of blood and there are 6 copies of 18S rRNA genes per *P. falciparum* cell, a median molecular parasitemia value of 1.2×10^5 copies/ μ l of blood would be expected for a median microscopic parasitemia value of <0.5%, consistent with what we observed. It also is not surprising that we detected a statistically significant correlation between microscopy and

qPCR results for *P. falciparum* but not for *P. vivax*, since the latter had a smaller sample size and highly restricted range of parasitemia levels (many cases at the lowest levels reported by clinical microbiology laboratories). Since the lowest microscopy category includes parasitemia levels that likely vary >1 order of magnitude, it is not surprising that molecular parasitemia levels were highly variable in this group. Finally, since all samples were obtained from patients with clinical malaria that had been diagnosed by microscopy, we could not challenge the lower limit of detection of our assay for the diagnosis of subclinical malaria or submicroscopic parasitemia.

Precise correlation of molecular and microscopic parasitemia findings could be assessed optimally in a large cohort of patients monitored longitudinally for symptomatic and asymptomatic parasitemia, with documentation of treatment and with 1 ml of blood being obtained at each visit. Although less clinically relevant, microscopic and molecular parasitemia levels ideally also would be correlated for other *Plasmodium* species. We have prospectively evaluated the described 18S rRNA gene *P. falciparum* target in a clinical trial of chemoprophylaxis for the prevention of infection with *P. falciparum* (26) and found it equivalent to microscopy (minimum of 200 oil-immersion fields per thick blood film reviewed). Our 18S rRNA gene *P. falciparum* target also was assessed in a >2,000-person cohort followed for 6 months in a hypoendemic area of Tanzania (8). Using latent-class analysis, we found it to be more sensitive than microscopy, RDTs, and a *CYTB* target for the detection of symptomatic and asymptomatic *P. falciparum* malaria, despite extraction of blood DNA from small volumes (~25 µl of blood on filter paper) rather than the large volumes (~1 ml) reported here.

In summary, we describe the development of multiplex qPCR assays to detect and to differentiate the four major species of malaria and emerging *P. knowlesi* and subsequent evaluation with a large panel of blood samples from symptomatic patients with clinical microbiology laboratory-confirmed microscopic malaria. The assays described have many potential applications, including support of large epidemiologic studies and trials of new vaccines or therapies, sensitive and specific detection of malaria with low parasitemia levels (such as clinical malaria in returning travelers and subclinical malaria in refugees) (27), and accurate identification to the species level of malaria diagnosed by another method (such as microscopy or RDT). Adapting our assays to other platforms, such as loop-mediated isothermal amplification, could make them cost-effective, point-of-care tests for use in poor regions in which malaria is endemic (28).

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