Simple Real-Time PCR and Amplicon Sequencing Method for Identification of *Plasmodium* Species in Human Whole Blood

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Malaria is the leading identifiable cause of fever in returning travelers. Accurate *Plasmodium* species identification has therapy implications for *P. vivax* and *P. ovale*, which have dormant liver stages requiring primaquine. Compared to microscopy, nucleic acid tests have improved specificity for species identification and higher sensitivity for mixed infections. Here, we describe a SYBR green-based real-time PCR assay for *Plasmodium* species identification from whole blood, which uses a panel of reactions to detect species-specific non-18S rRNA gene targets. A pan-*Plasmodium* 18S rRNA target is also amplified to allow species identification or confirmation by sequencing if necessary. An evaluation of assay accuracy, performed on 76 clinical samples (56 positives using thin smear microscopy as the reference method and 20 negatives), demonstrated clinical sensitivities of 95.2% for *P. falciparum* (20/21 positives detected) and 100% for the *Plasmodium* genus (52/52), *P. vivax* (20/20), *P. ovale* (9/9), and *P. malariae* (6/6). The sensitivity of the *P. knowlesi*-specific PCR was evaluated using spiked whole blood samples (100% [10/10 detected]). The specificities of the real-time PCR primers were 94.2% for *P. vivax* (49/52) and 100% for *P. falciparum* (51/51), *P. ovale* (62/62), *P. malariae* (69/69), and *P. knowlesi* (52/52). Thirty-three specimens were used to test species identification by sequencing the pan-*Plasmodium* 18S rRNA PCR product, with correct identification in all cases. The real-time PCR assay also identified two samples with mixed *P. falciparum* and *P. ovale* infection, which was confirmed by sequencing. The assay described here can be integrated into a malaria testing algorithm in low-prevalence areas, allowing definitive *Plasmodium* species identification shortly after malaria diagnosis by microscopy.

Malaria infections are a major cause of morbidity and mortality throughout the world, with 198 million cases and 584,000 deaths globally in 2013 and with the heaviest burden of disease seen in developing countries (1). Malaria is also the single most common etiologic agent of febrile illness in travelers returning to areas where malaria is not endemic (2). In a recent Geo-Sentinel surveillance study, *Plasmodium* infections accounted for 21% of 6,957 patients with fever in a cohort of 24,920 ill returned travelers (3). Malaria was most frequently diagnosed in febrile persons returning from areas where malaria is endemic such as Sub-Saharan Africa, South and Central Asia, and Latin America (3). Additionally, 33% of mortality among febrile travelers was attributed to malaria (3). Thus, the ability to promptly identify and treat malaria in nonendemic areas is critical. Furthermore, identification of the infecting species is essential for effective treatment of *P. vivax* and *P. ovale*. These *Plasmodium* species possess a dormant hypnozoite stage in the liver that can reactivate and cause disease months to years after the initial infection (4). Primaquine is the only antimalarial drug that can eradicate the hypnozoites and is, therefore, necessary for effective treatment of *P. vivax* and *P. ovale* (4). Additionally, timely identification of *P. knowlesi* is essential because of the high morbidity and mortality associated with this parasite; 7.5% to 10% of *P. knowlesi* infections progress to severe malaria in the absence of antimalarial treatment, with a mortality rate of ~2% (5). Importantly, *P. knowlesi* infections have been reported in returning travelers, although the majority of confirmed cases to date have occurred in a narrow region where *P. knowlesi* infection is endemic (6).

Microscopy is currently the reference standard for diagnosis and species identification of malaria. However, its sensitivity and specificity are compromised by morphological overlaps and

strongly depend on timely processing of blood specimens and on the technologist's expertise (7, 8). Rapid antigen-based diagnostic tests have aided diagnosis of malaria, particularly in resource-poor settings (9), but these tests have limited capability for species discrimination, as they cannot distinguish among non-*falciparum* *Plasmodium* species (10). More recently, various molecular assays have emerged for malaria species identification (5). These assays have increased specificity compared to microscopy and are superior for detecting mixed infections (11), which can account for >5% of malarial infections (12). However, most existing molecular assays employ PCR primers against the *Plasmodium* 18S rRNA gene, which have been reported to cross-react among species (13–15). For example, published primers targeting the *P. knowlesi* 18S rRNA gene have been shown to cross-react with some *P. vivax* isolates (13). Similarly, two different published *P. vivax* 18S rRNA primer sets have been shown to produce nonspecific amplification with other *Plasmodium* species (14). Thus, tar-

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TABLE 1 *Plasmodium* primers used in this study

Primer name	Target species	Target gene	Sequence	Product (bp)	<i>T_m</i> range (°C)
P-FWD P-REV	Pan- <i>Plasmodium</i>	18S rRNA	GGGGGCATTCGTATTACAGAT GCCCTTCGGTCAATTCTTTT	317	74.5–76.6
F-FWD F-REV	<i>P. falciparum</i>	Pfr364 ^a	CCGGAATTCGGGTTTATAGAC GAAGTGCATGTGAATTGTGC	220	74.7–76.9
V-FWD V-REV	<i>P. vivax</i>	Pv <i>dhfr</i>	ACCCGTGTGACGTCTTCTTC GGTGCCCTTGCTGTTGTAC	120	78.7–80.1
M-FWD M-REV	<i>P. malariae</i>	Pm <i>dhfr</i>	CAACTGCACGTCGTTAGACTTTG GCTGGTGTACTGCCTTTGTC	108	76.0–77.9
O-FWD O-REV	<i>P. ovale</i>	Po <i>dhfr</i>	GGKCTTGGTGTCCCTTCA TGTGRGCATTTCTCTAAAACG	113	72.8–74.8
K-FWD K-REV	<i>P. knowlesi</i>	Pkr140 ^a	CTAACACCTCATGTCGTGGTAG AGATCCGTCTCATGATTTCC	200	75.3–77.2

^a Species-specific primers targeting repetitive sequences in *P. falciparum* and *P. knowlesi* were described previously (17, 18) and were adopted with minor modifications.

getting a *Plasmodium* gene that is less conserved across species than 18S rRNA would improve the specificity of *Plasmodium* species discrimination by PCR.

Here, we present the performance characteristics of a laboratory-developed test for *Plasmodium* species identification from whole blood. The assay uses a panel of SYBR green real-time PCRs to detect non-18S rRNA gene targets specific to each of the five *Plasmodium* species known to infect humans. The assay also incorporates amplification of an 18S rRNA pan-*Plasmodium* target to allow species identification by sequencing. This component of the assay increases sensitivity and specificity for cases where intra-species genetic diversity may preclude amplification from the species-specific primers.

MATERIALS AND METHODS

Ethics. Per Stanford University Institutional Review Board, this study was exempt from ethical approval and written informed consent because the human-derived samples constituted nonidentifiable, residual clinical specimens. Samples from Karolinska University Hospital were collected with approval from the Ethical Review Board in Stockholm and with informed patient consent.

Study specimens. Assay validation was performed using 56 archived malaria-positive smear-confirmed specimens: 31 frozen EDTA whole blood samples from Stanford Health Care Clinical Microbiology Laboratory; 20 extracted DNA specimens from Karolinska University Hospital (Stockholm, Sweden); 1 extracted *P. ovale* DNA specimen from the Centers of Disease Control and Prevention (CDC), courtesy of A. J. da Silva; and 4 *P. malariae* specimens from the Mayo Clinic, courtesy of B. S. Pritt (1 whole blood and 3 extracted DNA specimens). Four of the samples were only assessed with the corresponding species-specific primers due to insufficient quantity of material (the *P. ovale*-positive DNA sample from the CDC and three *P. malariae*-positive DNA samples from the Mayo Clinic). The method of DNA extraction was not known for the four specimens for which only extracted DNA was received. *Plasmodium*-negative specimens consisted of 20 frozen EDTA whole blood specimens from patients who received care at the Stanford Health Care for nonmalarial illnesses. The specificity of species-specific primer sets was tested using these *Plasmodium*-negative specimens as well as specimens that were positive for a single *Plasmodium* species but negative for the other four. The lower limits of detection of the PCR assays for *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae*, and pan-*Plasmodium* were assessed by testing serial 1:5

dilutions of extracted DNA from patient specimens with morphologically and molecularly confirmed infection, diluted in water. The estimated parasitemia ranges (based on the number of serial dilutions) that were tested were 0.2% to 0.0000128% for *P. falciparum*, *P. ovale*, *P. malariae*, and pan-*Plasmodium* and 0.3 to 0.0000192% for *P. vivax*. Assuming red blood counts (RBC) of 4,000,000 RBC/ μ l (<http://www.cdc.gov/dpdx/diagnostic-procedures/blood/microexam.html>), these ranges are equivalent to 8,000 to 0.5 and 12,000 to 0.77 parasites/ μ l, respectively. The sensitivity of the pan-*Plasmodium* primers was tested using the *P. falciparum* specimen dilutions. Assay validation for *P. knowlesi* was performed by spiking different concentrations of genomic *P. knowlesi* DNA (MRA-456G; BEI Resources Repository, NIAID, NIH) into 10 *Plasmodium*-negative whole blood samples. Specifically, 5 μ l of three different dilutions of genomic *P. knowlesi* DNA (76,887, 7,689, and 769 genome copies/ μ l) were spiked into 200 μ l whole blood and DNA was extracted. The concentrations of the *P. knowlesi*-spiked specimens were estimated to be 1,922 copies/ μ l of whole blood (3 samples), 192 copies/ μ l (3 samples), and 19 copies/ μ l (4 samples). These specimens were tested with the *P. knowlesi* primers on two separate occasions. They were also tested for specificity with the four non-*knowlesi* species-specific primers.

Primer design. Primers targeting the dihydrofolate reductase (*dhfr*) gene were designed for *P. ovale*, *P. vivax*, and *P. malariae* (Table 1) using representative sequences for the five species: *P. vivax* (GenBank accession no. EU478864.1), *P. malariae* (EF198111.1), *P. ovale* (EU266602.1), *P. falciparum* (J03028.1), and *P. knowlesi* (GQ250089.1). Multiple-sequence alignment and pairwise comparisons were performed using the Clustal Omega tool (16) in order to identify candidate regions with species-specific signatures. Candidate primers were assessed for specificity *in silico* by querying with BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) against the representative species-specific *dhfr* sequences above. The *P. ovale* primers include ambiguous nucleotides in positions where the *P. ovale wallikeri* and *P. ovale curtisi* subspecies differ (Table 1). Primer sensitivities were evaluated *in silico* by querying with BLAST against all complete *dhfr* coding sequences available for the respective species in the NCBI nucleotide database: 19 sequences for *P. vivax*, 8 for *P. ovale*, and 9 for *P. malariae*. Species-specific primers targeting repetitive sequences in *P. falciparum* and *P. knowlesi* have been described previously (17, 18) and were adopted with minor modifications (Table 1). The pan-*Plasmodium* primers target highly conserved sequences in the 18S rRNA gene and encompass a region of ~320 bp with internal sequence divergence that is sufficient to allow for species discrimination by sequencing. The human beta actin primer set, which serves as an extraction control, has been described previously (19).

Nucleic acid extraction. Frozen whole blood specimens stored at -80°C were thawed at room temperature and mixed by vortexing for 15 s. DNA was extracted from 200 μl of whole blood using the QIAamp DNA Blood minikit according to the manufacturer's instructions (Qiagen, Germantown, MD) and eluted into 100 μl of manufacturer-supplied elution buffer. The DNA samples obtained from Karolinska Institute were extracted using the MagAttract DNA Mini M48 kit and BioRobot M48 automated nucleic acid purification workstation (Qiagen, Germantown, MD).

Real-time PCR conditions. Monoplex reactions were performed for each specimen with the five species-specific primer sets, the pan-*Plasmodium* primer set, and the human beta actin extraction control. The person performing PCR was blinded to the microscopy determined species identity of each sample. All reactions were performed on a Rotor-Gene 6000 real-time cycler (Qiagen) using 10- μl reaction volumes, 5 μl of $2\times$ Fast-Start SYBR green master mix (Roche Applied Science, Indianapolis, IN), 3 μl of DNA eluate, and forward and reverse primers at final concentrations of 500 nM each, except for *P. vivax* (350 nM). The final primer concentrations were optimized experimentally. The following cycling parameters were used: 95°C for 5 min and 45 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 40 s, followed by melting analysis with ramping from 60°C to 90°C in 0.2°C increments.

Sequencing of pan-*Plasmodium* amplicons. Thirty-three amplification products from the pan-*Plasmodium* 18S rRNA real-time PCRs were used to validate the amplicon sequencing portion of the assay. Amplicons were selected to represent all five species: 16 *P. falciparum*, 3 *P. ovale*, 2 *P. malariae*, 8 *P. vivax*, and 4 *P. knowlesi*. In some cases, the amplicons were derived from the same microscopy positive templates but independent real-time PCRs. Amplicons were purified with the ExoSAP-IT kit (Affymetrix, Santa Clara, CA). Bidirectional cycle sequencing was performed as previously described (20) on a DNA Engine thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the pan-*Plasmodium* P-FWD and P-REV primers and BigDye Terminator mix (Life Technologies, Grand Island, NY). Sequencing products were purified with a BigDye XTerminator purification kit (Life Technologies, Grand Island, NY). Sequencing data analysis was performed on an ABI 3730 genetic analyzer (Life Technologies, Grand Island, NY). DNA sequences were assembled with the Lasergene software (DNASTar, Madison, WI) and queried with NCBI BLAST in the GenBank database. A distance score of 0% to $<1\%$ was used as the criterion for species identification.

Control nucleic acids and reference material. The following specimen types were used for primer optimization: cultured *P. falciparum* (courtesy of E. Yeh, Stanford University), patient specimens confirmed positive by microscopy for *P. vivax*, *P. ovale*, and *P. malariae*, and genomic DNA from *P. knowlesi* H strain (MRA-456G; BEI Resources Repository, NIAID, NIH). Positive controls for each species were included in each PCR run and included extracted DNA from cultured *P. falciparum* at 1% parasitemia, *P. knowlesi* genomic DNA diluted in water to ~ 770 genome copies/ μl , extracted DNA from a patient with microscopy confirmed *P. vivax* infection, and plasmid clones of the target amplicons for *P. malariae* and *P. ovale* (DNA2.0, Menlo Park, CA) diluted to ~ 660 copies/ μl .

Statistics. The melting temperature (T_m) ranges for each reaction represent two standard deviations around the mean for all positives tested with that primer set (Table 1). Sensitivities, specificities, and 95% confidence intervals (CIs) were calculated using the VassarStats website (<http://vassarstats.net/>). Discrepant test results were resolved by testing additional specimens from the same patients, repeat testing, and/or sequencing the relevant amplicons as necessary.

RESULTS

Primer design and optimization. Given the potential for cross-reactivity among species with 18S rRNA *Plasmodium* species primers (13, 14), a search was performed in the NCBI nucleotide database for genes with divergent sequences that may be targeted by PCR. Genes with multiple sequences available for each species

were included and further analyzed by interspecies and intraspecies multiple sequence alignments. This led to the identification of *dhfr* as a gene with sequences that are divergent among species but conserved among strains of a single species. Pairwise comparisons of representative sequences revealed interspecies sequence identities of 66% to 76%, except for *P. knowlesi* and *P. vivax*, which were $\sim 85\%$ identical (data not shown). Candidate primer sets targeting *dhfr* were designed for *P. vivax*, *P. malariae*, and *P. ovale* and were accepted if they had 100% alignment to all available sequences of the target species and reduced alignment to other species. The species-specific regions targeted for *P. falciparum* and *P. knowlesi* were based on prior reports (17, 18), and the corresponding published primers were adopted with minor modifications (Table 1). Primer conditions were optimized using control nucleic acids for each species, and the expected melting temperature for each species amplicon was determined. Representative melting curves for each primer pair are shown in Fig. 1.

Real-time PCR assay analytical evaluation. Archived specimens from malaria-negative patients and patients positive for malaria by microscopy were used to assess the performance of the real-time PCR assay. The number of specimens tested for each species, as well as the calculated sensitivities and specificities, are shown in Table 2. For *P. falciparum*, 1 out of 21 microscopy positive specimens was not detected with the *P. falciparum* primers but was correctly identified by sequencing of the 18S rRNA amplification product. The calculated sensitivity for the *P. falciparum*-specific primers is 95.2% (95% confidence interval [CI], 74.1% to 99.7%). The calculated sensitivities for the other primer sets were 100% for pan-*Plasmodium* (95% CI, 94.1% to 100%), 100% for *P. vivax* (95% CI, 79.9% to 100%), 100% for *P. ovale* (95% CI, 62.9% to 100%), and 100% for *P. malariae* (95% CI, 52.7% to 100%). The *P. knowlesi*-specific primers, tested on 10 whole blood samples spiked with *P. knowlesi* genomic DNA at 1,922, 192, and 19 copies/ μl , detected all specimens on two separate occasions.

Analytical specificities for the individual primer sets were 100% for pan-*Plasmodium* (95% CI, 79.9% to 100%), 100% for *P. falciparum* (95% CI, 91.2% to 100%), 100% for *P. ovale* (95% CI, 92.7% to 100%), and 100% for *P. malariae* (95% CI, 93.4% to 100%). For *P. vivax*, 3 out of the 52 clinical samples that were presumed negative for *P. vivax* gave unexpected *P. vivax* products. There was insufficient sample quantity to repeat the PCR for these samples, and therefore, it was not possible to discriminate between nonspecific amplification and mixed infection. The calculated specificity for the *P. vivax*-specific primers is 94.2% (95% CI, 83.1% to 98.5%). When the *P. knowlesi*-spiked samples were tested with the other species-specific primer sets, there was nonspecific amplification in only one sample, which gave a product with the *P. vivax* primers.

Two specimens, obtained at different time points from the same patient, were positive for *P. falciparum* and *P. ovale*. However, the peripheral blood smear and BinaxNOW malaria test results were consistent with *P. falciparum* infection. Sequencing of the 18S rRNA amplification product detected only *P. falciparum*. To confirm the presence of *P. ovale*, we tested additional specimens from the same patient by real-time PCR and sequenced the *P. ovale* product, which aligned to *P. ovale dhfr* sequences in the GenBank database, using NCBI BLAST. This case demonstrates the utility of molecular methods for identification of mixed infec-

FIG 1 Representative melting temperature curves for the species-specific and pan-*Plasmodium* SYBR green-based real-time PCR assay. The curves were generated using control nucleic acids for each primer set. Data are presented as rate of change in relative fluorescence units (RFU) with time (T) (d(RFU)/dT) on the y axis versus melting temperature (T_m) (°C) on the x axis. The melting temperatures indicated in the boxes represent the temperature at which the curve crosses a d(RFU)/dT threshold set at 1.

tations since the finding of mixed infection led to treatment with primaquine.

The pan-*Plasmodium* primers were evaluated with 52 microscopy confirmed samples: *P. falciparum* (*n* = 19), *P. vivax* (*n* = 20), *P. ovale* (*n* = 8), *P. malariae* (*n* = 3), and 2 mixed *P. falciparum*-*P. ovale* infections, all of which amplified. The ability of the pan-*Plasmodium* primers to detect *P. knowlesi* was first assessed using the spiked whole blood samples described above. However, these samples rarely amplified from the pan-*Plasmodium* primers. The lower sensitivity may be due to lower primer efficiency, interfering substances, and/or nucleic acid fragmentation during the blood spiking and extraction limiting amplification of the larger amplicon size. To investigate the decrease in sensitivity of the pan-*Plasmodium* primers for *P. knowlesi*, we prepared serial dilutions of *P.*

knowlesi genomic DNA in water and repeated the real-time PCR with the *P. knowlesi* and pan-*Plasmodium* primers. The lowest concentration that was detected by the two primer sets was ~8 genome copies/μl; however, the pan-*Plasmodium* primers showed a larger cycle threshold (data not shown), indicating lower efficiency compared to *P. knowlesi*-specific primers.

In the limit of detection experiments, PCRs were positive down to the lowest estimated parasitemia levels that were tested: 0.0000128% for *P. falciparum*, *P. ovale*, *P. malariae*, and pan-*Plasmodium* and 0.0000192% for *P. vivax*. This is equivalent to ~0.5 and ~0.77 parasites/μl, respectively, assuming red blood counts (RBC) of 4,000,000 RBC/μl. The lowest tested concentrations of *P. knowlesi* spiked in whole blood or diluted in water also produced positive results (19 genome copies/μl or 8 genome copies/

TABLE 2 Performance of the real-time PCR *Plasmodium* primer sets in this study

<i>Plasmodium</i> target	Microscopy positive (no.)	Real-time PCR positive (no.)	Sensitivity (%)	True negative (no.)	Real-time PCR negative (no.)	Specificity (%)	Total samples tested (no.)
<i>Plasmodium</i> genus	52	52	100.0	20	20	100.0	72
<i>P. falciparum</i>	21	20	95.2	51	51	100.0	72
<i>P. vivax</i>	20	20	100.0	52	49	94.2	72
<i>P. ovale</i>	9	9	100.0	62	62	100.0	71 ^a
<i>P. malariae</i>	6	6	100.0	69	69	100.0	75 ^a
<i>P. knowlesi</i>	10	10	100.0	52	52	100.0	62 ^b

^a Four of 56 malaria smear-positive samples were tested with only the expected species-specific primers due to the limited quantity of DNA (one *P. ovale* and three *P. malariae* samples). Two mixed *P. falciparum*-*P. ovale* specimens were excluded from the *P. ovale* counts because the presence of *P. ovale* was not known prior to PCR testing and therefore were not counted as either true-positive or true-negative samples.

^b Only 52 of the 76 previously characterized samples were tested due to limited quantity of available DNA. The 10 *P. knowlesi*-spiked samples were not included because they are not true clinical samples.

μl, respectively). These results indicate that the species-specific and pan-*Plasmodium* real-time PCR primers described here are highly sensitive for detection of the five malaria species infecting humans in microscopy positive specimens.

Sequencing assay analytical evaluation. The ability to identify the five species by sequencing the 18S rRNA gene was assessed in 33 amplification products of the pan-*Plasmodium* real-time PCR assay described above. Samples for sequencing were selected so that all five species were represented by >1 amplification product. The person performing sequencing was blinded to the microscopy and PCR-determined species identity of each sample. The assay was able to correctly identify 16/16 *P. falciparum*, 3/3 *P. ovale*, 2/2 *P. malariae*, 8/8 *P. vivax*, and 4/4 *P. knowlesi* samples. As described above, we also sequenced a mixed *P. falciparum*-*P. ovale* infection and only detected *P. falciparum*. It is well established that the sensitivity limit of Sanger sequencing is ~20% for mixed sequences. Thus, we hypothesize that *P. falciparum* represents the dominant species in this case, while *P. ovale* nucleic acids are present at <20% of *Plasmodium* DNA. Overall, there was 94.1% agreement between the sequencing and real-time PCR results; 32 samples were concordant between each method and one true positive was missed by each of the methods (the mixed infection by sequencing and the *P. falciparum* sample missed by the species-specific PCR as discussed above).

DISCUSSION

The current study describes the design and evaluation of a real-time PCR assay for *Plasmodium* species identification using non-18S rRNA targets to increase specificity. An advantage over previously published molecular assays is the inclusion of a pan-*Plasmodium* 18S rRNA PCR whose amplicons can be sequenced if amplification with the species-specific PCR primers fails, for example, due to sequence divergence at the primer annealing sites. In such cases, the sequencing step of this assay will still allow species discrimination. It may also allow the detection of *Plasmodium* species previously unknown to infect humans similar to the recent discovery of *P. knowlesi* as a human pathogen (5, 6).

The majority of existing molecular assays for *Plasmodium* species identification target the 18S rRNA gene, including a widely used nested PCR (21) and a number of real-time PCR assays (22–24). Although highly sensitive and specific, nested PCR assays carry an inherent risk of contamination during transfer of amplicons between tubes (25). Real-time PCR has the advantage of a closed system and increased sensitivity relative to conventional PCR (25); however, there is evidence that assays targeting the *Plasmodium* 18S rRNA gene may not be optimal for species discrimination due to primer cross-reactivity (13, 14). This is likely due to the high degree of conservation of the 18S rRNA gene among *Plasmodium* species. In fact, pairwise comparisons of representative 18S rRNA sequences for the five species revealed interspecies sequence identities of 77% to 87%, and the divergent regions of the gene were not optimal for primer design due to high A/T content (data not shown). Additionally, when we attempted to adopt several published 18S rRNA targeting primers, we were not able to verify their species specificity in a SYBR green assay (data not shown), similar to what has been reported by others (15). Targeting less conserved genomic regions may improve specificity; however, for most non-*falciparum* species, there is a paucity of publicly available sequences, which compromises the ability to adequately assess intraspecies sequence conservation. Thus, for

non-18S rRNA targets, there is a risk of inadvertently designing primers in genomic regions that may be genetically divergent to an unanticipated level, leading to reduced sensitivity. It is also important to recognize that whereas initial studies may report high species specificities when tested with relatively small numbers of clinical samples, those may not be verifiable as additional strains are tested, and this applies for 18S and non-18S primers (13–15).

The assay described here overcomes these potential pitfalls for species identification by employing non-18S rRNA real-time PCR targets and an 18S rRNA sequencing target. The non-18S rRNA primers demonstrated high specificity for the intended species, with the following exceptions. Three clinical specimens that were microscopy positive for other species and one *knowlesi*-spiked sample gave low-level products with *P. vivax* primers (Table 2). This low-level *P. vivax* amplification was observed only in patient samples for which the extracted DNA had been stored for prolonged periods of time and subjected to multiple freeze-thaw cycles and also in one of the diluted *P. knowlesi*-spiked samples, suggesting that low-frequency nonspecific annealing to fragmented DNA may be occurring in such partially compromised specimens. The performance of existing 18S rRNA-targeting *P. vivax* assays in similar specimens has not been assessed. Importantly, the non-18S rRNA primers targeting each *Plasmodium* species demonstrated overall specificity and sensitivity exceeding 94%, although a larger sample size with broad global distribution is necessary to confirm their sensitivity.

The sequencing step of the assay employs consensus primers to generate a relatively large amplification product (~320 bp) that contains the A/T-rich divergent regions described above, allowing species discrimination by sequencing. All of the 18S rRNA amplification products that were evaluated by sequencing identified the same species as microscopy and/or real-time PCR. In fact, the single *P. falciparum* sample missed by the species-specific PCR was correctly identified upon sequencing of the 18S rRNA product. However, the sequencing assay had limited ability to resolve mixed infections: it is expected to produce either a mixed sequence, requiring further investigation, or one sequence representing the dominant species. The latter outcome was observed in the case with mixed *P. falciparum*-*P. ovale* infection sequenced in this study. This indicated that the sequencing assay should not be used alone for *Plasmodium* species determination but as a follow-up to the real-time PCR to resolve cases of absent amplification from the species-specific primers or cases where microscopy and the PCR are discrepant.

A potential issue with 18S *Plasmodium*-targeting primers is nonspecific annealing to *Babesia* nucleic acids, as reported recently (26), due to a high degree of sequence homology in the 18S rRNA genes of the two parasites. The pan-*Plasmodium* primers in our assay are not expected to yield false-positive results in *Babesia*-infected patients for the following reasons. First, there are multiple mismatches in the 3' end of the forward primer (3 out of 5 bp) with the *Babesia* 18S rRNA gene, which predicts poor annealing to the *Babesia* target (27). Second, even if amplification does occur with the 18S primers from a *Babesia* template, the non-18S species-specific primers would not produce amplicons, which would lead to sequencing of the 18S product and identification of *Babesia*. Thus, the design of our assay minimizes the possibility of the type of false-positive result reported in the study above.

The dilution experiments performed in this study indicate that the real-time PCR primer sets are sensitive to levels of at least 0.77

parasites/μl for the four common species and the pan-*Plasmodium* primer set, which is well below the sensitivity of microscopy, estimated at 5 to 20 parasites/μl for thick smears (28). Of note, we did not observe a decrease in sensitivity in these experiments when a multicopy genomic sequence was examined for *P. falciparum* (~40 copies of Pfr364 per genome [17]) versus the single-copy *dhfr* target for *P. ovale*, *P. malariae*, and *P. vivax*. However, these experiments were performed with serial dilutions of extracted nucleic acids rather than testing whole blood specimens with the corresponding levels of microscopy determined parasitemia. Nevertheless, during the assay validation, we tested 10 microscopy negative samples from patients with recent histories of malaria infection and antimalarial treatment, of which eight were positive by the real-time PCR assay. These results indicate that the assay is highly sensitive and detects either submicroscopic parasite burdens and/or circulating cell-free nucleic acids from lysed parasites.

One limitation of this assay is that the six *Plasmodium* PCRs and the one human real-time PCR are performed in monoplex. The SYBR green method is simple and widely used and eliminates concerns about fluorescent probe stability when an assay is performed infrequently; however, it does not allow a high degree of multiplexing. Although monoplexing may be acceptable in low-prevalence settings where the assay is performed infrequently, multiplexing will be required to make this assay adaptable to laboratories with larger testing volumes. Approaches that may be taken to allow multiplexing of the assay include high resolution melt analysis or incorporation of fluorescently labeled probes that can be detected at different wavelengths.

Another drawback of our study is the absence of clinical *P. knowlesi*-positive samples tested in the validation. This reflects the infrequent clinical infections with this species in returning travelers (6). It should also be noted that samples spiked with *P. knowlesi* nucleic acids were poorly detected with the pan-*Plasmodium* 18S rRNA primer set, although they were robustly detected with the *P. knowlesi*-specific primers. We found that pan-*Plasmodium* primers are less sensitive than *P. knowlesi*-specific primers based on cycle threshold values when we tested serial dilutions of *P. knowlesi* nucleic acids in water. One possible explanation for the lower sensitivity is nucleic acid fragmentation during preparation and extraction of the spiked samples. Since the pan-*Plasmodium* amplicon is relatively large (~320 bp), it will likely be more affected by DNA fragmentation than the *P. knowlesi* PCR (amplicon size of ~200 bp). As these are artificial samples, it will be important to assess the performance of the two primers in the future in clinical *P. knowlesi*-infected specimens.

In summary, we describe a highly accurate and simple real-time PCR assay for *Plasmodium* species identification, with a potential sequencing step to increase sensitivity if the species-specific primers do not amplify a product. The assay can be incorporated into a testing algorithm in low-prevalence areas, where microscopy is used to screen for malaria upon presentation.

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