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Validation of a Semiquantitative Real-Time PCR Assay for Malaria Diagnosis and Treatment Monitoring

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Microscopic examination of blood smears is the current gold standard for laboratory confirmation of malaria. However, it is time-consuming and lacks sensitivity while requiring highly skilled laboratory professionals. This study validated a multiplex real-time PCR test for the detection, speciation, and semiquantification of *Plasmodium* species in whole blood. Analytical results showed the assays were highly specific and sensitive, with a PCR efficiency of 100.21% and 101.99% for *Plasmodium falciparum* and *Plasmodium vivax*, respectively. The limit of detection was 0.0001% parasitemia for both *P. falciparum* and *P. vivax* with remarkable precision. Accuracy was validated by testing 128 EDTA—whole blood samples from patients suspected of having malaria, with only two discordant results (positive percentage agreement = 98.65%; negative percentage agreement = 98.15%). Moreover, the linearity between cycle threshold value and parasite density (R^2 = 0.941) was validated by testing samples serially collected over a 12-day period from a patient positive for *P. falciparum*, which enabled semiquantitative estimation of parasitemia. This malaria PCR test demonstrates high sensitivity, specificity, reproducibility with minimal hands-on time, and a quick technical turnaround time of <2 hours with capability of providing parasite density estimation to improve diagnosis and monitor treatment of malaria. *(J Mol Diagn 2026, ■: 1—9; https://doi.org/10.1016/j.jmoldx.2026.06.005)*

Despite progress in malaria control, the World Health Organization has estimated between 263 and 294 million malaria cases and close to 600,000 deaths occurred globally in 2023, averaging 60.4 cases per 1000 population at risk, exceeding that of 2022 by approximately 11 million.[1] Although 94% of the global cases occur in the African continents, approximately 2000 cases are reported yearly in the United States (US Centers for Disease Control and Prevention, *https://www.cdc.gov/malaria/php/surveillance-report/index.html*, last accessed July 30, 2025). Although most of the US cases are returning travelers from endemic counties, locally acquired cases have also been reported.[2]

Malaria is transmitted mainly through bites by female *Anopheles* mosquito carrying *Plasmodium* parasites. Although there are >150 known species, 5 are known to cause infection in human: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*. *Plasmodium falciparum* is responsible for the highest mortality and morbidity rate, whereas *P. vivax* is the most widely distributed across all endemic areas and the second largest cause of malaria.[3] The clinical presentations vary depending on the infecting species, the level of parasitemia, and the host factors, including the immune status.[4] In addition to some of the common symptoms, including fever, chills, myalgias, headache, weakness, vomiting, jaundice, splenomegaly, and thrombocytopenia, infections by *P. falciparum* can progress to central nervous system involvement, acute renal failure, severe anemia, or acute respiratory distress syndrome.[5,6]

Despite progress made in diagnosis, treatment, and prevention, the disease has remained endemic in many parts of the world. Therefore, the risk of outbreaks in nonendemic countries, where many immune-naïve populations live, is still high because of travel-related cases. Without accurate

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and rapid diagnosis leading to proper treatment, malaria can lead to complications and death. Identification of malaria at the species level with parasitemia is critical in determining disease severity and guiding treatment decisions. Microscopic examination of blood smears is the gold standard test for laboratory confirmation of malaria, but it requires highly skilled laboratory scientists and lacks sensitivity in cases of low parasitemia. Moreover, current antigen detection methods lack specificity and are often limited to identification of *P. falciparum* only.[7–9]

Molecular methods for malaria testing based on real-time PCR are being explored. However, progress has been slow. To date, there are two commercially available US Food and Drug Administration–approved malaria molecular tests: the cobas Malaria test (Roche Diagnostic, Indianapolis, IN), which is used for screening blood donors for malaria; and the BioFire Global Fever Panel (bioMerieux, Durham, NC). This study validated a malaria PCR test using primers and probes developed by Kamau et al[10] for the detection and semiquantification of *P. falciparum*, *P. vivax*, and other *Plasmodium* species in EDTA–whole blood on the BD MAX automated system.

Materials and Methods

Specimen Enrollment

Both fresh and frozen residual EDTA–whole blood specimens from patients with clinical suspicion for malaria were included in the study. All samples were previously tested for malaria by either blood smear examination or malaria PCR [performed by the ARUP Reference Laboratories (Salt Lake City, UT)].

Reagent and Testing

The BioGX Sample-Ready OSR (BioGx, Birmingham, AL) is formulated in lyophilized PCR mix containing previously described and published primers/fluorescent probes sets by Kamau et al[10] for the multiplex real-time PCR-based detection of rRNA gene from the DNA of *Plasmodium* species and the human RNase P gene, which serves as an endogenous extraction or sample processing control. The malaria PCR test is run on the BD MAX system (BD Diagnostic, Franklin Lakes, NJ) using the BD MAX ExK DNA-3 Extraction Strips and OSR reagent tube, which contains all PCR primers, probes, enzymes, dNTPs, $MgCl_2$, buffers, and other components required for PCR. The multiplex PCR detects and differentiates *P. falciparum*, *P. vivax*, and other *Plasmodium* species.

For testing, 50 μL of the EDTA–whole blood specimen was added to the BD MAX Sample Buffer Tube. After vortexing, the tube went through the automated process of DNA extraction, elution, real-time PCR amplification, and detection. The BD MAX ExK DNA-3 Extraction Strips contain a combination of lytic and extraction reagents designed to perform cell lysis and DNA extraction. Following cell lysis, the released DNA is captured by magnetic affinity beads. The beads with the bound DNA are washed and then DNA is eluted using 12.5 μL of Elution Buffer. A fraction of the eluted DNA is used for PCR amplification and detection on the BD MAX System. The eluted DNA is transferred to 25 μL BioGX Rehydration Buffer. From the 37.5 μL BioGX Rehydration Buffer/Eluate mixture, 12.5 μL is transferred to rehydrate the lyophilized BioGX Master Mix. The entire 12.5-μL volume of rehydrated master mix is transferred to the BD MAX PCR cartridge, and 4 μL of the 12.5 μL is monitored for PCR detection on the BD MAX System. The thermocycling conditions were as follows: 300-second hold at 97°C followed by 40 cycles of 12 seconds at 97°C, 25 seconds at 60°C, and 10.2 seconds at 70°C. A sample is negative for malaria when *P. falciparum*, *P. vivax*, and *Plasmodium* species DNA targets are not detected, and the internal control (RNase P) DNA target is detected. If none of the targets is detected, the sample is invalid. If both *P. falciparum* and *Plasmodium* species targets are detected, the sample is positive for malaria regardless if the internal control is detected or not. The same scenario is true for *P. vivax*. There are other circumstances that may require further analysis or a repeat testing for confirmation. For instance, if neither *P. falciparum* nor *P. vivax* targets are detected and *Plasmodium* species target is detected, it may be that the sample has a low parasite density, or the sample is positive for a *Plasmodium* species other than *P. falciparum* or *P. vivax*.

Analytical Evaluation

To determine the limit of detection (LoD), 10-fold dilutions of blood with known parasitemia for *P. falciparum* and *P. vivax* were prepared and tested in triplicate. The presumed LoD was defined as the last concentration at which all three replicates tested positive for the target species. The LoD was then confirmed by testing a total of 20 replicates for *P. falciparum* and 23 replicates for *P. vivax*, with at least 95% detection rate. Specificity was evaluated by testing EDTA–whole blood specimens spiked with *Candida albicans*, *Candida guillermondii*, *Candida glabrata*, *Candida auris*, *Cryptococcus neoformans*, and *Cryptococcus gatti* at 0.5 McFarland concentration as well as blood specimens positive for *Babesia*, *Ehrlichia*, and *Anaplasma* acquired from ARUP Reference Laboratories. This study also investigated whether the test could accurately detect mixed species with the same sensitivity by testing *P. falciparum* and *P. vivax* dual-positive samples compared with single-species positive samples. To validate the assay precision, a panel of controls that include *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and negative EDTA–whole blood was evaluated by three different operators (A.L., S.G.-F., and S.K.) on different BD MAX System instruments at different

time points. Moreover, specimen stability was evaluated by storing EDTA–whole blood specimens positive for *P. falciparum* and *P. vivax* at room temperature for up to 3 weeks and 4°C for up to 4 weeks. A set of three replicates were tested at each time point, and the variation in cycle threshold (C_T) values was analyzed. The effect of temperature <4°C was not assessed as a pilot study showed that specimens were stable at frozen temperature even after at least two freeze-and-thaw cycles.

Accuracy

A total of 128 EDTA–whole blood samples from patients with clinical suspicion for malaria were tested. Of those samples, 25 were kept frozen until testing, 49 were refrigerated for up to 3 weeks before testing, and 54 were stored at room temperature and tested within 48 hours of collection. Seventy-nine of those samples (53 negatives, 26 positives) were previously tested by blood smear examination, the standard of care testing in the laboratory. Another 49 samples were previously tested positive by malaria PCR performed at ARUP. Residual specimens that were collected more than a month before testing were kept frozen until testing. Specimens acquired from ARUP were kept refrigerated and tested within 3 weeks after collection. The rest of the specimens were tested within 48 hours of collection.

Concordance Analysis

The positive percentage agreement, negative percentage agreement, and overall percentage agreement between the malaria PCR and the reference methods were calculated for qualitative agreement. To determine parasite density, the correlation between C_T value and parasitemia was established by plotting the logarithmic values of the actual parasitemia from repeated sampling of the same patient and the logarithmic values of the calculated parasitemia using the linear regression curve equation established from the serial dilution experiments for LoD and assay efficiency.

Results

Analytical Validation

The analytical performance of the malaria PCR test was evaluated on the BD MAX system. Data showed excellent linearity over five orders of magnitude for *P. falciparum* with a slope = −3.3171, R^2 = 0.993, and PCR efficiency = 100.20% (Figure 1A). Likewise for *P. vivax*, a linearity was observed over the range of dilutions tested with a slope = −3.2759, R^2 = 0.9974, and PCR efficiency = 101.96% (Figure 1B). Linearity over four orders of magnitude was also observed for both *P. ovale* and *P. malariae* with a slope = −3.1083 and PCR

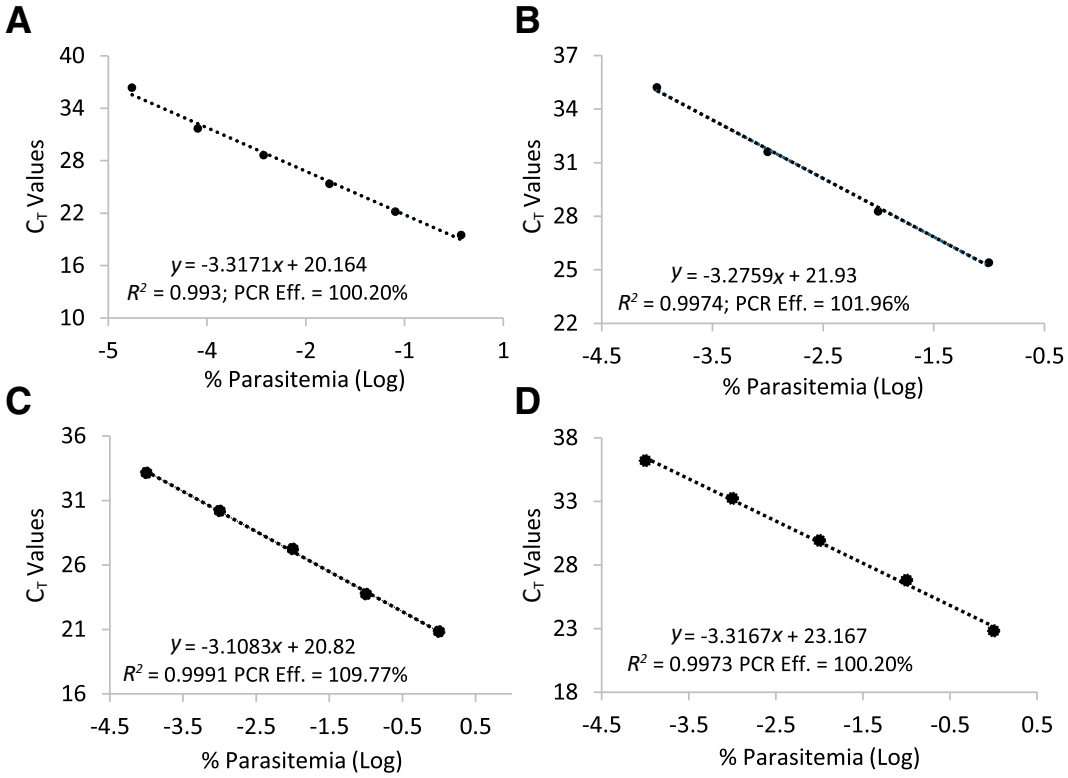


Figure 1 Linear regression plots generated from 10-fold serial dilution of EDTA–whole blood sample with known parasitemia tested in triplicate. The *y* intercept, slope, R^2 value, and PCR efficiency (Eff.) were determined for *Plasmodium falciparum* (**A**), *Plasmodium vivax* (**B**), *Plasmodium ovale* (**C**), and *Plasmodium malariae* (**D**).

efficiency = 109.77% (Figure 1C) and a slope = −3.3167 and PCR efficiency = 100.20%, respectively (Figure 1D).

The LoD for *P. falciparum* and *P. vivax* was established by using EDTA−whole blood specimen with known parasitemia, 2.3% and 0.1% parasitemia for *P. falciparum* and *P. vivax*, respectively. The presumed LoD was determined to be 0.0001% parasitemia or 5 parasites/μL (assuming a standard red blood cell count of 5,000,000/μL) for both *P. falciparum* and *P. vivax* (Table 1). The LoD was confirmed for both targets after testing a total of 20 replicates for *P. falciparum* and 23 replicates for *P. vivax*. The LoD of *P. ovale* and *P. malariae* was also assessed and estimated to be comparable to *P. falciparum* and *P. vivax* based on the serial dilution studies (data not shown).

The assay was shown to be specific as no cross-reactivity was observed when nontarget blood pathogens, such as *Babesia*, *Ehrlichia*, *Anaplasma*, and yeasts, were tested (data not shown). Because infection with multiple organisms, although rare, may affect detection and C_T value interpretation, samples positive for both *P. falciparum* and *P. vivax* were tested. The average C_T value differences between the dual-positive samples and single-species positive samples for *P. falciparum* and *P. vivax* were 0.35 and 0.75, respectively (data not shown), less than 2 SDs (0.78).

The within-run precision was determined by testing a panel that includes *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and negative EDTA−whole blood in triplicate in the same run on the same BD MAX System. The assay was shown to be reproducible with SD of the C_T values ranging from 0.25 to 0.75 and CV of the C_T values ranging from 0.01% to 0.04% (data not shown). Twenty-five total replicates were tested for between-run precision by three different operators on different BD MAX instruments at different time points. An SD of 0.27 to 0.39 C_T and a CV ranging from 1.2% to 1.8% were observed (data not shown). Moreover, the data showed that EDTA−whole blood samples were stable for at least 3 weeks at room temperature with SD from the mean C_T values varying from 0.15 to 1.06 and from 0.10 to 0.45 for *P. falciparum* and *P. vivax*, respectively (Figure 2, A and B). When refrigerated, samples were stable for at least 4 weeks with SD from the mean C_T values varying from 0.36 to 1.06 and from 0.10 to 0.56 for *P. falciparum* and *P. vivax*, respectively (Figure 2, C and D).

Accuracy Assessment

The clinical performance of the malaria PCR assay was evaluated by comparing the results with those from the microscopic examination of blood smears, the standard-of-care method in the laboratory, and those tested by a malaria PCR test performed at ARUP Reference Laboratories, together referred to reference methods. Of the 128 residual samples included in this study, 53 were negative and 73 were positive by the reference methods (Table 2). The positive samples included 42 *P. falciparum*, 24 *P. vivax*, 7 *P. ovale*, and 2 *P. malariae*. Of the 128 samples, 73 were tested positive by both tests, whereas 53 were tested negative. Only two samples were discordant. One smear-negative sample tested positive by the malaria PCR; the patient had consistent travel history and symptoms for malaria, and thus this case was determined to be truly positive for malaria. Another sample previously tested positive by the ARUP malaria PCR tested negative by the assay; this was most likely because of the sample stability and being weakly positive (original C_T = 38.9), as the threshold cutoff is 40 cycles. The overall concordance was 98.44% (n = 128). The positive percentage agreement was 98.65% (95% CI, 92.70%−99.97%), and the negative percentage agreement was 98.15% (95% CI, 90.11%−99.95%).

Case Presentation and Parasitemia Estimation by C_T Value

A 21-year-old man with no significant medical history presented to the emergency department with nausea, vomiting, diarrhea, extreme fatigue, fever, and chills for the past

Table 1 Limit of Detection for *Plasmodium falciparum* and *Plasmodium vivax*

Parasite	Concentration of parasitemia, %	Replicates, *N*	Detected		Mean C_T	SD
			N	%		
P. falciparum	2.3	3	3	100	19.47	0.23
	0.23	3	3	100	23.20	1.82
	0.023	3	3	100	25.33	0.15
	0.0023	3	3	100	28.60	0.26
	0.00023	3	3	100	31.67	0.40
	0.0001	20	20	100	32.64	0.40
	0.000023	23	16	68	36.32	1.05
P. vivax	0.1	3	3	100	25.40	0.36
	0.01	3	3	100	28.27	0.58
	0.001	3	2	67	31.60	0.14
	0.0001	23	22	96	35.21	1.49
	0.00001	3	0	0	0	0

The *P. falciparum* limit of detection (LoD) = 0.0001 with 100% detected with mean C_T = 32.64 ± 0.40. The *P. vivax* LoD = 0.0001 with 96% detected with mean C_T = 35.21 ± 1.49.

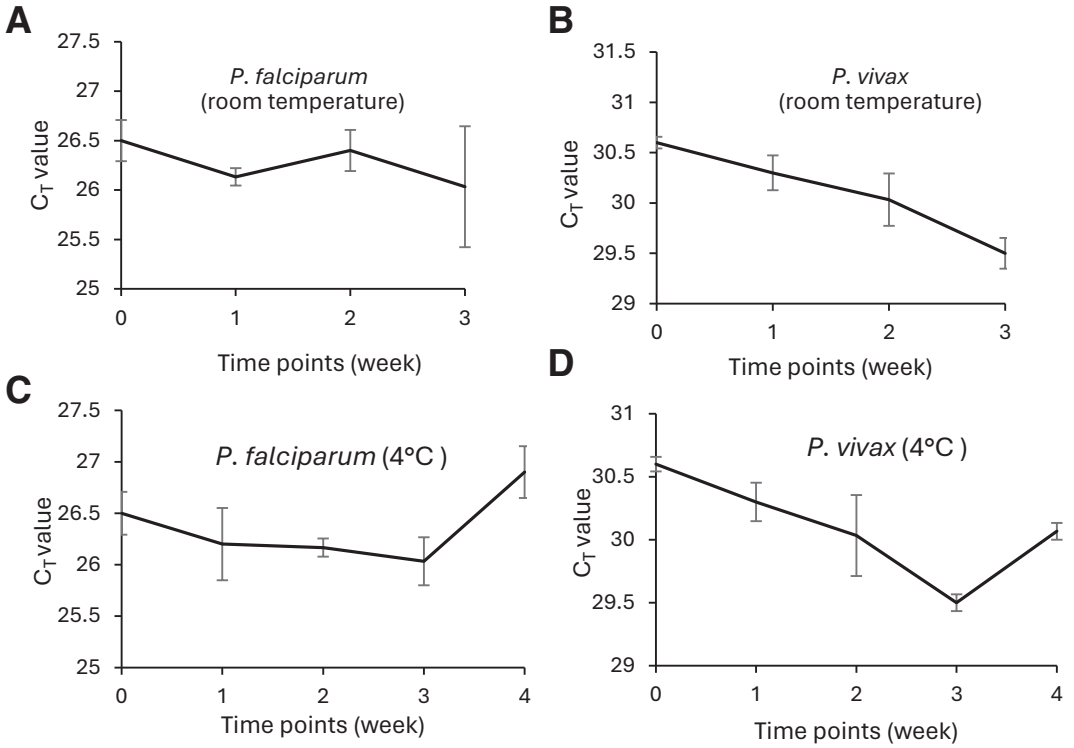


Figure 2 Sets of specimen positive for *Plasmodium falciparum* and *Plasmodium vivax* were incubated at room temperature for up to 3 weeks (**A** and **B**) and refrigerated (4°C) for up to 4 weeks (**C** and **D**). A set of three replicates were tested at each time point, and the mean C_T values and SD were plotted.

week. With worsening kidney failure and profound hyponatremia, the patient was admitted to the intensive care unit for continuous renal replacement therapy and closer monitoring for shock and transfusion needs. The patient lives in Los Angeles, California, but grew up in Tanzania. He visited Tanzania for 3 weeks before the symptom onset. The patient did not remember mosquito bites while in Tanzania but noted that his mom and his uncle had a fever that spontaneously resolved. No one else in the family was sick. He did not take malaria prophylaxis during this trip and has never been diagnosed with malaria before. Blood specimens were collected by the medical team and submitted to the Microbiology laboratory for malaria testing.

The patient blood sample was analyzed by microscopic smear and was positive for *P. falciparum* with 16% parasitemia. The correlation between parasitemia and C_T value from the malaria PCR was established by testing multiple samples collected over 12 days during the patient's hospitalization. The percentage parasitemia decreased from 16% to 0.01% after 4 days of treatment with i.v. artesunate followed by treatment with oral artemether-lumefantrine, corresponding to C_T values increasing from 16.1 to 26.7 (Table 3). However, parasites were no longer detectable by microscopy when parasitemia was <0.01%. In contrast, the malaria PCR assay continued to detect parasite DNA in subsequent samples collected on day 4 through day 12, with

Table 2 Accuracy

	Reference method (smear/real-time PCR)		
Testing method	Detected	Not detected	Total
Malaria PCR LDT			
Detected	73	1	74
Not detected	1	53	54
Total	74	54	128

The positive predictive value was 98.65% (95% CI, 92.70%–99.97%), and the negative predictive value was 98.15% (95% CI, 90.11%–99.95%). Accuracy was 98.44% (95% CI, 94.47%–99.81%).

LDT, laboratory-developed test.

Table 3 Clinical Case Parasitemia Burden and Real-Time PCR Results

Collection date	Collection time, h	Parasitemia, %	Real-time PCR, C_T
1/20/25	0	16	16.1
1/21/25	4	8.1	18.5
1/21/25	11	3.27	20
1/22/25	25	1.01	22
1/22/25	29	0.24	23.3
1/22/25	41	0.05	25.2
1/23/25	53	0.02	24.6
1/23/25	65	0.01	26.7
1/24/25	77	0	27.9
1/25/25	101	0	27.3
1/27/25	152	0	29.1
1/31/25	247	0	32.1

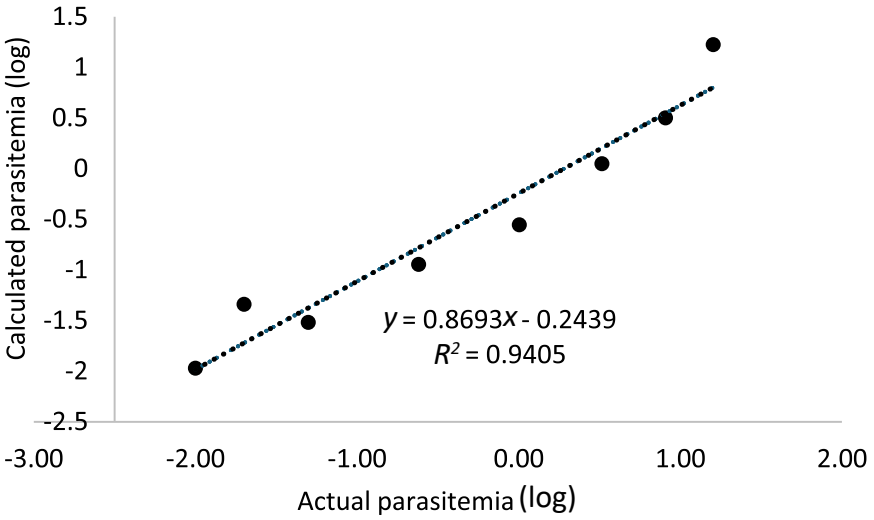


Figure 3 Correlation between the actual parasitemia and calculated parasitemia based on C_T values.

C_T values continuing to increase from 27.9 to 32.1. Youden index analysis was performed to determine a C_T value cutoff that correlates with microscopy negative result, which was determined to be 27.5. The logarithmic values of the actual parasitemia versus the corresponding calculated parasitemia, which was determined using the equation resulting from the linear regression curve for PCR efficiency ($y = -3.3171x + 20.164$), was plotted (Figure 3). Linear correlation was established between parasitemia determined by microscopy and calculated parasitemia based on C_T value (slope = 0.8935 and $R^2 = 0.9405$). These results demonstrated that the parasite density can be calculated on the basis of C_T value by the test with acceptable accuracy even below the limit of detection by microscopy.

Implementation of the Malaria PCR in the Clinical Laboratory

This test has currently been implemented in routine diagnostic testing workflow for malaria and seamlessly integrated into the algorithm in conjunction with the conventional microscopy-based blood smear examination (Figure 4). When a patient was suspected of having malaria or presented with signs and symptoms and had traveled to an endemic area, a malaria test panel was ordered. Both

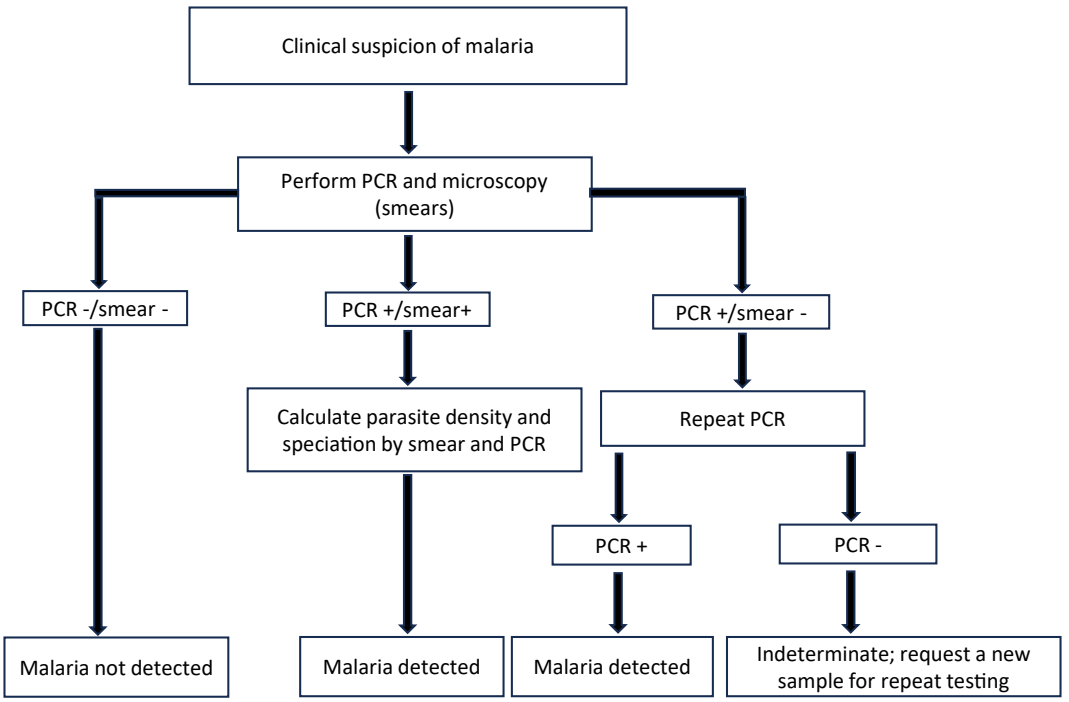


Figure 4 Malaria PCR algorithm.

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smear and PCR tests were performed simultaneously. If no parasite was observed by smear and PCR was negative for *Plasmodium*, the patient was negative for malaria. If parasite was observed and PCR was positive, malaria was detected and parasitemia was determined by both smear and PCR, but only the parasitemia from smear was reported to the patient medical record, while the PCR-based parasitemia was provided as a supplemental result on clinician request. If PCR was positive and smear was negative, the PCR test was repeated. If the repeat PCR remained positive, malaria was detected and the parasitemia was determined by PCR. However, if the repeat PCR was negative, the result was reported as indeterminate, and a new blood draw was requested for confirmation. A single negative smear or negative PCR did not rule out malaria, especially if there was a strong suspicion; therefore, repeat testing every 8 to 12 hours for 3 days was recommended. Also, PCR-positive, smear-negative results may represent previously treated infection as DNA may still be circulating in the blood after effective killing of the parasite.

Discussion

Malaria is a global public health problem with high mortality, especially in women and children aged <5 years in developing countries.[1,11,12] In addition to the host immune status, specific species of *Plasmodium* and the parasitic burden can directly correlate with the disease severity. Therefore, it is essential to develop rapid and accurate diagnostic tests that can speciate the organism and provide accurate parasitemia but do not require a high level of expertise to perform.

Microscopic analysis of thin and thick Giemsa-stained blood smear has been the gold standard method for malaria diagnosis. It has the diagnostic capability of speciating the parasite, providing parasitemia as well as evidence of treatment responsiveness.[13] However, it requires well-trained and experienced microscopists. Mixed infections are difficult to diagnose by microscopy; it lacks sensitivity, which can lead to false-negative results in asymptomatic carriers with low parasitemia. It can also be tedious and time-consuming to rule out malaria in a negative sample from patients who present with symptoms mimicking malaria.

Rapid diagnostic tests have significant impact on malaria diagnosis and treatment, especially in endemic countries and those with limited resources, because of their ease of use and providing quick results.[7,14] However, false-positive results have been frequently observed in patients with other infectious conditions and immunologic factors, such as rheumatoid factor, leishmaniasis, Chagas disease, schistosomiasis, toxoplasmosis, human African Trypanosomiasis, dengue, and hepatitis C infection.[7] False-negative results have also been reported in cases of histidine-rich protein 2 (*HRP*2) gene deletion, low parasitemia, as well as prozone effect due to high parasitemia.[8,9,15] In addition, the rapid diagnostic tests are not effective in monitoring treatment because of its persistent positivity for weeks after effective treatment and resolution of clinical symptoms.[16]

There has been some progress made in molecular testing of malaria in the past decade. However, despite their high sensitivity and specificity, the application of molecular diagnostic tests for malaria have been lagging in the clinical laboratories. So far, there have been only two US Food and Drug Administration−approved malaria molecular tests, the cobas Malaria test by Roche and the Global Fever Panel by BioFire, neither of which is widely adopted in the clinical laboratory testing; public health or reference laboratories may offer these tests, but the results may not turn around in a timely manner. There have been ongoing efforts by research groups and clinical laboratories to develop and validate their own laboratory-developed PCR malaria tests. Highly sensitive and specific multiplex real-time PCR assays to detect and sometimes quantify *Plasmodium* species by amplifying RNA and DNA of the 18S rRNA genes have been described.[10,17−19] The LoD for those assays varies between 0.5 and 3 parasites/μL for *P. falciparum*, 0.25 and 1.13 parasites/μL for *P. vivax*, 1 parasite/μL for *P. ovale*, and 0.8 and 5 parasite/μL for *P. malaria*.[10,17−19] Many of those assays use a separate extraction platform followed by a real-time PCR instrument for nucleic acid extraction and PCR analysis, respectively.

In this study, a real-time multiplex semiquantitative PCR was validated for the detection and quantification of *P. falciparum*, *P. vivax*, and other *Plasmodium* species in EDTA−whole blood from patients suspected of having malaria on a sample-to-answer molecular platform. The assay is highly sensitive, with LoD as low as 0.0001% parasitemia (5 parasites/μL) for both *P. falciparum* and *P. vivax*, which is 100 times more sensitive than the conventional microscopic blood smear examination. The assay is shown to be specific as no cross-reactivity was observed among different *Plasmodium* species and other blood parasites, such as *Babesia*. This is important because *Babesia* can often be mistaken for *P. falciparum* as they are both intraerythrocytic parasites that share morphologic characteristics and disease symptoms, which include fever, chills, nausea, vomiting, headache, and disseminated intravascular coagulation.[20,21] Although pre-analytical parameters, such as hemolysis and lipemia, were not directly assessed, visually lysed negative samples used in pooling for serial dilution or positive samples used in assay precision did not seem to affect the assay. This study ran a pilot experiment in which lipemic blood spiked with malaria-positive samples versus nonlipemic blood was tested; the difference in C_T values was negligeable (<0.5).

The assay is highly accurate, with an overall concordance of 98.37%. The standard procedure for malaria diagnosis includes the BinaxNOW Malaria test (Abbott, Abbott Park, IL) in addition to microscopic smear analysis. Of the 128 samples tested, one resulted in false positive by BinaxNOW

Malaria test but was negative by both microscopic smear and malaria PCR test, highlighting the limitation of the antigen test and superior performance of the malaria PCR assay. Determining parasitemia for malaria is extremely important for assessing disease severity and monitoring treatment efficacy. A good correlation was observed between actual parasitemia determined by microscopy and calculated parasitemia by the malaria PCR; this allows for the estimation of parasitemia with as low as 0.0001%, which is not possible by microscopy.

One of the limitations of this study is that parasitemia correlation was derived from a single patient and, therefore, serves only as a representative rather than a validated semiquantitative relationship across patients. Also, the malaria PCR test does not provide an absolute quantification, which would have required a set of standards with known parasitemia density, which are not available in this study. The other limitation is that the accuracy of the C_T value–based parasitemia for *P. vivax* using uncontrived clinical samples could not be validated because of lack of positive *P. vivax* cases. This was expected because of the low rate of *P. vivax* infection in the United States. One specific caveat of malaria PCR test is that it may amplify DNA from extracellular parasites and DNA from nonviable parasites, leading to overestimation of parasitic burden and, hence, representing a case of PCR positive, smear negative. Prior studies have demonstrated that parasite DNA may remain detectable by PCR for some time after parasite clearance, which can complicate interpretation in post-treatment monitoring.[22–24] Therefore, it is important to corroborate clinical presentations following treatment.

It is also acknowledged that factors, such as gametocytemia, multiple infected erythrocytes, and host-related variables, may influence parasite clearance dynamics and C_T values. Infections consisting predominantly of gametocytes may lead to lower parasitemia estimates as gametocytes represent the transmissible reservoir stage rather than the asexual stages responsible for clinical disease.[25] Patients with gametocyte-only infections may, therefore, less frequently seek medical attention, potentially limiting detection in routine diagnostic workflows. Studies detecting gametocytes use real-time PCR targets *Pfpeg4*, *Pfg27*, and *Pfs25* for *P. falciparum* and *Pvs25* for *P. vivax* as specific markers to detect early and late sexual stages (gametocytes) of the malaria parasite,[26,27] whereas the present PCR uses only the 18S rRNA regions.[17] Additionally, host factors, such as multiple infected erythrocytes, their circulation time, or adhesiveness,[28,29] and host variability [ie, iron availability, sickle-cell trait, and glucose-6-phosphate dehydrogenase (G6PD) deficiency] in immune-mediated parasite clearance, may influence the relationship between parasitemia and C_T values.[28,30,31] Moreover, because of its high sensitivity, good molecular laboratory practice and quality control must be in place to avoid contamination leading to false-positive results.

In summary, this study developed and validated a highly sensitive molecular test that can not only speciate *Plasmodium* species but also provide the semiquantitative estimation of parasitemia objectively. This test requires <5 minutes hands-on time and 1.5 hours run time. In addition to its use in clinical diagnosis, the malaria PCR test may also be used for donor blood screening and malaria outbreak investigations. Most notably, this test reduces the reliance on the experience of microbiologist performing the test and provides added confidence and speed for accurate malaria diagnosis. Further studies are required to evaluate the true clinical utility of the malaria PCR in both diagnosis and treatment monitoring.

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Disclosure Statement

None declared.

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