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Advances in Malaria Testing: Screening and Identification of Carriers from Saliva

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Abstract

Plasmodium is a parasite that can infect red blood cells and cause flu-like symptoms with malaria infection. Traditional diagnostic methods do not include counting or testing for gametocytes, which can reservoir in the liver for long periods of time and recirculate. These carriers may have no symptoms, but they can transmit infection to others or to mosquitos. Currently, no diagnostic tests have been approved to detect *Plasmodium* gametocytes in either symptomatic or asymptomatic whole blood samples. Therefore, we developed real-time PCR assays to detect active and carrier states of malaria. The first is a traditional screening test that can detect any of the five *Plasmodium species* that cause malaria infection. The second is a companion test to differentiate and quantitate *Plasmodium falciparum* and *P. vivax* gametocytes in samples of whole blood from patients who may be asymptomatic and present negative results from screening tests. The screening test showed amplification of *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi* in saliva with an overall detection limit of 565 copies/µL. The gametocyte test showed no cross-reactivity between *P. falciparum* and *P. vivax* with a limit of detection of RNA at 1000 copies/µL.

Keywords: Malaria, *Plasmodium falciparum, Plasmodium vivax, Plasmodium,* Real-Time PCR

1. Introduction

Half of the world's population is at risk for malaria infection. Malaria is a bloodborne infection caused by *Plasmodium falciparum, P. vivax, P. malariae, P. ovale, or P. knowlesi.* In 2021, there were an estimated 247 million cases of malaria with 619,000 deaths and in some areas about four in five of those deaths were children under 5 years of age[1]. Although testing and treatment have successfully increased worldwide, gaps exist in access, quality, and affordability of care and testing. Current testing approaches consist of light microscopy examination of blood smears, Rapid Diagnostic Tests (RDTs), and Polymerase Chain Reaction (PCR) based testing[2]; however, these tests are designed to detect circulating, active infections of *Plasmodium* and do not take the *Plasmodium* life cycle into account.

The lifecycle of this parasite continues when a mosquito from the *Anopheles* genus bites a human host and injects saliva contaminated with *Plasmodium* sporozoites into the host's bloodstream. These sporozoites travel through the blood until they reach the liver, where they invade the cells. Some of these parasites will continue to replicate into more sporozoites, while others will differentiate into merozoites and enter the blood again, entering Red Blood Cells (RBCs) and continuously multiplying into multinucleated cells called schizonts[3]. These cells can either commit to dividing back into mononucleated merozoites or differentiate and divide into mononucleated gametocytes. These gametocytes attach themselves to the walls of capillary beds until they are taken back into an Anopheles mosquito, where they will complete their life cycle and return to their sporozoite state. What is noteworthy about this lifecycle is that there can be three distinct populations of Plasmodium in a host at any given time: merozoites that cause the primary symptoms of the infection, sporozoites that can reintroduce infection after treatment, and gametocytes that can spread and infect

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other members of the community, each of which require different treatments to eliminate[4]. These populations are not eliminated by the medications designed to clear merozoites, and they can remain in the blood for up to several months after symptoms clear[3]. If any of these populations are not eliminated, the patient may relapse or infect members of the community after being discharged from the hospital.

Tests that can detect the contagious, differentiated gametocytes of the parasite are necessary to achieve the long-term goal of eradication, but this cannot be achieved through available diagnostic tests. Light microscopy cannot distinguish between merozoites and gametocytes, as gametocytes are not significantly different in appearance and often at densities too low to detect with microscopy. Blood used for microscopy is collected and stored in vacutainers containing potassium EDTA as an anticoagulant. Prolonged exposure of the parasites to EDTA can alter the morphology of the RBCs or the parasite[5]. RDTs have limited sensitivity to low-level infections, and several tests have been rendered obsolete due to mutation of currently used antigens[6]. Parasites with histidine-rich protein 2 deletions or those that can no longer express histidine-rich protein 2 or 3 can cause false negative results in rapid detection tests[1,7]. PCR approaches have the highest potential due to their high sensitivity and specificity, but currently there are no WHO-prequalified tests that can detect and distinguish between *P. falciparum* and *P. vivax*[1]. Additionally, PCR tests require complex laboratory equipment and trained technicians to run the tests and interpret the data adequately. Our aim was to develop a real-time PCR assay to detect *Plasmodium species* that cause malaria in saliva samples and differentiate *P. falciparum* and *P. vivax* gametocytes as a companion test.

2. Materials and Methods

2.1 Samples

Double-stranded DNA gene blocks from a 135bp region of *P. falciparum, P. vivax, P. ovale,* and *P. malariae* (Integrated DNA Technologies) were utilized for malaria screening assay experiments. A single-stranded 158bp DNA Ultramer™ (Integrated DNA Technologies) of *RNaseP* was utilized as a control. A 1:1 mixture of each *Plasmodium species* and *RNaseP* was spiked into healthy control saliva specimens after lysis. A companion assay was developed to differentiate and quantify gametocytes. Single-stranded RNA Ultramers™ from a 119bp mRNA genomic region of *Pfs25* and 110bp region of *Pvs25* were ordered from Integrated DNA Technologies. The RNA was utilized for gametocyte species differentiation testing. *Pfs25* and *pvs25* are effective biomarkers to detect *P. falciparum* and *P. vivax* female gametocytes, respectively, in carriers who were asymptomatic or had parasitemia[8-12]. A single-stranded 119bp RNA Ultramer (Integrated DNA Technologies) of *GAPDH* was utilized as a reference gene. For specificity testing, a universal control of RNA was created using a 1:1:1 mixture of *pfs25, pvs25,* and *GAPDH.*

2.2 DNA Isolation

The Classic™ Genomic DNA Isolation Kit (Lamda Biotech) and Viral DNA/RNA Kit (CW Bio) were used to isolate DNA and RNA from saliva samples. The Lamda Biotech protocol was modified to accommodate saliva and increase the final sample concentration. 500µL of saliva was digested with 500µL of Solution A and 20µL of Proteinase K (20mg/mL). The samples were incubated at 55°C in a dry bath for 60 minutes. Saliva samples were spiked with 100uL of *Plasmodium* at 6.76E07copies/µL and 100µL of *RNaseP* at 1.15E07copies/µL. Each species of *Plasmodium* was tested using the same procedure. The Lamda Biotech protocol was followed until the final step. We resuspended all DNA/RNA samples in 100uL of nuclease-free water, vortexed for 10 seconds to mix, and incubated the final DNA at 37°C for 2 minutes.

2.3 Primer Design

Genomic sequences for *Plasmodium species* were obtained from the National Center for Biotechnology Information. A multiple sequence alignment was performed to determine a highly conserved mitochondrial *cytochrome c oxidase III* (*cox3*) genomic region shared by *P. falciparum, P. vivax, P. ovale, P. malariae,* and *P. knowlesi.* This target sequence area was used to design primers and fluorescence-labeled TaqMan probes for real-time Polymerase Chain Reaction (qPCR) to screen for the presence of *Plasmodium.* Using the same procedure, primers and TaqMan probes for *RnaseP* were designed as a control of amplifiability and false negativity on PCR results. As a secondary test design, two orthologues *pfs25* (AF19376931) and *pvs25* (GU256271.1) transcripts previously described by Wampfler *et al* 2013 were used to design primers and fluorescence-labeled Taqman

probes to differentiate *P. falciparum* and *P. vivax* gametocytes using a single step, reverse transcription qPCR. A conserved region of *GAPDH* was used to design a reference gene for messenger RNA (mRNA) amplification. Primers and probes were purchased from Integrated DNA Technologies.

qPCR assays were performed on the Q Real-Time PCR Cycler (Quantabio) and Eco™ (Illumina). To detect *Plasmodium* in saliva specimens, the following reagents were added to a total 10µL reaction: 5µL of qPCR 2X MasterMix Universal for TaqMan Assay (Lamda Biotech), 0.04µL of *cox3* and *RnaseP* forward primers at 50µM, 0.04µL of *cox3* and *RnaseP* reverse primers at 50µM, 0.03µL of *cox3* and *RnaseP* TaqMan probes at 50µM, and 5µL of genomic DNA or PCR control. Amplification and detection were performed under the following conditions: 95°C for 2 minutes and 45 cycles of 95°C for 5 seconds and 60°C for 30 seconds with fluorescence capture. Gametocyte differentiation and quantification were run in separate 10µL real-time PCR reactions of 5µL of qPCR 2X MasterMix Universal for TaqMan Assay (Lamda Biotech), 0.08µL of *pfs25/GAPDH* or *pvs25/GAPDH* forward primers at 50µM, 0.08µL of *pfs25/GAPDH* or *pvs25/GAPDH* reverse primers at 50µM, 0.04µL of *pfs25/pvs25/GAPDH* TaqMan probes at 50µM, 1uL of GoScript™ reverse transcriptase (Promega), and 5µL of RNA or PCR RNA control. Amplification and detection of RNA was performed under the following conditions: 10 minutes of 45°C for reverse transcription, 95°C for 2 minutes for hot-start activation, and 40 cycles of 95°C for 10 seconds and 55°C for 30°C with fluorescence capture. Each experiment included two negative controls without genomic DNA and two positive controls with sample replicates.

2.4 Analytical Sensitivity of the Screening Assay

The sensitivity of the *Plasmodium* screening assay was assessed by testing 10-fold serial dilutions of spiked saliva specimens. Two saliva samples were tested with each: *P. falciparum, P. malariae, P. ovale,* and *P. vivax.* All dilutions were tested in duplicate or triplicate reactions using the *Plasmodium* screening qPCR assay.

2.5 Analytical Reproducibility

The reproducibility of the *Plasmodium* screening qPCR assay was assessed by testing a 4-point serial dilution of *Plasmodium falciparum* and *RNaseP* 1:1 mixture. Materials tested on the QuantaBio at Bob Jones University's Cancer Laboratory were compared to materials tested on the Eco at Bosem. All dilutions were tested in triplicate reactions.

2.6 Analytical Specificity of the Screening Assay

We obtained 34 DNA and RNA samples to test with the *Plasmodium* screening assay. The samples were run on the Eco in single reactions with negative and positive controls.

2.7 Analytical Sensitivity of the Gametocyte Assay

The sensitivity of the Gametocyte differentiation and quantification assay was assessed by testing 10-fold dilutions of RNA ordered from IDT for *pfs25, pvs25,* and *GAPDH*. All dilutions were tested in duplicate using qPCR.

2.8 Analytical Specificity of the Gametocyte Assay

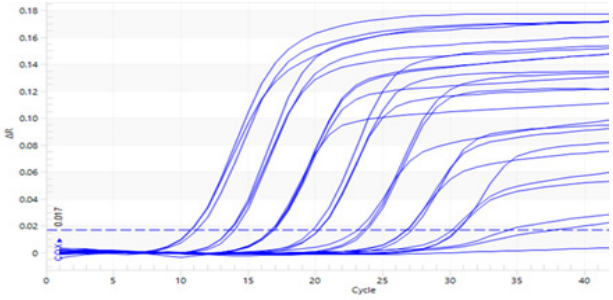
A universal RNA control mix along with individual RNA control samples was run with combinations of primer sets to determine if any of the analytes would cross-react.

3. Results

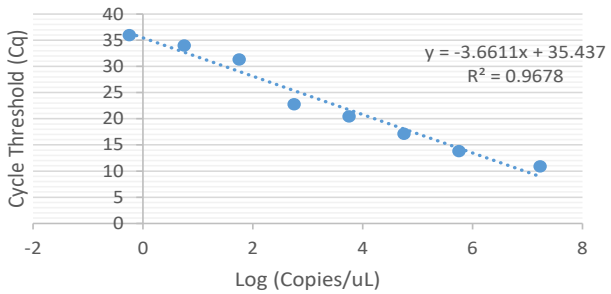
3.1 Analytical Sensitivity of the Screening Assay

The amplification and linear fit plots of the *Plasmodium* screening qPCR assay provided by Eco48 for *P. falciparum, P. vivax, P. ovale,* and *P. malariae* are shown in Figure 1. A positive amplification curve passing the threshold with an associated cycle threshold (Cq) value indicated the presence of the target gene. High Cq values represented low amounts of gene copies per reaction, whereas low Cq values represented high amounts of gene copies present in the reaction. Each reaction was run in duplicates for two contrived saliva samples per species. A probit analysis calculated the assay's detection limit to 8.43 copies/µL, the lowest concentration of analyte that can be detected at least 95% of the time or greater. The detection limit was confirmed by running 20 replicates for each species, and the highest LOD across species was used for the final

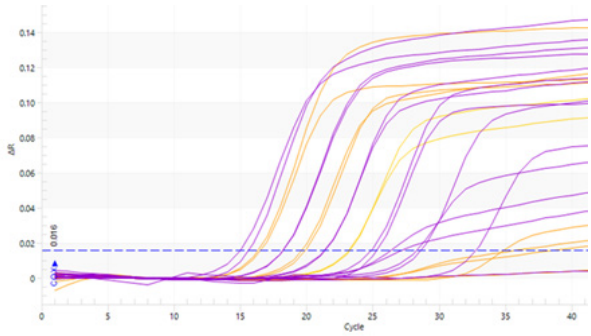
A. Amplification Plot of *Plasmodium falciparum*



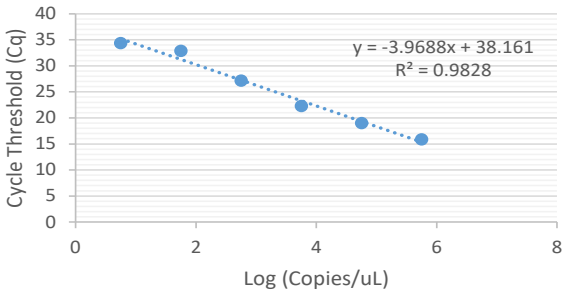
B. Limit of Detection for *Plasmodium falciparum*



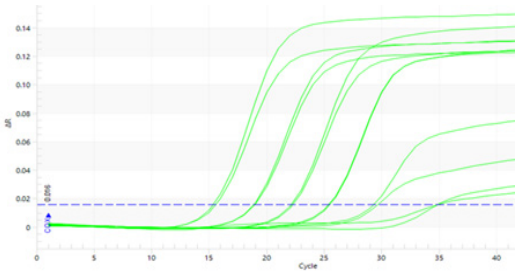
C. Amplification Plot of *Plasmodium vivax*



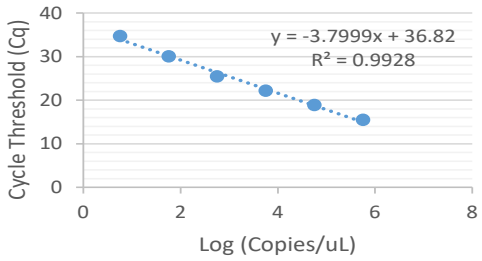
D. Limit of Detection for *Plasmodium vivax*



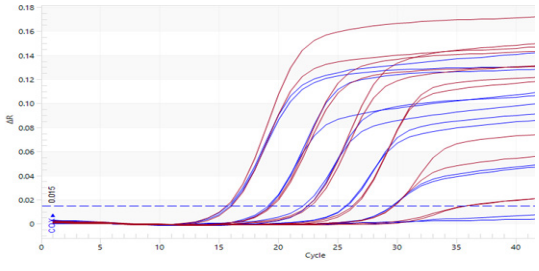
E. Amplification Plot of *Plasmodium malariae*



F. Limit of Detection for *Plasmodium malariae*



G. Amplification Plot of *Plasmodium ovale*



H. Limit of Detection for *Plasmodium ovale*

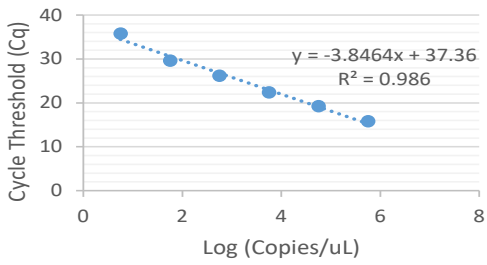


Figure 1. Amplification and Linear Plots for Each of the *Plasmodium* species.

overall assay. The actual detection limit in saliva samples was 565 copies/µL (94/95 hits; 98.95% for all species). The mean Cq values were calculated for each *Plasmodium species*. The mean Cq values for *P. falciparum* ranged from 10.90 (1.69E07 copies/µL) to 35.99 (0.565 copies/µL), those for *P. vivax* ranged from 15.87 (5.65E05 copies/µL) to 34.35 (5.65 copies/µL), those for *P. malariae* ranged from 15.48 (5.65E05 copies/µL) to 34.73 (5.65 copies/µL), and those for *P. ovale* ranged from 15.83 (5.65E05 copies/µL) to 35.79 (5.65 copies/µL). The range of the *Plasmodium* screening qPCR assay showed reproducible linearity from 565-5.65E6 copies/µL.

Figures 1a-h show the paired amplification and linear regression analysis plots for each species using averaged Cq values across contrived saliva samples and replicates. The amplification plots were acquired from Eco and analyzed in Eco Study software v5.0.4890 using default settings. Figures 1a-1b show *P. falciparum* from 0.565 to 5.65E05 including a starting concentration of 1.69E07 copies/µL. Figures 1c-d show *P. vivax* from 5.65 to 5.65E05 copies/µL. Figures 1e-f show *P. malariae* from 5.65 to 5.65E05 copies/µL. Figures 1e-f show *P. ovale* from 5.65 to 5.65E05 copies/µL. The linear regression plots calculated a very high correlation coefficient between parasite load and Cq values. The average primer efficiency for all species was 82.86%, which passes the acceptance criteria between 80-110 % with a slope between -3.1 to -3.9.

3.2 Reproducibility between Labs and Machines

The Cq values of *Plasmodium* Screening qPCR assay from QuantaBio and Eco are shown in Table 1. A one-way ANOVA was performed to compare the effect of instrument type on Cq values. A one-way ANOVA revealed no statistically significant difference in Cq values between at least two groups ($F(1, 22) = 0.0997$, $p = 0.7552$).

3.3 Analytical Specificity of the Screening Assay

The expected and actual results were recorded from testing 34 samples of DNA and RNA with the *Plasmodium* screening qPCR assay from Eco. The results show a 100% agreement between results with no cross-reactivity and detection of *P. falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi* (Table 2).

3.4 Analytical Sensitivity and Specificity of the Gametocyte Assay

The amplification and linear fit plots of the *Plasmodium* gametocyte qPCR assay provided by Quantabio for *pfs25* (*P. falciparum)* and *pvs25 (P. vivax)* are shown in Figure 2. Each reaction was run in triplicates, and the limit of detection was confirmed by running 10 replicates. A

Table 1. Instrument Comparison of *P. falciparum*

	Cycle Threshold Values (Cq)	
Copies/uL	Quantabio	Eco
1.69E+06	12.49	13.35
	12.49	13.35
	12.32	13.38
1.69E+05	15.97	16.6
	15.84	16.64
	16.36	16.63
1.69E+04	19.28	19.9
	19.63	20.18
	21.17	19.67
1.69E+03	22.53	23.06
	23.47	23.59
	22.18	23.59

Table 2. Specificity Results

Sample	Expected Result and Sample Type	ActualResult
Sample 1	Negative saliva	Negative
Sample 2	Negative saliva	Negative
Sample 3	Negative saliva	Negative
Sample 4	Negative saliva	Negative
Sample 5	Positive (*P. falciparum*)	Positive
Sample 6	Negative saliva	Negative
Sample 7	Positive (*P. falciparum*)	Positive
Sample 8	Positive (*P. vivax*)	Positive
Sample 9	Positive (*P. vivax*)	Positive
Sample 10	Positive (*P. malariae*)	Positive
Sample 11	Positive (*P. malariae*)	Positive
Sample 12	Positive (*P. ovale*)	Positive
Sample 13	Positive (*P. ovale*)	Positive
Sample 14	Negative saliva	Negative
Sample 15	Negative saliva	Negative
Sample 16	Negative (*pfs25* RNA gametocyte only)	Negative
Sample 17	Negative (*pfs25* RNA gametocyte only)	Negative
Sample 18	Negative (*pvs25* RNA gametocyte only)	Negative
Sample 19	Negative (*pvs25* RNA gametocyte only)	Negative
Sample 20	Positive (*P. falciparum*)	Positive
Sample 21	Positive (*P. vivax*)	Positive
Sample 22	Positive (*P. malariae*)	Positive
Sample 23	Positive (*P. ovale*)	Positive
Sample 24	Negative (*RNaseP*)	Negative
Sample 25	Negative (*GAPDH* RNA)	Negative
Sample 26	Negative (Monkey Pox Virus)	Negative
Sample 27	Negative (Aquaporin 3 RNA)	Negative
Sample 28	Negative Pooled Human genomic DNA from whole blood	Negative
Sample 29	Negative buccal swab	Negative
Sample 30	Negative buccal swab	Negative
Sample 31	Positive (*P. falciparum*)	Positive
Sample 32	Positive (*P. vivax*)	Positive
Sample 33	Positive (*P. malariae*)	Positive
Sample 34	Positive (*P. ovale*)	Positive

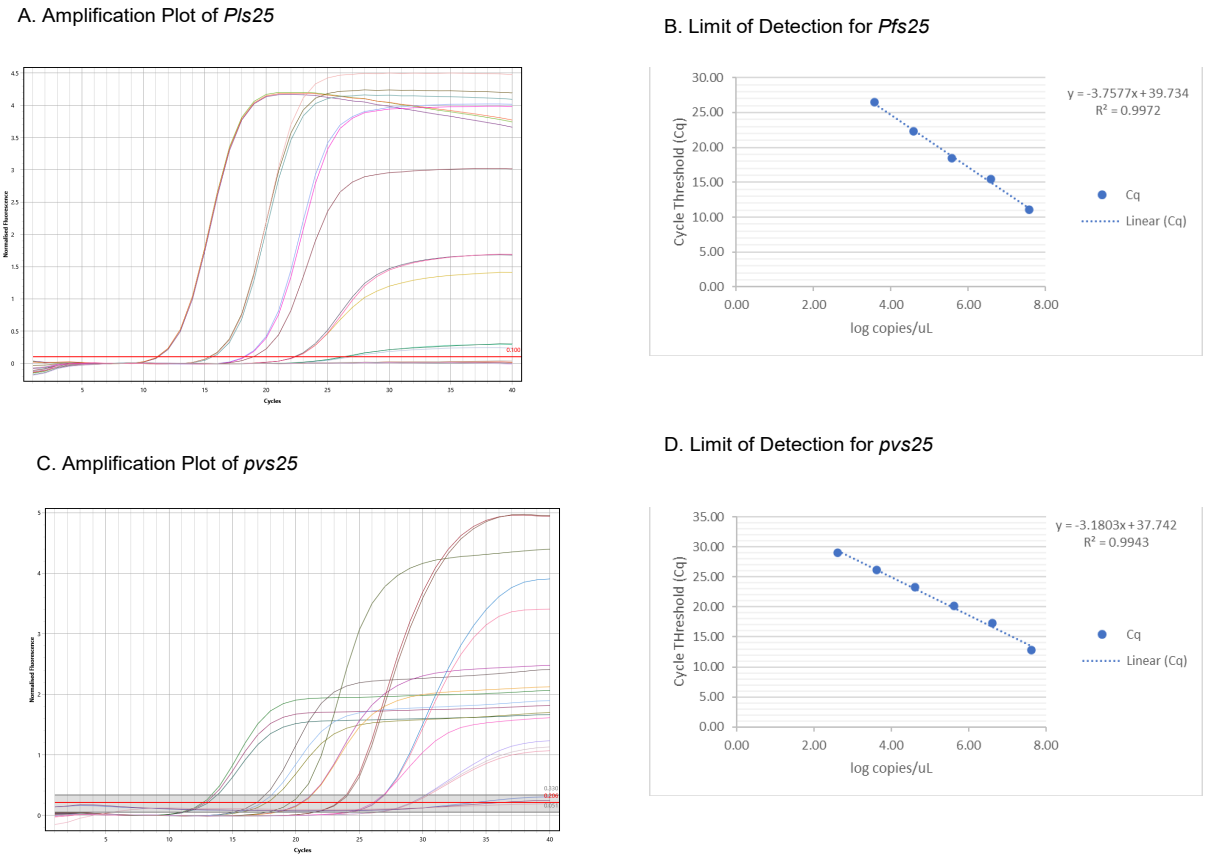


Figure 2. Amplification and Linear Plots for *Plasmodium falciparum* and *P. vivax* Gametocyte Targets.

probit analysis determined the detection limit to 6.36 copies/µL for *pfs25* and 5.95 copies/µL for *pvs25*. A confirmation test using *pvs25* RNA determined the actual limit of detection as 100 copies/µL (20/20 hits; 100%), and for *pfs25* RNA, a limit of detection at 1000 copies/µL (20/20 hits; 100%). The mean Cq values were calculated for each transcript: for *pfs25* 11.13 (3.83E07 copies/µL) to 25.83 (1.0E03 copies/µL) and for *pvs25P* 12.83 (4.15E07 copies/µL) to 30.24 (100 copies/µL).

Figures 2a-d show the paired amplification and linear regression analysis plots *P. falciarpum* mRNA transcript *pfs25* and *P. vivax* mRNA transcript *pvs25*. The amplification plots were acquired from Quantabio and analyzed in Q-qPCR software v1.0.4. Figures 2a-2b show *pfs25* results from testing RNA from 3.83 to 3.83E07 copies/ µL. Figures 2c-2d show *pvs25* results from testing RNA from 4.15 to 4.15E07 copies/µL. The linear regression plots calculated a very high correlation coefficient between gametocyte load and Cq values. The primer efficiency for *pfs25* was calculated at 84.55% and for *pvs25* was 106.3%.

Figure 3 shows the amplification plots of the gametocyte assay tested between each RNA sample and different combinations of primers. The specificity showed no cross-reactivity between the *pfs25* RNA and *pvs25 or GAPDH primers,* and likewise, the *pvs25* RNA showed no cross-reactivity with *pfs25 or GAPDH* primers.

Figure 3a-f shows the amplification plots from Quantabio using the universal control and individual RNA samples with differing combinations of gametocyte master mixes. Figure 3a shows the amplification of *pfs25* RNA using *pfs25/GAPDH* primers; *pvs25* did not amplify with this mix. Figure 3b shows the amplification of *pfs25* RNA using *pfs25/pvs25* primers. Figure 3c shows the amplification of *pvs25* RNA using *pvs25/GAPDH* primers; *pfs25* RNA did not amplify. Figure 3d shows the amplification curve of *pvs25* RNA using *pfs25/pvs25* primers. Figure 3e shows the amplification of *GAPDH* RNA using *pfs25/GAPDH* primers. Figure 3f shows the amplification of *GAPDH* RNA using *pvs25/GAPDH* primers.

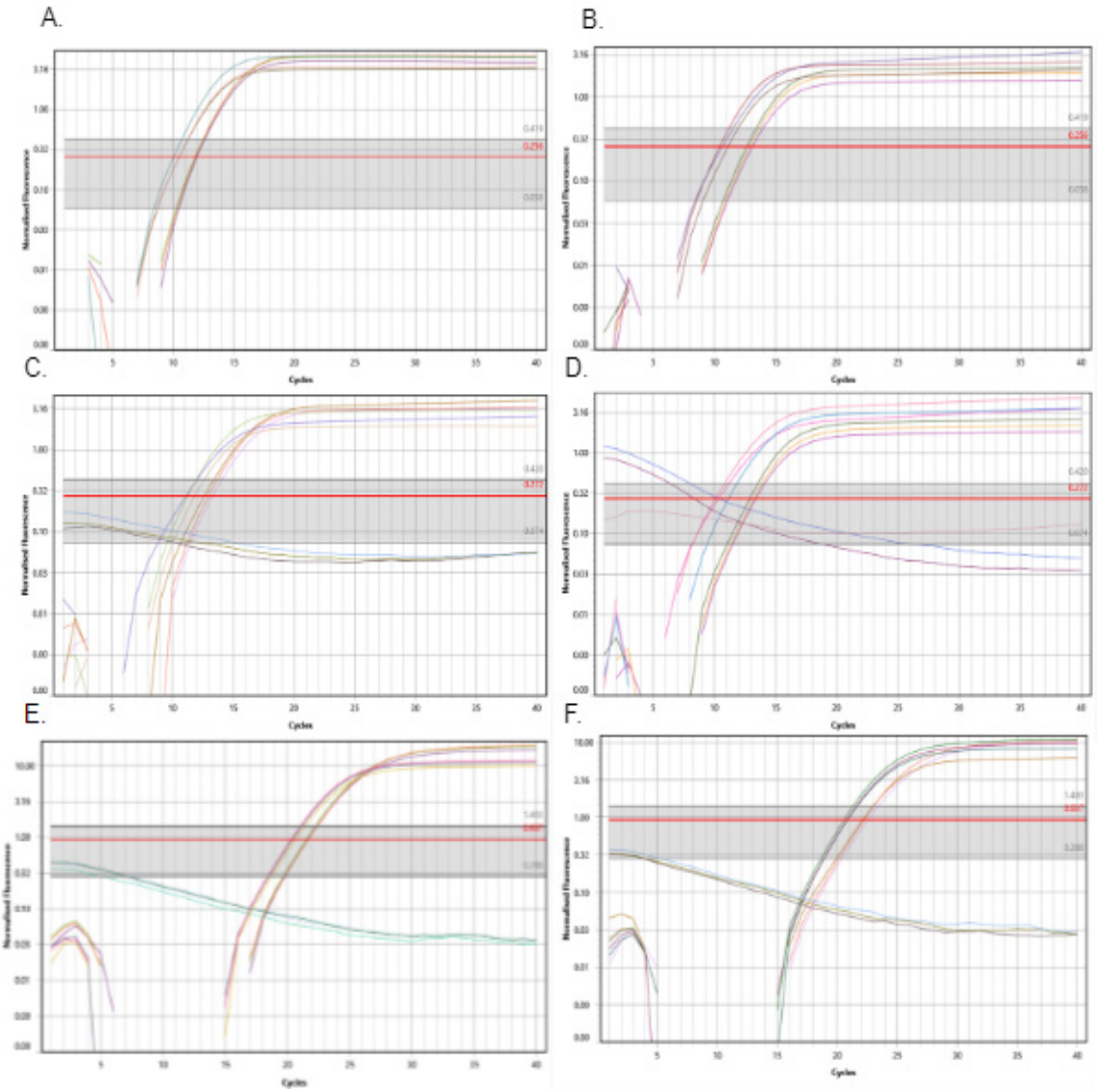


Figure 3. Gametocyte Assay Specificity.

4. Discussion

Although several real-time PCR assays exist to detect *Plasmodium,* microscopy continues to be the gold standard testing method due to its cost and rapid turnaround[13]. Most laboratories have access to light microscopes and slides with the ability to clean and re-use them, which reduces the need for expensive equipment and continued purchase of consumables. The Giemsa stain used for microscopy is the highest cost but is widely available and can be used for other types of identification. The downside to microscopy is that it requires a trained eye to screen and identify the parasites. The World Health Organization (WHO) guidelines recommend screening 100 viewing fields, and once malaria parasites are detected, the parasite density should be estimated after counting the infected Red Blood Cells (RBCs) among 500 to 2000 RBCs on thin smear, but not including gametocytes[13]. Typically, the ring stage or gametocytes are seen using microscopy and seldom trophozoites or schizonts with a reported positive blood smear containing 50-100 parasites/μL[13,14]. Visual interpretation requires capillary or venous blood collection, and it cannot determine if gametocyte or schizont forms remain reserved in the host.

Patients with severe malaria or children may demonstrate respiratory distress and anemia in addition to the common symptoms of fever, chills, headache, myalgia, vomiting, and anorexia[14]. Parasitaemia is considered only when the number of parasites is >10,000/μL of blood[15]. These symptoms make it difficult to collect a blood sample and may result in false negative results due to low red blood cell counts. A host with gametocytes in reservoir or low-level circulation may transmit parasites through direct contact with body fluids or mosquito bites. Gametocyte densities are higher in chronic, asymptomatic cases versus incident infections, leading to increased incidents of mosquitoes becoming infected[16,17]. The human malaria infectious reservoir is not fully understood but points toward the ability of gametocytes to carry infection within a population by continuing the life cycle. Currently, there are no approved screening assays for malaria designed to detect gametocytes and no assays that can detect malaria from a sample other than whole blood.

In this study, we designed a qPCR assay to detect *P. falciparum, P. vivax, P. malariae, P. knowlesi,* and *P. ovale* and a companion qPCR assay to differentiate *P. falciparum* and *P. vivax* gametocytes. *Cox3* was detected down to 565 copies/μL in saliva samples. *Pfs25* and *pvs25* were detected at 1000 copies/μL and 100 copies/μL, respectively. The sensitivity was confirmed with 20 replicates in both assays. The specificity of the screening assay was confirmed by testing 34 samples including the five malaria-causing *Plasmodium species,* to ensure no cross-reactivity, and the specificity of the gametocyte test used RNA to confirm no cross-reactivity between the *Plasmodium* species. The absence of contamination was confirmed by including positive and negative controls in each run for both assays.

The qPCR described here did not show a detection limit as low as previously described analytical sensitivities. We believe this is due to the sample type and gene target. Previously, an 18S ribosomal RNA gene PCR detected *P. falciparum* in saliva with a sensitivity of 95% and blood at a sensitivity of 100% compared to microscopy, but when comparing saliva results directly to blood results, the sensitivity of saliva was 82%[15]. The high detection limits of our tests may be the result of our sample type or extraction method modifications and may not represent the optimal performance of the assay. We confirmed the limit of detection for *P. falciparum* as low as 16.9 copies/μL (19/20; 95%) in one saliva sample. We noticed a significant difference in Cq values from samples tested directly after extraction compared to repeated results collected after storage at -20°C for at least 24 hours. Also, we noted differences in Cq results from samples with different NanoDrop concentrations and 260/280 readings. These differences suggest that extraction quality and yield affect the limit of detection and other parameters of downstream applications. To account for differences in extraction or inherently attributed to population diversity, we used the averaged Cq values among all replicates and samples to determine the final detection limit. In the future, we plan to re-evaluate the detection limit from whole blood samples compared to saliva and microscopy. Additionally, most molecular assays target the 18S ribosomal RNA gene, which has 4-8 copies per *P. falciparum* genome, whereas *cox3* has 20-150 copies per *P. falciparum* parasite[18]. We chose the *cox3 gene* as the target due to its stability to withstand mutation. We explored several other gene target areas for design, but the primers could not detect all five of the *Plasmodium species* like the current design. We also tried to use species-specific mutations to create a differentiation assay using the *cox3* target area, but due to the 100% similarity between *P. vivax* and *P. knowlesi* in the target area, it was not possible. This led us to explore the utility of using the mRNA gametocyte test to differentiate between *P. falciparum* and *P. vivax,* which proved successful with no cross-reactivity. The gametocyte assay shows promise in being used for detection, but limited time prohibited us from optimizing further and exploring gene expression characteristics.

5. Conclusion

Real-time PCR applications for screening and differentiation such as these developed show advantages for high throughput testing, differentiation of species, and potentially the ability to detect carriers of *Plasmodium*. More clinical evidence and optimization are needed. Evaluation of the gametocyte tests suggests that it may be beneficial in identifying carriers, but it should not be a replacement for screening. Both tests provide essential information needed to control the spread of malaria and lead to better treatment options. As we begin to understand the role gametocytes play in disease

transmission, the closer we will come to eradicating the disease.

6. Acknowledgments

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7. References

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