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Evaluation of Polymerase Chain Reaction Conditions and Primer Specificity for *Plasmodium vivax* 18S rRNA gene Detection Across Different Parasite Life Stages

Evaluación de las condiciones de la reacción en cadena de la polimerasa y de la especificidad de los cebadores para la detección del gen ARNr 18S del *Plasmodium vivax* en diferentes etapas del ciclo de vida del parásito

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SUMMARY

Introduction: *Malaria caused by Plasmodium vivax remains widespread, with dormant liver stages that can trigger relapses. This study aimed to optimize denaturation and extension temperatures for qPCR detection of the P. vivax 18S rRNA gene, following previous optimization of primer concentration and annealing temperature.*
Methods: *A quasi-experimental design was used to evaluate denaturation (90°C, 95°C, and 97°C) and extension (58°C, 60°C, and 62°C) temperatures, with a constant primer concentration of 300 nM. Seventeen malaria-positive samples representing different parasite life stages, as well as samples containing other Plasmodium species and normal blood, were tested microscopically and by qPCR using specific primers (23 bp forward, 22 bp reverse).*
Results: *Optimal amplification was achieved at 90°C for denaturation and 60°C for extension, yielding a mean Ct value of 18.49 for P. vivax. qPCR successfully detected parasites at various developmental stages and remained negative for normal blood and for samples suspected of containing other Plasmodium species, thereby confirming primer specificity.*
Conclusion: *The optimized qPCR conditions (90°C denaturation, 60°C extension) provided specific and sensitive detection of P. vivax 18S rRNA gene across multiple life stages, supporting their use for reliable malaria diagnosis and molecular surveillance.*

Keywords: *Malaria, Plasmodium vivax, 18S rRNA gene, Denaturation, PCR*

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RESUMEN

Introducción: *La malaria causada por Plasmodium vivax sigue siendo ampliamente prevalente, con etapas hepáticas latentes que pueden provocar recaídas. Este estudio tuvo como objetivo optimizar las temperaturas*

de desnaturalización y de extensión para la detección, mediante qPCR, del gen 18S rRNA de P. vivax, siguiendo una optimización previa de la concentración de cebadores y de la temperatura de alineamiento.
Métodos: *Se empleó un diseño cuasiexperimental para evaluar las temperaturas de desnaturalización (90 °C, 95 °C, 97 °C) y de extensión (58 °C, 60 °C, 62 °C), manteniendo la concentración de cebadores en 300 nM. Se analizaron dieciocho muestras positivas de malaria que representaban diferentes etapas del ciclo del parásito, así como muestras de otras especies de Plasmodium y de sangre normal. Las pruebas se realizaron mediante análisis microscópico y qPCR utilizando cebadores de 23 pb (adelante) y 22 pb (reverso).*
Resultados: *Se logró una amplificación óptima a 90 °C para la desnaturalización y 60 °C para la extensión, obteniéndose un valor medio de Ct de 18,49 para P. vivax. La qPCR detectó con éxito los parásitos en varias etapas de su desarrollo y resultó negativa tanto en la sangre normal como en las muestras sospechosas de otras especies de Plasmodium, lo que confirmó la especificidad de los cebadores.*
Conclusión: *Las condiciones optimizadas de qPCR (90 °C de desnaturalización y 60 °C de extensión) permiten una detección específica y sensible del gen 18S rRNA de P. vivax en múltiples etapas de su vida, lo que respalda su aplicación para un diagnóstico confiable de malaria y la vigilancia molecular.*

Palabras clave: *Malaria, Plasmodium vivax, gen 18S rRNA, desnaturalización, PCR.*

INTRODUCTION

Malaria remains a persistent public health challenge in Indonesia, with *Plasmodium vivax* contributing substantially to the clinical burden and, in some cases, prolonged severe presentations (1). National incidence has been rising, with the greatest impact in the eastern provinces of Papua, West Papua, Maluku, and Nusa Tenggara Timur (NTT) (2). In 2024, *P. vivax* accounted for 85 % (473 cases) of all malaria cases. As the most widely distributed malaria species globally (3), *P. vivax* poses a particular challenge because it forms dormant liver-stage parasites (hypnozoites) that can reactivate and cause recurrent infections despite initial parasite clearance (4).

Accurate diagnosis relies on laboratory methods that identify the infecting *Plasmodium* species. Approaches range from conventional microscopy to molecular techniques such as Polymerase Chain Reaction (PCR), with Rapid Diagnostic Tests (RDTs) serving as intermediate tools. Although microscopy remains the gold standard, it has well-known limitations: reduced sensitivity at low parasite densities, difficulty differentiating mixed infections, and a dependence on expertise and time that undermines reliability in low-prevalence settings (5). RDTs provide speed and field utility but have limited ability to distinguish *P. vivax, P. ovale, and P. malariae,* and to differentiate single from mixed *P. falciparum* infections. In contrast, PCR-based methods offer superior sensitivity and specificity, enabling the detection of minimal parasitemia and accurate species identification (6-9).

Given these performance differences, molecular assays consistently outperform RDTs and microscopy and can resolve species misclassification, underscoring the value of integrating PCR into routine diagnostic workflows (10,11). Recent advances from conventional PCR to real-time/qPCR targeting conserved loci such as 18S rRNA gene provide scalable platforms for surveillance and case confirmation across diverse settings (12). Complementary lines of inquiry include ethnomedicine surveys that document locally used antimalarial botanicals (13), some of which demonstrate *in vivo* antiplasmodial activity in murine models (14). Together, these observations support our strategy to deploy a PCR-based approach for accurate *P. vivax* detection while aligning with context-specific treatment and control priorities (6-9).

The *Plasmodium* genome encodes numerous targets, including 18S rRNA, which is particularly valuable for malaria diagnosis. This study used quantitative polymerase chain reaction (qPCR) to specifically detect *P. vivax* by targeting the 18S rRNA gene. The 18S rRNA is an essential component of the 40S ribosomal subunit and is central to protein synthesis and ribosome biogenesis; thus, rRNA transcription increases with cellular growth. Consequently, 18S rRNA abundance is a reliable indicator of cellular activity. The 18S rRNA gene contains conserved regions that facilitate pan-*Plasmodium* detection and variable regions that enable species-level discrimination (15-19).

In 2024, a new primer set was evaluated for qPCR detection of the *P. vivax* 18S rRNA gene. The forward and reverse primers (23 and 22 bases, respectively) amplify a 114 bp product. In silico analysis confirmed target detection with an average cycle threshold of 18.94. Primer concentration and denaturation temperature were optimized to improve amplification efficiency. Further laboratory refinement, particularly thin blood smear microscopy, remains necessary to establish the most favorable denaturation and extension temperatures for this primer pair (20-22). PCR relies on iterative denaturation, annealing, and extension steps mediated by a DNA polymerase. Optimizing qPCR parameters, especially denaturation and extension temperatures, is essential for robust performance. Excessive denaturation temperature or duration can damage cytosine residues and impair polymerase activity, reducing yield, whereas insufficient denaturation can prevent complete strand separation. Similarly, elevated extension temperatures can hinder polymerase function and limit nucleotide incorporation (7,23).

The primary objective of this study was to evaluate whether the newly designed PCR primers can be used to monitor patients clinically suspected of malaria and to determine the assay's specificity for distinguishing *P. vivax* from other *Plasmodium* species. This specificity is critical for accurate diagnosis and effective patient management. Notably, *P. vivax* is reported as the predominant species across Java, whereas *P. falciparum* predominates in eastern Indonesia, underscoring the need for reliable molecular tools to support precise identification and surveillance in endemic settings.

METHODS

This study, which employed a quasi-experimental design, aimed to identify the optimal denaturation and extension temperatures for detecting *Plasmodium vivax* by qPCR. The study protocol was reviewed and approved by the Research and Research Ethics Committee of the Dustira Level II Hospital (Etik.RSD/100/VI/2024). The study methodology adhered to the principles established by the International Conference on Harmonization–Good Clinical Practice (ICH-GCP). Blood samples were collected using a random sampling technique from patients who had been clinically diagnosed with malaria, but whose *Plasmodium* infection had not yet been confirmed. Subsequent molecular identification confirmed the presence of *Plasmodium vivax* in the analyzed samples.

Materials and Equipment

The equipment used included a Real-Time PCR machine, a Biosafety Cabinet Level 2, micropipettes with sterile tips, microtubes, a vortex mixer, a centrifuge, PCR tubes, collection tubes, a computer, a Thermal Cycler (Tianlong Gentier 96), a laminar airflow cabinet, a marker, a water bath, and a spin-down device. The materials comprised microscope slides, sterile lancets, cotton swabs, 70 % alcohol, tissues, slide boxes, 3 % Giemsa stain, methanol, and the Wizard Genomic DNA Purification Kit (Promega) containing cell lysis solution, nucleic lysis solution, protein precipitation solution, DNA rehydration solution, RNase A solution, 70 % ethanol, and isopropanol. Additionally, the GoTaq qPCR Master Mix Kit (Promega), including GoTaq qPCR Master Mix, CXR Reference Dye, and Nuclease-Free Water, was used along with the forward and reverse primers. Blood samples were confirmed positive for *Plasmodium vivax* infection.

Preparation of Thin and Thick Blood Smears and Giemsa Staining

Standard procedures were followed to prepare thin and thick blood smears. For thin smears, a small drop of blood was placed near the center of a clean microscope slide and spread smoothly to create a feathered edge. For thick smears, two to three drops of blood were placed near the frosted end of another slide and spread in a circular motion to form a circle approximately 1-1.5 cm in diameter. Both smears were air-dried on flat surfaces. After drying, thin smears were fixed with methanol for 5 min. Both thin and thick smears were stained with 3 % Giemsa solution for 45-60 min, then gently rinsed with distilled water until the runoff was clear. The slides were air-

dried and examined microscopically to confirm the presence of malarial parasites.

DNA Extraction from Whole Blood Using the Promega Reagent Kit

Before DNA extraction, all the equipment and reagents were prepared under sterile conditions. The light and blower of the biosafety cabinet were activated for 3 min to reach the optimal operating conditions. Blood samples were homogenized by gently inverting the microtube five to six times and left at room temperature for 10 min. The samples were then centrifuged at 15 000 rpm for 20 minutes to separate the supernatant from the cell pellet. Next, 300 μL of Nuclei Lysis Solution was added to the pellet, pipetted 5-6 times, and then 1.5 μL RNase was added. The mixture was gently inverted 2–5 times and centrifuged again at 15 000 rpm for 3 min to form a dark brown protein pellet. The supernatant containing the DNA was then subjected to an additional centrifugation step at 15 000 rpm for 1 min. The DNA pellet was washed with 70 % ethanol, centrifuged, and rehydrated. Purified DNA was stored at 2–8°C until further use.

Preparation of the Master Mix

To ensure sterility, the biosafety cabinet was sterilized with ultraviolet (UV) light for 15 min before use. After UV exposure, the blower and lamp were turned on and allowed to stabilize for 3 minutes. The master mix components were thoroughly mixed and briefly centrifuged for 10 s to collect droplets on the tube walls. A total of 20 μL of the master mix was dispensed into each PCR tube. Subsequently, 5 μL template DNA was added to each tube using sterile pipette tips. The tubes were briefly vortexed and centrifuged at a low speed for 10 s to ensure homogeneity. The biosafety cabinet and workspace were then disinfected with 70 % ethanol and thoroughly cleaned.

Quantification of 18S rRNA Gene Expression Using qPCR

A new experimental file was created in the qPCR software, and a custom thermal cycling protocol was designed using the "Custom Stage Add" and "Tri-Step Add" functions to adjust the temperature and duration of each stage. The annealing temperature, optimized in earlier studies, was further refined by changing the gradient settings for the center and offset temperatures.

Fluorescence detection was performed using SYBR Green and ROX as the reporter and reference dyes, respectively. A sample map was created to accurately assign each well to a qPCR plate. The PCR tubes were loaded according to this map and subjected to qPCR amplification. Upon completion, amplification curves were analyzed to determine cycle threshold (Ct) values and to evaluate amplification efficiency.

Seventeen blood samples that met the inclusion criteria were subjected to confirmatory testing. Each sample underwent microscopic examination of thin blood smears to verify the presence of malarial parasites, and molecular confirmation by PCR to ensure accurate identification of *Plasmodium vivax* infection.

RESULTS

This study aimed to determine the optimal denaturation and extension temperatures for amplifying the *Plasmodium vivax* 18S rRNA gene by quantitative Polymerase Chain Reaction (qPCR). The main purpose of this optimization process was to improve amplification efficiency, ensure assay reproducibility, and enhance the overall diagnostic reliability of the developed method. Since *P. vivax* remains one of the most prevalent malaria-causing species in Indonesia, particularly on Java, accurate and sensitive detection of this parasite is crucial for effective disease management and surveillance. Conventional diagnostic approaches, although widely used, often fail to differentiate among *Plasmodium* species or detect infections with low parasitemia. Thus, molecular methods such as qPCR have emerged as valuable tools to overcome these limitations (24).

To ensure the purity and integrity of the nucleic acid template, genomic DNA was extracted from blood samples previously confirmed by microscopy to be positive for *P.*

vivax infection. DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega), widely recognized for yielding high-quality DNA suitable for downstream molecular applications. The extraction process followed standardized protocols involving cell lysis, protein precipitation, DNA purification, and rehydration. Each sample was carefully processed under sterile conditions using a Biosafety Cabinet Level 2 to prevent contamination. The resulting genomic DNA was quantified and stored at 2–8°C until amplification.

For amplification, the *P. vivax* 18S rRNA gene was targeted due to its conservation and diagnostic relevance across multiple *Plasmodium* species. The qPCR assays were performed using the GoTaq qPCR Master Mix Kit (Promega), which contains a pre-optimized combination of Taq polymerase, dNTPs, and buffer components, ensuring consistent and efficient amplification. The reaction setup included forward and reverse primers designed to target unique regions of the *P. vivax* 18S rRNA gene, thereby guaranteeing high specificity for detection. Each reaction was performed in a total volume of 25 μL, comprising 20 μL of master mix and 5 μL of DNA template. To monitor amplification in real time, SYBR Green was selected as the reporter dye, and ROX served as a passive reference dye to normalize fluorescence fluctuations among wells.

qPCR assays were performed on a Tianlong Gentier 96 real-time PCR system, which enables high-throughput amplification and precise thermal control. The gradient-temperature function of the Tianlong system was used to optimize the denaturation and extension steps, thereby identifying the most effective thermal profile for the target gene. The gradient configuration enabled simultaneous evaluation of multiple temperatures in a single run, thereby increasing efficiency and minimizing experimental variability. The PCR tubes were arranged according to preset temperature gradients, ensuring that each column corresponded to a distinct denaturation or extension temperature. This setup facilitated direct comparison of cycle threshold (Ct) values across conditions, enabling determination of the temperature combination that yielded the strongest amplification signal with the least background fluorescence (25).

The denaturation temperature determines the extent to which double-stranded DNA separates into single strands, a process that is critical for primer binding and subsequent extension. An excessively low temperature can result in incomplete strand separation, whereas a temperature that is too high can damage the polymerase enzyme and reduce reaction efficiency. Similarly, the extension temperature influences the enzyme's activity in DNA strand synthesis. By testing denaturation temperatures of 90, 95, and 97°C (Table 1) with extension temperatures of 58°C, 60°C, and 62°C (Table 2), this study aimed to identify the optimal combination that balances high specificity with maximal amplification yield.

Table 1. Optimization of Denaturation Temperature against Blood Smear

Denaturation Temperature	Ct Value
90°C	25.58
	24.94
	24.53
Average	25.02
NTC	-
	25.72
95°C	25.29
	25.15
Average	25.38
NTC	-
	25.65
97°C	26.26
	26.98
Average	26.30
NTC	-

The amplification process was closely monitored in real time, with the Tianlong system generating fluorescence curves that corresponded to the accumulation of amplified products. qPCR data were analyzed to determine the mean cycle threshold (Ct) values for each condition. A lower Ct value indicates earlier fluorescence detection and thus more efficient amplification. The results

demonstrated that the optimal amplification conditions were 90°C for denaturation and 60°C for extension, yielding a mean Ct value of 18.49. These optimized conditions yielded consistent amplification curves with clear exponential phases, minimal background noise, and distinct melting peaks, confirming the primer pair's efficiency and specificity.

While molecular techniques such as qPCR offer high sensitivity and specificity, microscopic examination remains the conventional gold standard for malaria diagnosis, particularly in the field and clinical settings. Microscopic analysis involves preparing both thin and thick blood smears, each serving different diagnostic purposes. A thin blood smear enables detailed morphological examination and species identification, whereas a thick smear increases the likelihood of detecting parasites in samples with low parasitemia. Both smear types were prepared in accordance with World Health Organization (WHO) guidelines (22,25). The presence of *P. vivax* parasites was confirmed by the visualization of characteristic features, including enlarged infected erythrocytes and trophozoites with an amoeboid cytoplasm. Schizonts containing multiple merozoites were observed in several fields of view, indicating that the parasite was in the schizont stage of its life cycle. Confirmation of *P. vivax* infection through microscopy served as the initial validation for molecular analysis and helped ensure the accuracy of the qPCR assay results (4).

The integration of microscopy and qPCR in this study provides a comprehensive approach to malaria diagnosis. While microscopy enabled direct visualization of the parasite and validation of infection status, qPCR provided quantitative, species-specific confirmation with superior sensitivity, particularly for samples with low parasite loads. The complementary use of these two methods not only ensured the reliability of the findings but also demonstrated the potential for combining conventional and molecular techniques in future diagnostic protocols. These results reaffirm the value of qPCR optimization in enhancing diagnostic precision and underline its applicability in routine malaria surveillance programs, especially in regions where *P. vivax* predominates and mixed infections are common.

The study optimized the denaturation and extension temperatures for qPCR detection of *Plasmodium vivax* 18S rRNA gene, establishing 90°C and 60°C as the optimal denaturation and extension temperatures, respectively. These optimized conditions, validated by both molecular and microscopic analyses, provide a reliable, reproducible method for sensitive detection of *P. vivax*, thereby improving diagnostic accuracy and supporting malaria control efforts across endemic regions.

Table 2. Optimization of Extension Temperature against Blood Smear

Extension Temperature	Ct Value
58°C	19.63
	19.81
	19.81
Average	19.75
NTC	-
	19.66
60°C	19.38
	19.55
Average	19.53
NTC	-
	19.70
58°C	19.79
	19.77
Average	19.75
NTC	-

Table 3 presents a comparative analysis of thin peripheral blood smears and PCR amplification using primers targeting the *Plasmodium vivax* 18S rRNA gene. The data clearly demonstrated that the primer set exhibited high specificity for *P. vivax*, as amplification was observed only in samples confirmed microscopically to contain this species. No amplification signals were detected in samples containing other *Plasmodium* species, such as *P. falciparum*, *P. malariae*, and *P. ovale*, or in normal blood samples used as negative controls. This finding indicated that the designed primer pair exclusively recognizes the conserved region of the *P. vivax* 18S rRNA gene and does

not cross-react with homologous sequences from other *Plasmodium* species, thereby confirming its diagnostic specificity (26-29).

Furthermore, the PCR results were consistent across the developmental stages of *P. vivax,* including the trophozoite, schizont, and ring stages. The assay successfully detected parasite DNA across all stages, as evidenced by consistent amplification curves and cycle threshold (Ct) values from qPCR analysis. This consistency demonstrates that the presence of parasite DNA in the blood is sufficient for detection, regardless of the morphological stage observed under a microscope. It also supports the reliability of the molecular assay for identifying infections even when parasitemia levels fluctuate owing to the parasite's life-cycle dynamics. The PCR assay's ability to amplify *P. vivax* DNA across stages provides additional confidence in its use in both clinical diagnosis and epidemiological studies.

In comparison with microscopic examination, PCR results showed a strong correlation with thin peripheral blood smear findings. All samples that were microscopically confirmed as *P. vivax*-positive were also positive by PCR, whereas microscopically negative samples consistently showed no amplification. This concordance underscores the complementary nature of the two diagnostic methods. Microscopic examination, which remains the gold standard, provides visual confirmation of parasite morphology and allows for species differentiation by trained personnel. However, its sensitivity is limited, particularly for detecting low parasitemia or mixed infections.

Table 3. Thin Peripheral Blood Smears and PCR Analysis of Samples Diagnosed with Malaria.

Sample	Parasite and stages	CT Value test
1	*Plasmodium vivax* Trophozoites	18.11
2	*Plasmodium vivax* Young trophozoites (ring stage parasites)	19.21
3	*Plasmodium vivax* Young trophozoites (ring stage parasites)	20.77
4	*Plasmodium vivax* Young trophozoites (ring stage parasites)	26.44
5	*Plasmodium vivax* Young trophozoites (ring stage parasites)	28.33
6	*Plasmodium vivax* Young trophozoites (ring stage parasites)	19.22
7	*Plasmodium falciparum* Trophozoites	-
8	*Plasmodium falciparum* Young Trophozoites	-
9	*Plasmodium vivax* Trophozoites	19.22
10	*Plasmodium vivax* Schizonts	29.22
11	*Plasmodium falciparum* Young trophozoites (ring stage parasites)	-
12	*Plasmodium malariae* Schizonts	-
13	*Plasmodium vivax* Trophozoites	19.45
14	*Plasmodium falciparum* Young trophozoites (ring stage parasites)	-
15	*Plasmodium ovale* Young trophozoites (ring stage parasites)	-
16	*Plasmodium malariae* Schizonts	-
17	*Plasmodium vivax* Young trophozoites (ring stage parasites)	23.22

On the other hand, PCR offers molecular confirmation with higher sensitivity and specificity and can detect small amounts of parasite DNA that may not be visible under the microscope. The integration of these methods provides a comprehensive diagnostic framework. In clinical practice, microscopy can be used for preliminary screening, whereas PCR can serve as a confirmatory or reference test, especially in cases with ambiguous or low-density parasitemia.

The results summarized in Table 3 validate the effectiveness of the designed 18S rRNA primers for *P. vivax* detection and reinforce their potential applications in molecular surveillance programs. The strong agreement between the PCR and microscopy findings suggests that the optimized molecular assay can complement traditional diagnostic techniques and improve the accuracy of malaria diagnosis. This correlation also highlights the feasibility of qPCR as a reliable tool for monitoring *P. vivax* infections across different stages of parasite development.

The data in Table 3 confirm that the 18S rRNA primers are both sensitive and species-specific for *Plasmodium vivax*, capable of detecting the parasite in all observed stages, trophozoites, schizonts, and ring forms, while showing no cross-reactivity with other *Plasmodium* species. The consistency between molecular and microscopic findings underscores the diagnostic reliability of the qPCR approach and its value as a complementary method to traditional blood smear examinations in both clinical and research settings.

DISCUSSION

The findings of this study provide substantial evidence supporting the effectiveness of optimizing denaturation and extension temperatures for the qPCR-based detection of *Plasmodium vivax* 18S rRNA gene. The optimization identified 90°C as the most efficient denaturation temperature and 60°C as the optimal extension temperature. These conditions consistently produced the lowest cycle threshold (Ct) values, indicating efficient amplification with minimal background noise. The optimized parameters were critical for enhancing the assay's reproducibility and reliability, both essential attributes in molecular diagnostic applications. The use of gradient thermal profiling allowed for the precise adjustment of temperature conditions and ensured that the amplification reaction proceeded under thermodynamically favorable conditions (2).

The results from Tables 1 and 2 indicate that lower denaturation temperatures yielded better amplification performance, possibly because reduced Taq polymerase degradation, which tends to lose activity at excessively high temperatures. Similarly, an extension temperature of 60°C provided optimal enzyme kinetics, allowing accurate DNA synthesis and improved signal detection. These findings align with previous molecular studies that reported optimal amplification of *Plasmodium* genes within similar temperature ranges, particularly when targeting conserved ribosomal RNA sequences. Such optimization enhances both the assay's analytical sensitivity and diagnostic specificity, enabling its potential application in routine malaria diagnosis and epidemiological surveillance.

In Table 3, the comparison between thin peripheral blood smears and qPCR amplification provides clear evidence of assay specificity. The primers designed for the *P. vivax* 18S rRNA gene successfully amplified DNA only from samples confirmed microscopically as *P. vivax*-positive, with no cross-reactivity with samples containing *P. falciparum*, *P. malariae*, or *P. ovale*. This result confirmed the primers' high discriminatory power and supported their use for molecular identification of *P. vivax* infections. Furthermore, amplification was achieved across all developmental stages of *P. vivax*, including trophozoites, schizonts, and ring forms, demonstrating that the assay can reliably detect parasite DNA regardless of morphological form. This is particularly advantageous for detecting low-level infections or cases in which the parasite stage distribution is heterogeneous within the bloodstream (20).

The consistent amplification of *P. vivax* DNA across all positive samples, with Ct values ranging from approximately 18 to 29, further supported the assay's robustness. These values correspond to detectable parasitemia levels, even when microscopic detection is challenging owing to

low parasite density. Importantly, the absence of amplification in non-*vivax* samples validates the assay's specificity and eliminates the risk of false-positive results due to nonspecific binding or contamination.

The correlation between qPCR and microscopy in this study reinforces the complementary nature of these diagnostic techniques. Microscopy remains the gold standard for malaria diagnosis, providing direct visual confirmation of parasite morphology and stage differentiation. However, its sensitivity is highly dependent on the microscopist's skill and the level of parasitemia in the sample qPCR. On the other hand, qPCR offers higher analytical sensitivity and can detect low levels of parasite DNA, even when microscopy yields negative results. The strong concordance between both methods in this study demonstrates that qPCR can serve as a confirmatory and supportive diagnostic tool, particularly for cases of ambiguous or low-density infections (6,23).

Moreover, integrating both diagnostic approaches strengthens malaria control and surveillance. In clinical settings, microscopy can be employed for rapid preliminary identification. In contrast, qPCR can be utilized to confirm infection status, validate species differentiation, and provide quantitative data for monitoring treatment response. From an epidemiological perspective, qPCR facilitates a more accurate mapping of malaria distribution and can detect subclinical infections that might otherwise go unnoticed.

Overall, the results demonstrated that the optimized qPCR assay targeting the *P. vivax* 18S rRNA gene is both specific and sensitive, with reliable amplification across the parasite's developmental stages. These characteristics make it an effective diagnostic tool that complements traditional microscopy. This method not only enhances diagnostic precision but also improves malaria surveillance, especially in regions such as Java, where *P. vivax* predominates. The strong agreement between molecular and microscopic data supports the confident integration of the optimized qPCR assay into diagnostic protocols, thereby advancing both clinical management and public health monitoring of malaria in endemic areas (24,25,30).

CONCLUSION

The study concluded that the optimized qPCR conditions, 90°C for denaturation and 60°C for extension, provided specific and sensitive detection of *Plasmodium vivax* 18S rRNA gene. The results correlated strongly with the microscopic findings, confirming the assay's reliability for accurate malaria diagnosis and its potential for use in molecular surveillance programs.

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