Le Infezioni in Medicina, n. 1, 49-56, 2026
doi: 10.53854/liim-3401-5

Optimization of Nested-PCR for the detection of *Plasmodium malariae* in laboratory settings in Southern Vietnam

Kim Trung Nguyen[1], Ngoc Hieu Nguyen[1], Quoc Long Dinh[1], Hoang Linh Vu Nguyen[1], Minh Anh Thi Nguyen[1], Hong Quang Huynh[2], Hue Van Thi Tran[1]

[1] Department of Medical Laboratory Technology, School of Nursing and Medical Technology, University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam;
[2] Institute of Malariology, Parasitology, and Entomology, Quy Nhon, Vietnam.

Article received 9 October 2025 and accepted 7 January 2026

SUMMARY

Nested Polymerase Chain Reaction is a highly effective technique for detecting infectious diseases, particularly in cases involving low level of parasitemia infections such as malaria.
Objective: This study aimed to optimize a Nested-PCR protocol for the specific detection of *Plasmodium malariae*, with a defined scope focused on refining cycling conditions, annealing parameters, and procedural steps to ensure reliable performance in laboratory settings in Southern Vietnam.
Methods: The assay targeted the *P. malariae* 18S rRNA gene using PLU5/PLU6 and MAL1/MAL2 primers. Cycle numbers were optimized at 25, 30, and 35 in the first round, and 25 and 30 in the second round. The annealing temperature for MAL1/MAL2 primers were evaluated from 54 to 60°C, and PCR products were visualized using the ChemiDoc-It² system.
Results: The MAL1/MAL2 primer pair demonstrated high specificity for *P. malariae*, with no amplification observed for other *Plasmodium* species (including: *P. falciparum*, *P. vivax*, *P. ovale* and *P. knowlesi*) or common microbial contaminants. The optimal conditions were determined to be 35 cycles in the first PCR round, 25 cycles in the second round, with an annealing temperature of 54°C for the MAL1/MAL2 primers. The protocol achieved a limit of detection as low as 0.5 parasites/µL.
Conclusions: The optimized protocol is well-suited for laboratories in Southern Vietnam.

Keywords: malaria, *Plasmodium malariae*, Nested-PCR, protocol optimization.

INTRODUCTION

Malaria is a life-threatening infectious disease caused by *Plasmodium* parasite, transmitted to humans through the bites of infected female *Anopheles* mosquitoes [1]. It remains a significant health burden in numerous tropical and subtropical countries [2]. The initial symptoms of malaria are often nonspecific, including fever, chills, and muscle pain, which can easily be mistaken for other acute conditions like influenza, leading to delays in diagnosis and treatment [1]. If not promptly treated, malaria can rapidly progress, causing severe anemia, multi-organ failure, and death. Specifically, infections caused by *Plasmodium malariae* tend to have a chronic, asymptomatic course lasting several years, and in rare cases, can result in serious complications such as nephrotic syndrome or chronic renal damage [1]. In Vietnam, three primary species of *Plasmodium* are prevalent: *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium malariae*, with *P. falciparum* and *P. vivax* accounting for approximately 98% of total infections

Corresponding author
Hue Van Thi Tran
E-mail: huevan@ump.edu.vn

[3]. *P. malariae* infections are comparatively rare, constituting only 1-2%, thus increasing the risk of being overlooked in diagnostic procedures and epidemiological surveillance [3]. However, between 2023 and 2024, several malaria outbreaks were recorded in central Vietnam, with *P. malariae* prevalence rising significantly to 7.89%–52.8% in certain areas [4-6].

The clinical diagnosis of malaria remains challenging due to overlapping and non-specific symptoms among *Plasmodium* species and the frequent occurrence of asymptomatic infections. Consequently, diagnostic techniques with high sensitivity and specificity have received considerable attention, as they are critical for guiding appropriate therapeutic interventions, minimizing the risk of antimalarial resistance, and enhancing disease control. Light microscopy of Giemsa-stained blood smears remains the gold standard for malaria diagnosis; however, it has a detection threshold of approximately 50-100 parasites/μL and is highly dependent on microscopist expertise [7]. Molecular diagnostic techniques, particularly PCR, have emerged to overcome these limitations, offering superior sensitivity and species-level specificity. Studies in three distinct populations in Papua New Guinea demonstrated that the prevalence of *P. malariae* increased 2.6-10.9-fold when diagnosed by PCR compared with microscopy (microscopy: 1.1-14.4%; PCR: 10-37%) [8–10]. Similarly, in three African studies, PCR detected *P. malariae* in 7.6%, 23.3%, and 39.2% of samples, whereas microscopy identified only 1.1%, 0%, and 18.7% of infections, highlighting the underestimation of *P. malariae* prevalence by conventional methods [11–13].

In Southeast Asia, a study in Thailand and Myanmar reported *P. malariae* prevalence of 2.2% by microscopy versus 24.3% by PCR, further demonstrating the superior sensitivity of molecular approaches [14]. Although PCR is increasingly applied to human blood samples, the detection of sporozoite infections in mosquitoes has largely relied on circumsporozoite protein-based ELISA. Recent analyses indicate that ELISA lacks sufficient sensitivity to detect low-density *P. malariae* sporozoites, a limitation observed across multiple Plasmodium species, including *P. falciparum* [15]. Consequently, PCR is considered a more reliable tool for accurately determining *P. malariae* sporozoite infection rates in mosquito salivary glands. A recent study in Guinea-Bissau using PCR on mosquito salivary glands and midgut oocysts detected *P. malariae* in 1-5% of specimens, demonstrating its potential for vector surveillance [16]. These findings underscore the necessity of molecular diagnostics to accurately estimate both human and mosquito infection prevalence, particularly for low-density and subclinical *P. malariae* infections.

Nested Polymerase Chain Reaction (Nested-PCR) is a DNA amplification method that utilizes two sets of primers in successive rounds of amplification, offering superior sensitivity and specificity compared to conventional PCR. This method enables detection of parasitemia as low as 0.5–5 parasites/μL and is currently recommended as a confirmatory assay to support the exclusion of malaria [7]. However, employing a single set of Nested-PCR conditions for all five *Plasmodium* species may result in diagnostic inaccuracies, such as false-negative results. At present, reports focusing specifically on the use of Nested-PCR for the diagnosis of *P. malariae* remain scarce in the literature. Moreover, the method is often perceived as technically complex, time-consuming, and costly, which limits its routine implementation in resource-constrained settings. Therefore, optimization of the protocol to accommodate the practical constraints of specific laboratories or regions is essential. In this context, the present study aimed to optimize a Nested-PCR protocol, with a focus on improving diagnostic performance, reducing assay time, and ensuring practicality under the laboratory conditions of Southern Vietnam, particularly in light of the evolving epidemiological landscape of malaria in the region.

MATERIALS AND METHODS

Study population

Dried Blood Spot (DBS) samples were collected from 30 patients in Khanh Vinh District, Khanh Hoa Province, October 2024 to April 2025. All samples were microscopically confirmed as *P. malariae*-positive using Giemsa-stained thick and thin blood smears examined under light microscopy.

Reference samples of *P. vivax* (Specimen 2503), *P. malariae* (Specimen 2504), *P. falciparum* (Specimen 2505), *P. ovale* (Specimen 2506), and *P. knowlesi* (Specimen 2510) were provided by the Institute of Malariology Parasitology and Entomology Quy Nhon.

Control DNA samples of other organisms, includ-

ing *Toxoplasma gondii* (ATCC 50839), *Giardia lamblia* (ATCC 30888), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10231), and *Staphylococcus aureus* (ATCC 25923) were provided by the Ho Chi Minh City Biotechnology Center.
Negative control: UltraPure™ DNase/RNase-Free Distilled Water is designed for use in all molecular biology applications were provided by Thermo Fisher Scientific, USA.

DNA Extraction
Genomic DNA was extracted from DBS samples using the TopPURE® Blood DNA Extraction Kit, following the manufacturer's protocol. The extracted DNA was subsequently used as the template for PCR amplification.

Nested Polymerase Chain Reaction

First PCR Round
Each reaction was prepared in a 25 µL volume, consisting of 5 µL of DNA template, 12.5 µL of 2X MyTaq™ HS Mix, 5.5 µL of DEPC-treated water, and 1 µL each of the PLU5 and PLU6 primers (10 µM) in *Table 1*. Thermocycling conditions included initial denaturation at 95°C for 5 minutes; followed by 25, 30, or 35 amplification cycles (each comprising 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute); and a final extension step at 72°C for 5 minutes.

Second PCR Round
Each reaction was prepared in a 25 µL volume, consisting of 2 µL of the primary PCR product, 12.5 µL of 2X MyTaq™ HS Mix, 8.5 µL of DEPC-treated water, and 1 µL each of the MAL1 and MAL2 primers (10 µM) in *Table 1*. Thermocycling conditions included initial denaturation at 95°C for 5 minutes; followed by 25 or 30 amplification cycles (each comprising 95°C for 1 minute, primer annealing at either 54°C, 56°C, 58°C, or 60°C for 1 minute, and 72°C for 1 minute); and a final extension at 72°C for 5 minutes.

Table 1 - Primer names and sequence used in the present study [7, 17].

Primer	*Sequence (5′ - 3′)*
PLU5	CCT GTT GTT GCC TTA AAC TTC
PLU6	TTA AAA TTG TTG CAG TTA AAA CG
MAL1	ATA ACA TAG TTG TAC GTT AAG AAT AAC CGC
MAL2	AAA ATT CCC ATG CAT AAA AAA TTA TAC AAA

Gel Electrophoresis
PCR products from the second round were mixed with 10X DNA Loading Buffer and electrophoresed on a 2% Agarose gel in 0.5X TAE buffer at 110 V for 30 minutes. GelRed™ 3X in Water (Biotium, USA) was diluted to a 1X working concentration and incorporated directly into the Agarose gel prior to casting to enable fluorescence-based detection. Band patterns were visualized using the ChemiDoc-It² Imaging System (UVP) under UV illumination at 302 nm. A sample was considered positive when a sharp, specific band corresponding to the expected size of 145 bp for *P. malariae* was observed, whereas samples showing no band, smearing, or non-specific amplification were classified as negative.

Data Analysis
Data were analyzed using Microsoft Excel 2016. A p-value < 0.05 was considered statistically significant.

RESULTS

Optimization of thermocycling conditions for Nested-PCR targeting P. malariae
Optimization was performed by assessing different thermocycling parameters across ten confirmed *P. malariae*-positive samples, repeated in triplicate. The electrophoretic results under each tested condition are summarized in *Table 2*.
The results (*Figure 1a-c*) indicated that, within the same sample, protocols D, E, and F exhibited greater amplification efficiency compared to the other tested conditions. Among the four annealing temperatures evaluated in the second PCR round, 54°C consistently yielded sharper and more specific bands. To enhance the quantity of DNA template entering the second round, protocol E.1, which comprise 35 cycles in the first round, 25 cycles in the second round, and an annealing temperature of 54°C, was selected for further evaluation of its diagnostic applicability.
This optimized protocol was further validated across 30 positive samples. As shown in *Figure 1d*, all tested samples exhibited a distinct, sharp band at the expected size for *P. malariae*. No smearing or

Table 2 - Evaluation of electrophoresis results under different amplification conditions.

Protocol		*Amplification conditions*			*Results*
		Amplification cycles		*Annealing temperature 2nd round (°C)*	
		First PCR round (cycles)	*Second PCR round (cycles)*		
A	1	25	25	54	0
	2			56	0
	3			58	0
	4			60	0
B	1		30	54	1
	2			56	0
	3			58	0
	4			60	0
C	1	30	25	54	1
	2			56	0
	3			58	0
	4			60	0
D	1		30	54	1
	2			56	1
	3			58	1
	4			60	1
E	1	35	25	54	1
	2			56	1
	3			58	1
	4			60	0
F	1		30	54	1
	2			56	1
	3			58	1
	4			60	1

0 = No band, unclear band, smeared or non-specific amplification.
1 = Sharp, specific band at expected size.

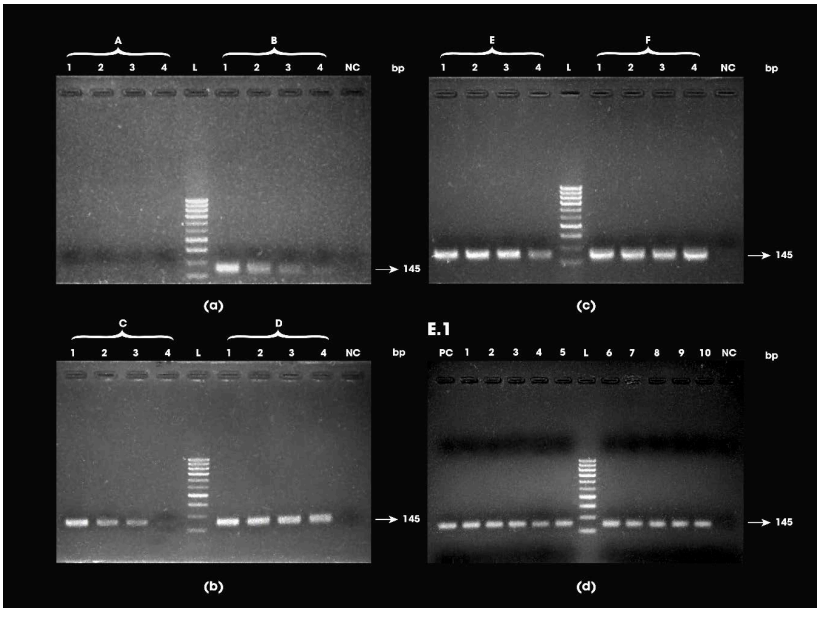

Figure 1
Electrophoretic results of *P. malariae* under various amplification conditions. **(a)** protocol A and B, **(b)** protocol C and D, **(c)** protocol E and F, **(d)** electrophoretic results of ten samples using procedure E.1. (L: DNA ladder 100bp; NC: negative control; PC: positive control; 1–10: samples).

secondary bands were observed, indicating high specificity. Additionally, negative controls showed no amplification, confirming the absence of cross-contamination.

Assessment of Primer Specificity

The MAL1/MAL2 primer pair was tested for specificity using DNA from five *Plasmodium* species (*P. malariae, P. falciparum, P. vivax, P. ovale, P. knowlesi*). As shown in *Figure 2a*, amplification occurred exclusively in the *P. malariae* sample, with no bands detected in the other species, confirming species-level specificity without cross-reactivity.

To further assess cross-reactivity with non-*Plasmodium* organisms, the primer pair was also tested on DNA from *Toxoplasma gondii, Giardia lamblia, Escherichia coli, Candida albicans*, and *Staphyloccocus aureus*. No amplification was observed in any of these samples (*Figure 2b*), indicating high primer specificity toward *P. malariae*.

Limit of Detection (LOD) determination

The limit of detection (LOD) is a critical parameter in any clinical diagnostic assays it reflects the assay's ability to reliably detect the presence of diseases, pathogens, or relevant biological indicators at low concentrations. In this study, the LOD of the optimized Nested-PCR protocol was evaluated using serial dilutions of *P.malariae*-positive samples to obtain final parasite concentrations of 0.25,

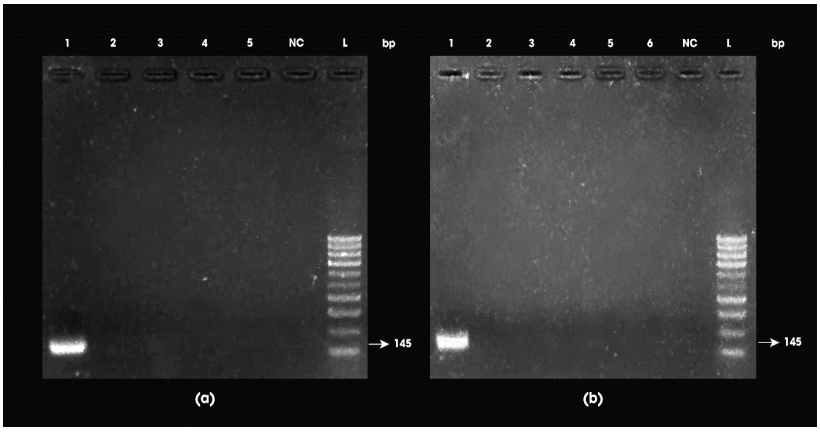

Figure 2
Electrophoretic assessment of primer specificity for MAL1/MAL2.
(a) specificity among *Plasmodium* species
(1: *P. malariae*; 2: *P. falciparum*; 3: *P. vivax*; 4: *P. ovale*; 5: *P. knowlesi*; NC: negative control; L: DNA ladder 100bp).
(b) specificity against non-*Plasmodium* species
(1: *P. malariae*; 2: *Toxoplasma gondii*; 3: *Giardia lamblia*; 4: *Escherichia coli*; 5: *Candida albicans*; 6: *Staphylococcus aureus*; NC: negative control; DNA ladder 100bp).

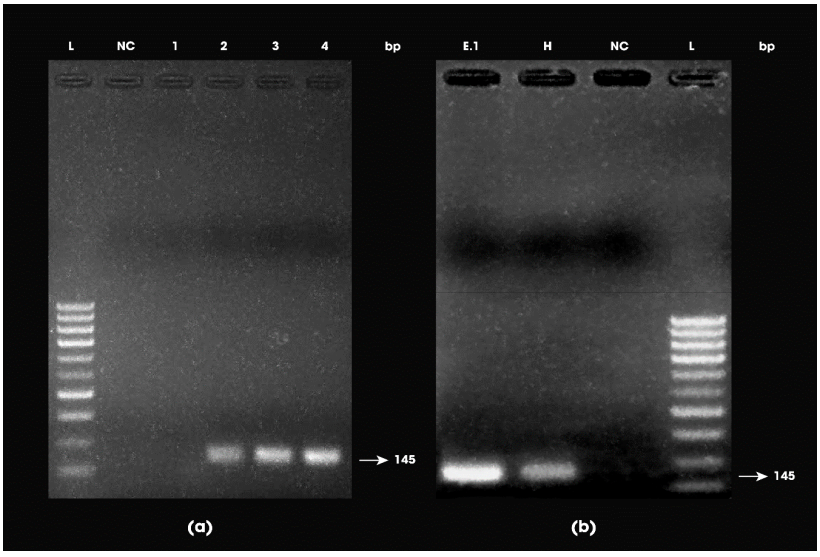

Figure 3
Electrophoretic results of *P. malariae*
(a) in diluted concentrations
(L: DNA ladder 100bp; NC: negative control; 1: 0,25 parasites/µL; 2: 0,5 parasites/µL; 3: 1 parasites/µL; 4: 2 parasites/µL).
(b) comparing two DNA amplification protocols
(E.1: protocol E.1; H: protocol recommended by the MOH of Vietnam; NC: negative control; L: DNA ladder 100bp).

0.5, 1, and 2 parasites/µL. The result of 20 samples for each dilution level shown in *Figure 3a*, at the lowest concentration of 0.25 parasites/µL, 8 out of 20 samples (40%) yielded positive results. When the concentration was increased to 0.5 parasites/µL, 19 out of 20 samples (95%) were positive, reaching statistical significance ($p < 0.05$). At concentrations ≥1 parasite/µL, all samples exhibited strong and distinct amplification bands, with no appreciable difference in intensity between 1 and 2 parasites/µL, suggesting that 0.5 parasites/µL is the optimal detection threshold for the method.

To further assess the performance of the optimized protocol, its amplification efficiency was compared to that of the standard Nested-PCR protocol recommended by the Ministry of Health (MOH) of Vietnam. As shown in *Figure 3b*, the optimized protocol (E.1: 35 cycles in the first round, 25 cycles in the second round, annealing temperature is 54°C) produced clearer and more intense bands across all tested samples. This comparison confirms the enhanced sensitivity and efficiency of the optimized conditions.

DISCUSSION

Nested PCR is a widely used molecular technique in the diagnosis of infectious diseases with limited DNA template, particularly protozoan parasites such as *Plasmodium* spp. By employing two consecutive rounds of amplification using a primary primer pair followed by an internal (nested) primer pair, this method significantly enhances both the sensitivity and specificity of DNA detection relative to conventional PCR. However, the performance of Nested-PCR is determined by several factors, including annealing temperature, number of amplification cycles, template concentration, and primer specificity. Without appropriate optimization, the protocol may generate non-specific bands, secondary amplification products, or insufficient sensitivity to detect low-parasitemia infections potentially leading to false-negative results that complicate clinical practice. Moreover, it is essential to optimize the protocol specifically for each laboratory, country, or region, as implementation is dependent on operational realities unique to each setting.

In this study, we systematically evaluated three factors, including the number of amplification cycles in the first round (25, 30, and 35 cycles), the number of cycles in the second round (25 and 30 cycles), and the annealing temperature for MAL1/MAL2 (54°C, 56°C, 58°C, and 60°C). Each condition was tested in triplicate using 10 *P. malariae*-positive DNA samples. Among all tested combinations, protocol E.1 (35 cycles in round one, 25 cycles in round two, and 54°C annealing temperature) produced the best amplification performance. The total time required for this optimized protocol was 3 hours and 50 minutes, compared to 7 hours and 5 minutes under the standard protocol recommended by the Ministry of Health of Vietnam [7]. Furthermore, multiple studies have reported longer assay times for Nested-PCR (electrophoresis is not included). For instance, Snounou *et al.* (2002) reported a runtime of over 5 hours per reaction [17], and Lardeux *et al.* (2008) required more than 4 hours [18]. These comparisons underscore the time-efficiency of our optimized protocol, highlighting its practical suitability for routine diagnostic workflows targeting *P. malariae.*

The primers must be designed to ensure specificity to the target DNA sequence and the expected amplicon length. Annealing temperature plays a critical role in enabling specific and complete binding of primers to the template strand. In this study, the annealing temperature was evaluated at four levels: 54°C, 56°C, 58°C, and 60°C. The results indicated that at 54°C, the MAL1/MAL2 primer pair yielded more distinct and specific amplification products. In contrast, the annealing temperature used in the Ministry of Health of Vietnam guidelines [7] and in the protocol published by Snounou *et al.* (2002) was 58°C [17]. This discrepancy may be attributed to the fact that previous protocols were designed for simultaneous detection of all five *Plasmodium* species, whereas the present study focused specifically on optimizing conditions for the primer pair targeting *P. malariae*.

Testing primer specificity is a crucial step in the design and standardization of a PCR assay, aiming to ensure that only the target sequence is amplified while minimizing the risk of cross-reactivity. In this study, the specificity of the MAL1/MAL2 primer pair was assessed, and the results confirmed that amplification occurred only in *P. malariae* samples. No amplification was observed in other *Plasmodium* species (*P. falciparum, P. vivax, P. ovale* and *P. knowlesi*) or in non-*Plasmodium* organisms commonly encountered in laboratory set-

tings, including *T. gondii, G. lamblia, E. coli, C. albicans,* and *S. aureus*. These findings demonstrate the high specificity of the MAL1/MAL2 primers and confirm the diagnostic reliability of the optimized protocol in both clinical and epidemiological applications.

Although *P. malariae* typically presents with mild or absent clinical symptoms, its extended pre-patent period, ability to maintain chronic low-grade parasitemia, and capacity for silent circulation render it an important cryptic reservoir within transmission networks. Such low-density infections often fall below the detection threshold of light microscopy, resulting in substantial underestimation of true prevalence and missed opportunities for case containment. In this context, a highly sensitive nested-PCR assay is operationally valuable, as it enables detection of submicroscopic parasite loads and improves the resolution of surveillance systems, particularly in settings approaching elimination. However, the method is limited by its higher operational cost, multi-step workflow, and the requirement for laboratory infrastructure, restricting its utility in routine field diagnostics.

In conclusion, this study demonstrates that optimizing the number of amplification cycles and the annealing temperature can significantly improve the diagnostic efficiency of Nested-PCR for detecting *P. malariae*, particularly in cases with low level of parasitemia or asymptomatic cases. The optimized protocol is compatible with current laboratory conditions in Southern Vietnam. However, further validation is required to evaluate its long-term stability. We recommend additional assessments using independent sample sets to evaluate its sensitivity, specificity, accuracy, positive predictive value, and negative predictive value.

CONCLUSIONS

The optimized Nested-PCR protocol in this study, which involved adjusting the annealing temperature and amplification cycle numbers, demonstrated outstanding performance in detecting *P. malariae*, especially in samples with low level of parasitemia. Moreover, the protocol was time-efficient, making it suitable for laboratories with limited resources and well-suited to the practical conditions of developing countries.

Funding
This study did not receive any specific funding from public, commercial, or not-for-profit funding agencies.

Conflict of interest
No benefits have been or will be received from a commercial party related directly or indirectly to the subject matter of this article.

Acknowledgements
The authors would like to give thanks for the support of the Institute of Malariology Parasitology and Entomology Quy Nhon and Department of Laboratory Medicine, Faculty of Nursing and Medical Technology, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam for creating conditions for the research team to carry out the research at the laboratory.

REFERENCES

[1] Collins WE, Jeffery GM. *Plasmodium malariae*: parasite and disease. *Clin Microbiol Rev*. 2007; 20(4): 579-592.
[2] Fuehrer H-P, Campino S, Sutherland CJ. The primate malaria parasites *Plasmodium malariae*, *Plasmodium brasilianum* and *Plasmodium ovale* spp.: genomic insights into distribution, dispersal and host transitions. *Malar J*. 2022; 21(1): 138.
[3] Ministry of health portal (Vietnam). Things to know about Malaria. Ministry of Health Portal Information; 2015 Nov 3 [cited 2025 Apr 19]. Available from: https://moh.gov.vn/chuong-trinh-muc-tieu-quoc-gia//asset_publisher/7ng11fEWgASC/content/nhung-ieu-can-biet-ve-benh-sot-ret-thuong?inheritRedirect=false.
[4] National Institute of Malariology - Parasitology - Entomology in Ho Chi Minh city. Report on malaria prevention and elimination work in 2023. 37/BC-VSR.
[5] Khanh CV, Thanh PV, Ha NTH, et al. Unprecedented large outbreak of *Plasmodium malariae* malaria in Vietnam: epidemiological and clinical perspectives. *Emerg Microbes Infect*. 2025; 14(1): 2432359.
[6] National Institute of Malariology Parasitology and Entomology, Vietnam. Complicated developments in the fluctuating malaria situation in the malaria-endemic area of Khanh Vinh district, Khanh Hoa province, 2023-2024 (Part 2 - End). Available from: https://www.impe-qn.org.vn/trong-nuoc/dien-tien-phuc-tap-ve-tinh-hinh-sot-ret-bien-dong-tai-vung-sot-ret-luu-hanh-huy/ctmb/18/11917.
[7] General Department of Preventive Medicine. Handbook of guidelines for infectious disease testing, vol-

ume I, issued together with Decision No. 217/QD-D; 2016 [cited 2025 Apr 19].
[8] Kasehagen LJ, Mueller I, Kiniboro B, et al. Changing patterns of *Plasmodium* blood-stage infections in the Wosera region of Papua New Guinea monitored by light microscopy and high throughput PCR diagnosis. *Am J Trop Med Hyg*. 2006; 75(4): 588.
[9] Mehlotra RK, Lorry K, Kastens W, et al. Random distribution of mixed-species malaria infections in Papua New Guinea. *Am J Trop Med Hyg*. 2000; 62(2): 225-231.
[10] Mehlotra RK, Kastens W, McNamara DT, et al. Malaria infections are randomly distributed in diverse holoendemic areas of Papua New Guinea. *Am J Trop Med Hyg*. 2002; 67(6): 555.
[11] Snounou G, Viriyakosol S, Zamora MR, et al. Importance of sensitive detection of malaria parasites in human and insect hosts in epidemiological studies: field samples from Guinea Bissau. *Trans R Soc Trop Med Hyg*. 1993; 87(6): 649-653.
[12] Rubio JM, Benito A, Roche J, et al. Semi-nested multiplex PCR for detection of human malaria parasites and evidence of *Plasmodium vivax* infection in Equatorial Guinea. *Am J Trop Med Hyg*. 1999; 60(2): 183-187.
[13] Walker-Abbey A, Djokam RR, Eno A, et al. Malaria in pregnant Cameroonian women: effect of age and gravidity on submicroscopic and mixed-species infections. *Am J Trop Med Hyg*. 2005; 72(3): 229-235.
[14] Zhou M, Liu Q, Wongsrichanalai C, et al. High prevalence of *Plasmodium malariae* and *Plasmodium ovale* along the Thai-Myanmar border revealed by acridine orange staining and PCR. Trop Med Int Health. 1998; 3(4): 304-312.
[15] Beier JC, Koros J, Onyango F, et al. Quantitation of malaria sporozoites in salivary glands of wild Afrotropical Anopheles. *Med Vet Entomol*. 1991; 5(1): 63-70.
[16] Arez AP, Pinto J, Palsson K, et al. Transmission of mixed Plasmodium species and Plasmodium falciparum genotypes. *Am J Trop Med Hyg*. 2003; 68(2): 161-168.
[17] Snounou G, Singh B. Nested PCR analysis of Plasmodium parasites. *Methods Mol Med.* 2002; 72: 189-203.
[18] Lardeux F, Tejerina R, Aliaga C, et al. Optimization of a semi-nested multiplex PCR to identify Plasmodium parasites in wild-caught Anopheles in Bolivia. *Trans R Soc Trop Med Hyg*. 2008; 102(5): 485-592.