RESEARCH ARTICLE

A comparative study of the use of standard and Pv-Rv primers in regular nest 2-PCR and touchdown nest 2-PCR to detect *Plasmodium vivax*

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Malaria is a disease caused by *Plasmodium* infection. Of the 5 species that infect humans, *Plasmodium vivax* and *Plasmodium falciparum* are the two most common species found in Indonesia. Early and accurate diagnosis is crucial for effective treatment and disease management. Conventional diagnostic methods, microscopy, and rapid diagnostic tests (RDTs) have limitations in sensitivity and specificity. PCR-based assays have undergone significant enhancement such as the development of touchdown PCR and primer designing. However, the use of PCR-based assays in *Plasmodium vivax* infections still has several limitations including the formation of dimers and cross reactivity. Two isolated *Plasmodium vivax* DNA samples taken from malaria confirmed patients and stored in the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya were tested with both standard *Plasmodium vivax* (rViv) primers and new (Pv-Rv) primer for *Plasmodium vivax* 18SSU RNA gene detection. Each pairs of primers underwent both regular nest 2-PCR and touchdown nest 2-PCR. The PCR products were checked by 3% agarose gel electrophoresis, and then quantified by using GelDoc Imaging System and ImageJ software. The paired t-test was used to analyze the results statistically. The results showed that, using rViv primers, there was no statistically significant difference in the ratio of band intensity to background intensity between regular nest 2-PCR and touchdown nest 2-PCR ($P > 0.05$). However, when the background intensity was analyzed, a statistically significant difference was discovered ($P < 0.05$). By using Pv-Rv primers, a significant difference in the background intensity as well as in the ratio of band intensity to background intensity between regular nest 2-PCR and touchdown nest 2-PCR was observed ($P < 0.05$). The results indicated that application of touchdown nest 2-PCR had an effect in reducing background noise by using either rViv primers (standard primer) or Pv-Rv primers (new primer). Further research and modification of Pv-Rv primers were required for *Plasmodium vivax* identification.

Keywords: touchdown PCR, Pv-Rv primer, *Plasmodium vivax*.

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Introduction

Malaria is a parasitic infection caused by the protozoan *Plasmodium* species. Malaria in humans is caused by 5 species of the *Plasmodium genus* including *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium knowlesi* [1, 2]. Of the 5 species that infect humans, *Plasmodium vivax* and *P. falciparum* are the two most common species found in Indonesia, comprising up to 95% of all cases with

Plasmodium vivax responsible for more than 30% and *P*. *falciparum* responsible for more than 60% especially in the eastern regions of the archipelago [3]. Early and accurate diagnosis is crucial for malaria effective treatment and disease management [4]. Conventional diagnostic methods such as microscopy and rapid diagnostic tests (RDTs) have limitations in terms of sensitivity and specificity, especially when dealing with low-level parasitemia or mixed infections. In recent years, polymerase chain reaction (PCR) has emerged as a powerful diagnostic tool, revolutionizing malaria diagnosis through its high sensitivity, specificity, and ability to detect low parasite densities [5, 6]. Over the years, PCR-based assays for malaria diagnosis have undergone significant advancements, leading to increased efficiency, cost-effectiveness, and accessibility. PCR amplifies specific DNA or RNA sequences in a sample, allowing for the identification and quantification of pathogens including *Plasmodium* species. PCR enables the detection of extremely low parasite loads, providing crucial information for accurate diagnosis, surveillance, and epidemiological studies [7, 8]. The development of novel PCR-based techniques including quantitative PCR (qPCR), nested PCR, multiplex PCR, and real-time PCR have further refined the diagnostic capabilities for malaria. These techniques not only enhance sensitivity and specificity but also allow for simultaneous detection of multiple *Plasmodium* species, differentiation of drug-resistant strains, and identification of genetic markers associated with disease severity [9].

Touchdown (TD) PCR is a variant of the PCR, which involves a stepwise decrease in the annealing temperature during the initial cycles of amplification. The primer sequences used in touchdown PCR typically consist of a higher melting temperature (Tm) region at the 3' ends followed by a lower Tm region towards the 5' ends. The function of TD PCR is to enhance specificity by promoting annealing of the primers to the target DNA template. The theory behind this method is that the initial higher annealing temperature favors binding of the primers to template sequence with a high complementarity, while reducing non-specific annealing to off-target sites. As the cycles progress, the annealing temperature is gradually lowered, allowing the primers to anneal to sequences with lower complementarity. This temperature gradient helps to minimize the formation of non-specific amplification products, leading to increased specificity and efficiency of the PCR reaction [9-10]. The current PCR primers utilized for the detection of *Plasmodium vivax* exhibit certain limitations that warrant consideration. While PCR has proven to be a highly sensitive and specific method for detecting this parasitic infection, the existing primer design may lead to reduced accuracy in certain scenarios. The primer's sequence specificity may result in false negatives due to genetic variations or mutations within the target DNA region of different *Plasmodium vivax* strains. Additionally, the primer's binding affinity may be influenced by variations in the parasite's genome, potentially compromising the test's reliability across diverse geographical regions [11]. There are significant limitations in PCR-based *Plasmodium vivax* detection assays by using standard primers, including dimer formation and cross reactivity with other *Plasmodium* species [5, 7]. Using standard primers, *Plasmodium vivax* will be detected at 121 base pairs, making it difficult to distinguish from dimers. To address these limitations, an improved primer design informed by comprehensive genomic analyses and broader sequence coverage could enhance the accuracy and applicability of PCR-based *Plasmodium vivax* detection methods.

This study focused on the comparison of current regular nest 2-PCR assay with the touchdown nest 2-PCR assay by using two sets of different primers that could be potentially employed in PCR detection of *Plasmodium vivax*. The results of this study could be potentially used to develop PCR assays and primers for *Plasmodium vivax* detection.

Materials and methods

Table 1. The primer sequences of the standard and Pv-Rv primers [12].

Identification	Primer	Sequence 5' to 3'
Nest 1: Genus-specific *Plasmodium*	rPlu1	TCAAAGATTAAGCCATGCAAGTGA
	rPlu5	CCTGTTGTTGCCTTAAACTTC
Nest 2: Species-specific *Plasmodium vivax*	rViv1 (standard primer)	CGCTTCTAGCTTAATCCACATAACTGATAC
	rViv2 (standard primer)	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA
	fPv-Rv (new primer)	TCGGCTTGGAAGTCCTTGTT
	rPv-Rv (new primer)	TGCCCCCAAGCTACTCCTAT

Genomic DNA isolation and purification

Two clinical confirmed *P. vivax* positive patients were included in this study. The whole process of this study was approved by the Ethics Commission of the Faculty of Medicine, Universitas Brawijaya (Approval certification number 08/EC/KEPK/01/2020). The genomic DNA (gDNA) was isolated from 200 µL of whole blood from each patient by using Purelink Genomic DNA kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. The concentration of gDNA in 25 µL of elution buffer was determined by using BioDrop (Biochrom, Cambridge, United Kingdom).

Nested polymerase chain reaction (PCR) assays

The nested PCR consisted of two stages as genus-specific amplification reaction (nest 1) followed by species-specific amplification reaction (nest 2). Briefly, genus-specific oligonucleotide primers rPlu1 and rPlu5 would be applied to amplify 1.6 - 1.7 kb fragment of ssrRNA gene of any *Plasmodium* parasite in nest 1 step. And then, the PCR products of nest 1 were used as the template for regular nest 2-PCR and touchdown nest 2-PCR reactions. The human malaria species-specific primer sequences were obtained from the ssrRNA *Plasmodium vivax* A-type gene (accession no. U03079). The primer sequences for the standard primers and the Pv-Rv primers were shown in Table 1. All oligonucleotide primers were obtained from Integrated DNA Technologies (IDT) (Coralville, Iowa, USA) [12]. The PCR assays were carried out by using GoTaq® Colorless Master Mix (Promega, Madison, WI, USA) and Bio-Rad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) following the manufacturers' instructions.

(1) Nest 1-PCR assay

The total volume of a PCR reaction mixture was 25 µL including 1 µL of 20 ng/µL gDNA template, 12.5 µL of GoTaq master mix, 1 µL of each 10 µM genus-specific primers (rPlu1 and rPlu5), and 9.5 µL of nuclease free water. The PCR reaction program was set as 95°C for 5 minutes followed by 30 cycles of 94°C for 1 minute, 58°C for 2 minutes, 72°C for 2 minutes. A final extension at 72°C was added for 5 minutes.

(2) Nest 2-PCR assay

1 µL of nest 1-PCR product was mixed with 12.5 µL of GoTaq master mix, 1 µL of each 10 µM species-specific primers, and 9.5 µL of nuclease free water. Both *Plasmodium vivax* species-specific primers were tested with the reaction program of 95°C for 5 minutes followed by 35 cycles of 94°C for 1 minute, 60°C for 2 minutes, 72°C for 2 minutes. An additional 5 minutes was applied for final extension at 72°C. The expected sizes of PCR products were 121 bp for standard primers and 170 bp for Pv-Rv primers, respectively.

(3) Touchdown (TD) nested -PCR assay

The PCR reaction mixture for touchdown PCR assay was the same as regular nested PCR assays. The PCR programs in two phases of touchdown PCR assay were as follows. In phase 1, initial denaturation 97°C for 1 minute and 95°C for 3 minutes followed by 10 cycles of 95°C for 30 seconds, 70°C of annealing temperature with a gradual decrease in temperature (- 1°C/cycle) for 45 seconds, and 72°C for 1 minute. In phase 2, 25

cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 1 minute, and then 72°C for 5 minutes. The TD PCR program in phase 2 was a generic amplification by using the first annealing temperature of phase 1.

All the PCR products were checked by using 3% agarose gel electrophoresis for fragment bands, dimers, and background noise, and then quantified by using GelDoc Imaging System (Bio-Rad, Hercules, CA, USA) and ImageJ software (https://imagej.nih.gov/ij/) [8, 13] for the intensity of the formed bands and background intensity. The intensity ratio of the fragment band and background was assessed and compared. All experiments in this study were repeated at least three times.

Statistical Analysis

SPSS 26.0 (IBM, Armonk, NY, USA) was employed in this study. The normality of the data distribution was tested by using the Shapiro-Wilk method, while the homogeneity was tested by using the Levene statistic. The relationship between the two variables was evaluated by using a one-way ANOVA followed by a paired t-test. The *P* value less than 0.05 was defined as significant difference, while *P* value less than 0.01 was defined as very significant difference.

Results

The PCR assays using standard primers

Two sets of different primers were used to compare the effects of TD PCR product formation. The results of both regular nest 2-PCR and touchdown nest 2-PCR using standard primers were shown in Figure 1. The resulting bands were analyzed by using ImageJ and paired T-test and showed that there was no significant difference in the ratio of band intensity to background intensity between regular nest 2-PCR and touchdown nest 2-PCR ($P > 0.05$). However, when the background intensities were compared between regular nest 2-PCR and touchdown nest 2-PCR statistically, a significant difference was observed ($P < 0.05$) (Table 2).

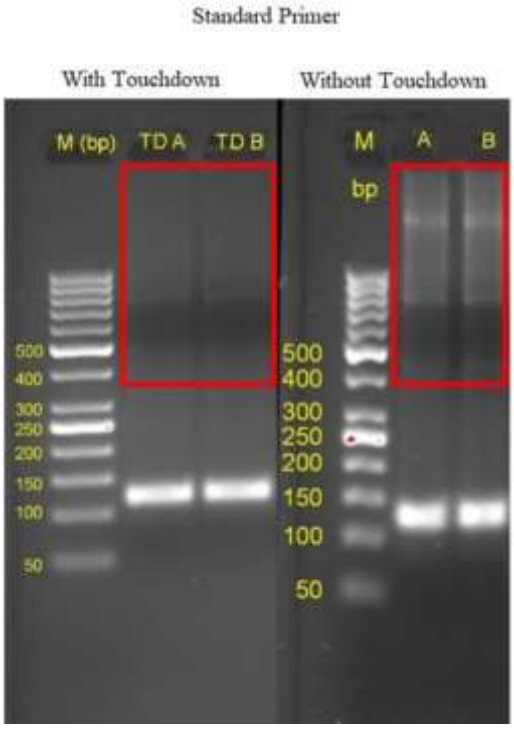

Figure 1. PCR products of both standard PCR (right panel) and touchdown PCR (left panel) using standard primers. The same DNA template was used in both PCR assays. Lanes TD A, TD B, A, and B were samples. Lane M was a DNA marker. The red box indicated the background intensity.

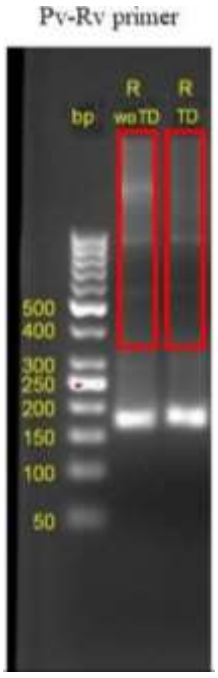

Figure 2. PCR products of both standard (R wo TD) and touchdown PCR (R TD) assays using Pv-Rv primers. The red box indicated the background intensity.

Table 2. Mean intensity of band formed with the use of standard primers under both standard PCR and touchdown PCR assays.

Standard primers						
PCR assay	Mean intensity					
	Product band	Background	*P* value	Difference between band and background	Ratio of band and background	*P* value
Standard	239.11	152.33	0.03	86.78	1.57	0.11
Touchdown	225.27	142.70		82.57	1.58	

Table 3. Mean intensity of band formed with the use of Pv-Rv primers under both standard PCR and touchdown PCR assays.

Pv-Rv primers						
PCR assay	Mean intensity					
	Product band	Background	*P* value	Difference between band and background	Ratio of band and background	*P* value
Standard	204.81	150.63	0.01	150.62	1.36	0.02
Touchdown	210.52	153.44		153.44	1.37	

The PCR assays using new primers (Pv-Rv)

The Pv-Rv primers were also applied in both regular and TD nested PCR assays (Figure 2). The results demonstrated a significant difference between the intensities of the product bands as well as a significant difference between the ratio of band intensity and background intensity in touchdown PCR product ($P < 0.05$). A very significant difference was also observed between the background intensities of both assays with Pv-Rv primers ($P < 0.01$) (Table 3).

Discussion

The current primers deemed as the standard to be used to detect *P. vivax* with the use of PCR were designed by using the sequences within the highly conserved section of the small subunit (SSU) rRNA of *Plasmodium vivax* [12, 14]. The results of this study showed that there was no significant difference in the ratios of PCR product band intensity and the background intensity between the touchdown PCR or standard PCR by using the standard primers for *P. vivax* detection. Such result suggested that the current PCR assay with the standard primer has been optimized and standardized to allow the most optimal results in detecting *Plasmodium vivax*. However, in terms of the background intensity, there was significant differences in both product intensity values and product-background intensity ratios between the groups using different PCR protocols, which confirmed that the application of touchdown PCR had an effect in reducing the background intensity, and hence reducing background noise when using both the standard and Pv-Rv primers.

The nested PCR method for *Plasmodium* detection was applied in this study because it was challenging to detect the *Plasmodium* species directly from the large size whole human gDNA, leading the primers tended to bind in unspecific target sequence. There are different primers aside from the current standard primers that are being developed to further increase the quality of PCR products and reduce cross reactions, especially in multiplex PCRs [13]. In this study, the Pv-Rv primers were developed by our research group, which targeted *Plasmodium vivax* small subunit rRNA gene (SSU rRNA) (accession no. U03079.1).

A different result was obtained when the TD PCR protocol was applied with the Pv-Rv primers, which showed that there was an increase in the product band intensity as well as a reduction in the background intensity resulting in a large ratio

of intensities compared to the current standard PCR protocol with Pv-Rv primers. The result demonstrated that there was still room for optimization of the Pv-Rv primer PCR protocol in the detection of *P. vivax* in blood samples. The increase in product band intensity as well as decrease in background intensity in theory was due to the higher annealing point used in the protocol to provide a much better formed amplicon which, as the assay runs was sufficient to prevent the formation of dimers and false priming [10, 15]. Ideally, the total number of cycles in both phases 1 and 2 should not exceed 30 to 35 cycles, as additional cycles beyond this risked the creation of nonspecific products and/or primer dimers. Positive and negative control reactions were set up by using plasmid template containing the sequence of interest as a positive control and a reaction lacking the template as a negative control [5-7]. Further studies are needed to optimize and integrate the use of TD PCR protocol with Pv-Rv primers and explore an optimal state to detect *Plasmodium vivax* in blood samples.

TD PCR also has applicability when the degree of complementarity between primers and templates is uncertain, especially when degenerated primers are used in reverse transcriptase-dependent PCR and appears particularly robust when being used in single nucleotide polymorphism typing. This method also has the potential to largely overcome the problems associated with high annealing temperatures required for some primer-template combinations and is particularly useful to those difficult to amplify templates, such as those with extensive secondary structures and high % of G+C islands in genomes and targets from organisms with 46% G + C content. Phase 2 procedures of the TD PCR program is a generic amplification stage with 20 to 25 cycles by using the first annealing temperature in phase 1. Ideally, the total number of cycles in both phases 1 and 2 should not exceed 30 to 35 cycles as additional cycles may cause the creation of primer nonspecific binding and/or dimers [5-7, 10, 12].

Conclusion

The results of this investigation revealed that touchdown nest 2-PCR outperformed regular nest 2-PCR and recommended using touchdown nest 2-PCR with a newly developed primers (Pv-Rv primers) to produce a high-quality product band with little background noise. *Plasmodium vivax* will be recognized as the presence of 170 bp size product band by using the novel primers, making it easier to distinguish from dimers. Further studies are required to optimize Pv-Rv primer PCR protocols.

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