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Original Research Article

## Molecular Detection Challenges of Human *Plasmodium knowlesi* infection by Polymerase Chain Reaction

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### Article Info

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### Abstract

**Background:** *Plasmodium knowlesi* is the fifth *Plasmodium sp.* causing malaria in humans. There were 545 *P. knowlesi* malaria cases reported in Indonesia in 2010-2021 period. The first case was reported from South Kalimantan, and more cases were reported in Sumatra and Kalimantan Island. The morphology of *P. knowlesi* is difficult to distinguish from other *Plasmodium* species, especially with *P. falciparum* and *P. malariae*. Therefore, molecular identification is still the most promising method for diagnosing *P. knowlesi* infection.

**Objective:** This study aimed to analyze the molecular detection method of human *P. knowlesi* infection using Polymerase Chain Reaction (PCR) and sequencing techniques.

**Methods:** DNA was isolated from malaria blood samples. *P. knowlesi* detection was conducted by nested PCR using primer rPLU1 and rPLU5 for nested 1 and Kn1f and Kn3r for nested 2. The PCR products were directly sequenced. The sequences were analysed using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI).

**Results:** Blood samples from ten malaria patients from Maluku province were collected after informed consent. The *P. knowlesi*-specific PCR amplification resulted in a band of approximately 420 bp in all samples. Sequence analysis showed the highest similarity (89-92 %) with many global *P. falciparum* strains. However, BLAST analysis for part of sequences also showed high similarities with several *P. knowlesi* H strains 18s rRNA from Peninsular Malaysia. Primer analysis using BLAST demonstrated the specificity of Kn3r-nested 2 primer, however, Kn1f primer showed a cross-reactive with other *Plasmodium sp.*, including *P. falciparum* and *P. vivax*.

**Conclusion:** Molecular detection of *P. knowlesi* infection is challenging. A new target gene for primer design and detection method with higher specificity for human *P. knowlesi* examination is needed to develop.

**Keywords:** Malaria, PCR, *Plasmodium knowlesi*, Primer.

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### INTRODUCTION

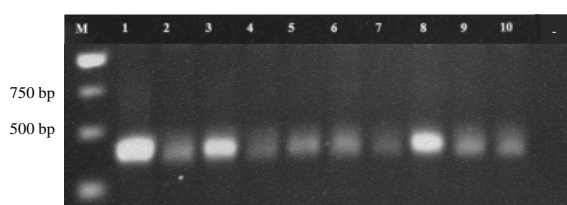
Malaria is an infectious disease caused by *Plasmodium sp.* and transmitted by *Anopheles* mosquito. In 2020, the World Health Organization (WHO) reported 241 million malaria cases with 627,000 deaths.<sup>1</sup> The

Indonesia Ministry of Health reported 254,05 thousand malaria cases in 2020.<sup>2</sup>

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**Table 1.** Patients Characteristics, clinical symptoms, and microscopic examination

Patient	Sex	Age	Clinical symptom	Microscopic examination
P1	M	23	fever, chills, headache	<i>P. falciparum</i>
P2	F	9	fever, chills, headache	<i>P. falciparum</i>
P3	M	15	fever, chills, headache	<i>P. falciparum</i>
P4	M	40	fever, chills, headache	<i>P. falciparum</i>
P5	M	16	fever, chills, headache	<i>P. falciparum</i>
P6	M	33	fever, chills, headache	<i>P. falciparum</i>
P7	M	48	fever, chills, headache	<i>P. falciparum</i>
P8	M	17	fever, chills, headache	<i>P. falciparum</i>
P9	F	32	fever, chills, headache	<i>P. falciparum</i>
P10	F	27	fever, chills, headache	<i>P. falciparum</i>



**Figure 1.** Visualization of the PCR in 1% agarose gel electrophoresis resulted in a single band of  $\pm 420$  bp. M: 1 kb DNA ladder; 1-10: PCR result from ten samples; -: negative control.

Twenty types of *Plasmodium* infect primates, and five of them can cause malaria in humans, including *Plasmodium knowlesi*. *P. knowlesi* is a type of *Plasmodium* that naturally infects long-tailed macaques (*Macaca fascicularis*) and pig-tailed macaques (*Macaca nemestrina*), whose transmission is mediated by the *Anopheles leucosphyrus* group mosquito.<sup>3,4</sup> In Indonesia, natural hosts of *P. knowlesi* are dispersed on the islands of Sumatra, Kalimantan, Java, Bali, Lingga Islands, Riau Islands, Bangka, Belitung, Banyak, Tambelan Islands, Natuna Islands, Simalur, Nias, Matasari, Bawean, Maratua, Timor, Lombok, Sumba and Sumbawa.<sup>5,6</sup> In addition, the vectors are spread on the islands of Sumatra, Java, Bali, Kalimantan, Sulawesi, and Maluku, along with several other small islands in Indonesia.<sup>3,7</sup>

Several Asian countries has reported human *P. knowlesi* infection. The first large cases of human *P. knowlesi* was reported from Sabah, Malaysia in 2004, followed by other countries such as Cambodia, Indonesia, Myanmar, the Philippines, Thailand, Vietnam, Singapore, and Brunei.<sup>8</sup> The first case of *P. knowlesi* infection in Indonesia was found in 2010 in Kalimantan. The case was found in gold miners in the tropical forests of South Kalimantan.<sup>9</sup> The following case of *P. knowlesi* infection occurred in an Australian citizen who had a history of working in the forests of South Kalimantan for 18 months, and subsequently suffered from high fever and other malaria symptoms emerged after returning to his home country.<sup>10</sup> And several cases of human *P. knowlesi* infection were

reported from the western part of Indonesia, especially from Sumatra and Kalimantan island.<sup>7</sup>

Peripheral blood smear examination is one of the standard diagnostic examinations for malaria. However, *P. knowlesi* and other *Plasmodium* species are difficult to differentiate since their morphology is similar to *P. falciparum* and *P. malariae*. In the early stages of its life cycle, the trophozoites of *P. knowlesi* resemble the trophozoite form *P. falciparum*. In contrast, the later stages of *P. knowlesi*, including the trophozoites, schizonts, and gametocytes, are more similar to *P. malariae*.<sup>11,12</sup> Rapid Diagnostic Test (RDT) has shown low sensitivity and cannot be used to confirm the cases of *P. knowlesi* infection.<sup>13,14</sup> Previous studies stated that the nested Polymerase Chain Reaction (PCR) method has the highest sensitivity and specificity compared to other known methods.<sup>11,12</sup> Therefore, molecular identification in PCR is still the most suitable and applicable technique for diagnosing *P. knowlesi* infection.<sup>11,13,15</sup> This study aimed to analyze the molecular detection method of human *P. knowlesi* infection using PCR and sequencing techniques and the extent of human *P. knowlesi* infection in Indonesia.

## MATERIALS AND METHODS

### Study Design

The study was an explorative laboratory study and conducted by serial sample collection followed by laboratory research. Samples were collected from the blood of malaria patients from Tiakur Health Center, South-West Maluku Regency, Maluku Province, Indonesia. Blood was dropped on a Whatman filter paper, dried at room temperature (RT), and stored at -20 °C until it was used.

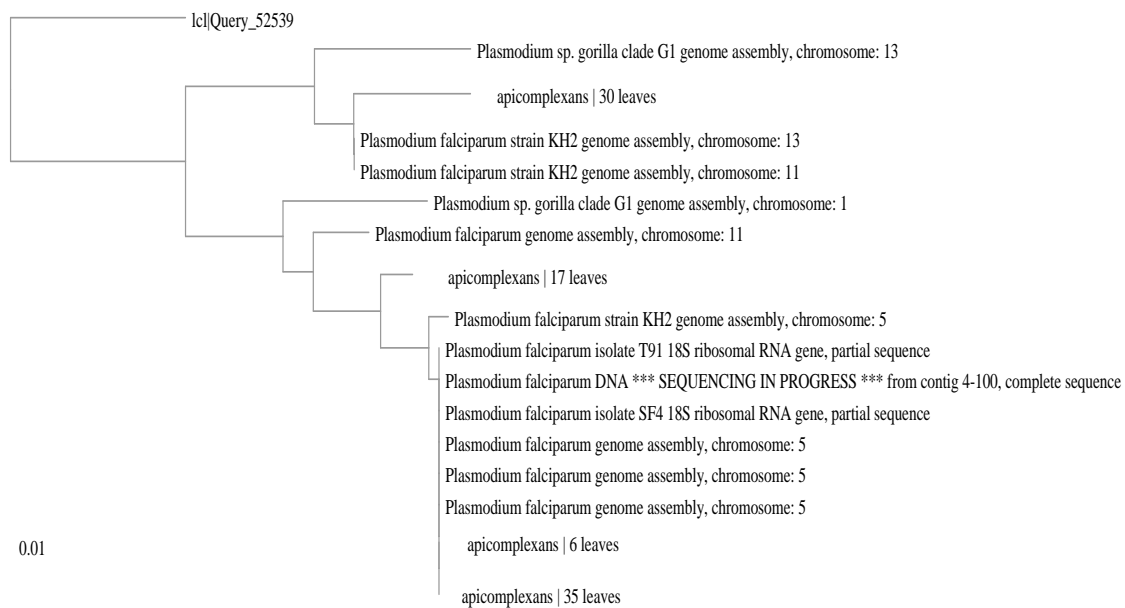
Patients were recruited based on inclusion criteria, i.e., patients with complaints of headache, fever, chills, nausea, vomiting, and other symptoms of malaria, show *Plasmodium sp.* infection on microscopic examination, and do not take malaria treatment for the last month.

### Ethical Approval

The study has been approved by the Ethical Committee of the Faculty of Medicine University of Jember No. 1596/H25.1.11/KE/2022.

Sequences producing significant alignments		Download	Select columns	Show	100	?			
<input checked="" type="checkbox"/> select all 100 sequences selected		GenBank	Graphics	Distance tree of results	MSA Viewer				
	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1374611	<a href="#">LR131487.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1378381	<a href="#">LR131485.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 6</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	2531954	<a href="#">LR131471.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 4</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1384506	<a href="#">LR131469.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1363299	<a href="#">LR131456.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1408578	<a href="#">LR131454.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1418881	<a href="#">LR131440.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1406692	<a href="#">LR131438.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum strain KH1 genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1340112	<a href="#">LR131424.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum strain KH1 genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1432942	<a href="#">LR131422.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum strain GB4 genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1481909	<a href="#">LR131408.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum strain GB4 genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1401438	<a href="#">LR131406.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 7</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1369659	<a href="#">LR131392.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1377278	<a href="#">LR131390.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1405381	<a href="#">LR131374.1</a>
<input checked="" type="checkbox"/>	<a href="#">Plasmodium falciparum genome assembly, chromosome: 5</a>	<a href="#">Plasmodium falciparum</a>	490	490	90%	7e-134	93.39%	1373600	<a href="#">LR131358.1</a>

**Figure 2.** BLAST analysis result. The sequences match with several *P. falciparum* sequences in the database.



**Figure 3.** The cladogram of the sequence. The sequence has a proximity with several *P. falciparum* sequences and *Plasmodium sp. gorilla* clade.

### DNA Isolation and PCR Amplification

The DNA isolation was carried out according to the mini QIAamp DNA kit protocol (Qiagen, Hilden, Germany). Five pieces of 3 mm-filter paper containing the patient's blood were added with the 180 µl buffer and incubated at 85°C for 10 mins. Then proteinase-K was added and incubated at 56°C for 1 h. The sample was washed twice with buffer and centrifuged before the DNA was extracted using a spin column.

The isolated DNA was amplified using MyTaq HS Red Mix (Bioline, London, UK) with nested PCR. The nested 1 PCR used genus-specific primers, i.e., rPLU1 (TCA

AAG ATT AAG CCA TGC AAG TGA) and rPLU5 (CCT GTT GTT GCC TTA AAC TTC), with a total volume of 50 µl and was performed with the following settings: initial denaturation at 94°C for 4 mins, followed by 35 cycles of 94°C for 30 secs, 55°C for 1 min, and 72°C for 1 min, and finalized by 72°C for 4 mins. The nested 2 PCR were carried out using species-specific primers, i.e., Kn1f (CTC AAC ACG GGA AAA CTC ACT AGT TTA) and Kn3r (GTA TTA GGT ACA AGG TAG CAG TAT GC), in a total volume of 20 µl with the similar setting with the nested 1, except for annealing

temperature of 62°C. Nested 2 PCR products were analyzed with agarose gel electrophoresis.

#### Direct Sequencing and Sequence Analysis

The PCR products were directly sequenced in both directions using BigDye® Terminator v3.1 cycle sequencing kit and ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems). The nucleotide sequences were analyzed using the Nucleotide Basic Local Alignment Search Tool (BLAST) tool on the National Center for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to perceive the nucleotide sequence's identity.

## RESULTS

Ten malaria patients were recruited after written informed consent. The patients were seven males and three females. The patients' characteristics are described in Table 1.

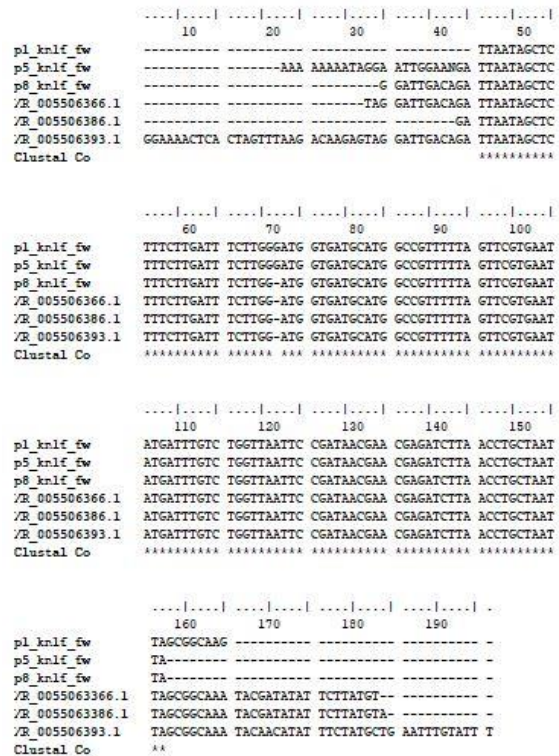
DNA isolation from all samples yielded 1.7-50 ng/μl. Nested PCR amplification resulted in a single band of approximately 420 bp in all samples. The negative control showed no band. Unfortunately, there was no positive control due to difficulty finding a positive sample (Figure 1).

Direct sequencing of PCR product read for approximately 402 nucleotides. The sequence will be deposited at GenBank. BLAST analysis demonstrated that the sequences matched for *P. falciparum* sequences (LR131487.1, LR131485.1, LR131471.1, LR131469.1, LR131456.1), as shown in Figure 2. It has 90% sequence coverage and 93.39% similarity with *P. falciparum* sequences. Sequence analysis using the Neighbour-joining method to design a cladogram demonstrated its proximity to several *P. falciparum* sequences and *Plasmodium sp.* gorilla clade, as presented in Figure 3.

Further analysis using BLAST and multiple alignments was conducted to investigate the relation of the sequences with *P. knowlesi* sequences. The 119 nucleotide upstream sequences from samples (P1\_Kn1f\_fw, P5\_Kn1f\_fw, P8\_Kn1f\_fw) were aligned with *P. knowlesi* strain H (XR\_005506393.1; XR\_005506386.1; XR\_005506366.1), as shown in Figure 4. However, BLAST analysis for those sequences showed 99.16% similarities with many *Plasmodium* species, such as *P. knowlesi*, *P. delichoni*, *P. gonderi*, *P. sp. gorilla clade*, *P. inui*, *P. sp.—DRC-Itaito*, *P. vivax*, *P. gaboni*, *P. cynomolgi*, and *P. coatneyi* (supplementary 1).

## DISCUSSION

The study isolated DNA from the patient's blood filter paper and yielded DNA in a concentration of 1.7-50 ng/μl. The use of blood filter paper has proven to be effective for DNA isolation to diagnose infection and determine molecular epidemiology.<sup>4,11,16,17</sup> Detecting malaria parasite DNA extracted from archival slides is relatively specific, though the sensitivity varies and does not depend on parasitemia. Previous epidemiological studies reported similar findings of the nested PCR method for malaria using DNA extracted from filter paper.<sup>11,16</sup>



**Figure 4.** Multiple alignments of 119 upstream sequences from samples (P1\_Kn1f\_fw, P5\_Kn1f\_fw, P8\_Kn1f\_fw) and several *P. knowlesi* strain H (XR\_005506393.1; XR\_005506386.1; XR\_005506366.1).

Microscopic examination as the gold standard for malaria diagnosis was applied in this study. The microscopic examination of patients in this study showed *P. falciparum* in all samples. Further determination by nested PCR to detect the possibility of *P. knowlesi* infection using *P. knowlesi*-specific primer, i.e., Kn1f and Kn3r yielded a single band of approximately 420 bp in all samples (Figure 1). The observed-single band of 420 bp in all samples except the negative control led to baffling questions since the reference reported a 290 bp as the *P. knowlesi* amplicon.<sup>14,18</sup> Moreover, direct sequencing of PCR product confirmed *P. falciparum* infection for all samples. Further analysis by designing a cladogram validates the *P. falciparum* sequence and its similarities (Figures 2 and 3).

The study result corroborates the previous studies on the difficulties of *P. knowlesi* detection.<sup>9,19,20</sup> PCR amplification followed by sequencing confirmed the *P. falciparum* infection as the result of microscopic examination. Another study reported mixed infection of *P. vivax* and *P. falciparum* or a single infection of *P. falciparum* in microscopic examination of thick and thin blood smears, but positive for *P. knowlesi* infection upon a molecular examination.<sup>21</sup> Therefore, suspected cases of *P. knowlesi* infection require a molecular examination to confirm the diagnosis.

Primer pair rPLU1 and rPLU5 for nested 1 are genus-specific primers, and Kn1f and Kn3r are species-specific primers designed based on the small subunit rRNA genes. BLAST analysis for Kn1f demonstrated that the ten most similar sequences with 100% coverage and 100% identity are *P. falciparum* (MN852862.1), *P. knowlesi* strain H

(LR701173.1), *P. falciparum* isolate Jr-01 (CP101625.1) dan *P. vivax* isolate P023VOO34 (OM033598.1). Meanwhile, Kn3r showed 96% coverage and 100% identity with all *P. knowlesi* clones (KY404060.1, MN535379.1), indicating its specificity. This result is similar to previous primer designs for *P. knowlesi* detection, such as Pmk8 and Pmkr9, with Pmk8 being highly specific while Pmkr9 cross-reacts with *P. vivax* S-type strain and other *Plasmodium* species, especially those related to *P. vivax*.<sup>9</sup> Another method using 5-plex malaria immunoassay containing pan-*Plasmodium* lactate dehydrogenase (pLDH), *P. falciparum*-LDH, *P. vivax*-LDH, *P. falciparum*-histidine-rich protein-2 (HRP2), and C-reactive protein showed cross-reactivity of *P. knowlesi* and *P. vivax*.<sup>22</sup> Furthermore, a new detection method using qPCR assay based on 18S rRNA gene with fluorescence-labeled probing showed high sensitivity compared to the conventional PCR method. However, the study was conducted in a malaria-endemic area, and further validation is needed for field settings, especially for mass screening and surveillance programs.<sup>23</sup>

The study also investigated the evolutionary relationship of *Plasmodium* species due to the high cross-reactivity of the sequences, especially between *P. knowlesi* and *P. vivax*. It found that the 119 upstream sequences have 99.16% similarities with many *Plasmodium* species. This result is similar to the previous studies.<sup>18,24</sup> This result supported our finding on the potential cross-reactivity of Kn1f primer with many *Plasmodium* species due to its similarity.

Molecular detection for *P. knowlesi* infection is still challenging. Several targets for detection, such as 18S ribosomal RNA and cytochrome-b (cytb gene), were found to be less specific and need sequencing for definite confirmation. Therefore, it is crucial for developing sensitive and specific *P. knowlesi* detection using new target genes and detection methods.

## CONCLUSION

This study highlights the challenges in detecting *P. knowlesi* at the molecular level. Previous molecular targets were found to have limited specificity, indicating a need for new target genes and detection methods. Developing such methods is crucial for preventing potentially severe cases of human malaria caused by *P. knowlesi* and designing effective control strategies.

## ACKNOWLEDGMENTS

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