Misidentification of *Plasmodium* Species by Cross-Reacting Primers and Cerebral Malaria Caused by *Plasmodium vivax*

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Abstract

Introduction The clinical presentation of a case as cerebral malaria with molecular identification confirming it as *Plasmodium vivax* underlines the importance of using molecular tools to identify the species and type of malaria. The possibility of the relationship between the complication observed during clinical diagnosis and the multifactorial molecular changes could likely be the reason for terming it cerebral malaria.

Methods We report four cases analyzed using the quantitative buffy coat technique followed by classical Giemsa stained thick-film microscopy, and nested polymerase chain reaction for the genus-specific region of *Plasmodium* targeting 18S rDNA followed by species-specific identification with a different set of primers and products confirmation with sequencing.

Results Primers targeting *P. knowlesi* generated the expected product size of 153 base pairs that, upon sequencing, matched with the *P. vivax* sequence reflecting the relatedness of the species. Likewise, primers targeting *P. ovale* generated a 456 product whose sequence matched the *P. vivax* sequence.

Conclusion Infection with *P. vivax* can potentially cause cerebral malaria, and *P. vivax* can cause severe malaria complications alone or mixed with other species and can show cerebral malaria signs, which are typically associated with *P. falciparum* infections. The sequence relatedness reflects the genome similarity between *P. knowlesi* and *P. ovale* with *P. vivax*. The need to reconfirm with an additional set of newly reported primers is mandatory.

Keywords
- *Plasmodium knowlesi*
- *P. falciparum*
- TFM
- QBC
- PCR

Introduction

Plasmodium vivax also referred to as "benign tertian malaria" in clinical practice, typically leads to a benign course with few complications.[1] The World Health Organization (WHO), in its 2021 World Malaria survey, registered 241 million malaria cases worldwide in 2020, resulting in the death of

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627,000 individuals, with 85% of them recorded from 20 countries and India being the only non-African country among them. Furthermore, 53% of *P. vivax*-related cases have been recorded in the WHO South-East Asia Region, with a high of 47% in India alone and about 82% of all malaria deaths.[2] Mangaluru, a city located in the state of Karnataka on the Southwest coast of India, is known for its rivers and tropical climate and is hypoendemic and seasonal to malaria. With the growing industrialization and a vast floating population of migrant workers at various construction sites, the city is prone to vector-borne diseases like malaria, mainly *P. falciparum* and *P. vivax*.[3] The monsoon season between late May and October coincides with a sharp increase in malaria cases, and the stagnant rainwater provides an ideal breeding ground for mosquitoes.[4] More than 50% of the cases reported in Karnataka are from Mangalore alone, [5] with the majority being positive for *P. vivax,* followed by *P. falciparum*. Apart from these species, *P. malariae*, *P. ovale*, and *P. knowlesi*, which are also responsible for malaria, are rarely recorded. *P. falciparum* is known to cause 90% of the world's malarial deaths,[6] with 50% of the cases in the South-East Asian region (SEARO of WHO). To date, *P. knowlesi* has only been recorded in India from the Andaman and Nicobar Islands.[7] *P. vivax* is regarded as less severe than *P. falciparum* malaria. Recent research and case reports, on the other hand, have conclusively proven that *P. vivax* infection may induce all of the complications associated with severe *P. falciparum* malaria.[8] More recently, reports on multiple infections, drug-resistant parasites, haplotypes within the species or subspecies and newer species of *Plasmodium* are being recorded. Against this background, molecular techniques become essential to dispel the ambiguity in reporting. Polymerase chain reaction (PCR) for detection followed by gene sequencing can offer the much-needed platform for diagnosis and epidemiology, help administer appropriate drugs, and prevent the spread of drug resistance. Febrile patients, positive for malaria upon microscopic, quantitative buffy coat (QBC) and other rapid diagnostic tests, are treated empirically with antimalarial medications without confirmed detection of species diagnosis. The test relies largely on microscopic analysis of blood smears,[9] which can present ambiguous results. Individuals with a low level of parasitemia, as in mild/early infection, can be missed due to difficulty in diagnosis by thick-film microscopy (TFM), QBC or rapid diagnostic test. However, DNA-based molecular test such as PCR facilitates the accurate identification of infection of low density, the species involved, and, more importantly, mixed infections that may be missed by TFM.[10] Infection with *P. falciparum* is most frequently associated with cerebral malaria (CM), leading to renal dysfunction, respiratory distress, and bleeding abnormalities. Also, of concern is the observation that clinical presentation as CM due to *P. vivax* has recently been reported.[11,12] Further, some oligonucleotide primers designed previously to amplify the regions in 18S specific to a particular *Plasmodium* species show cross-reaction by PCR, raising concerns about the diagnosis on species identification made hitherto. The PCR primers reported and commonly used to detect *P. knowlesi* infections in humans cross-reacted stochastically with *P. vivax* genomic DNA.[13] In this study, we report a case of severe malaria due to *P. vivax* that was characteristic in its clinical presentation and diagnosed as CM. Additionally, three cases that gave ambiguous results by PCR on the involvement of multiple *Plasmodium* species when retested with a different set of newly reported primers confirmed false positive reports of *P. knowlesi* and *P. ovale* due to overlapping of the primer binding regions with *P. vivax*. The sequencing of the PCR products confirmed them to be *P. vivax.*

Materials and Methods

Blood samples from four patients collected during 2017-2019 were confirmed as malaria according to symptoms outlined by National Vector Borne Disease Control Programme (NVBDCP), which included sweating, chills, shock, jaundice, convulsions, pulmonary oedema, and splenomegaly. Consent from these patients who had attended Justice K.S Hegde Charitable Hospital in Mangalore was obtained per the ethical guidelines. The institutional Ethical committee clearance was obtained vide NU/CEC/ICMR-05/2015, NU/CEC/2018/0183.

Hematological Analysis

Thick and thin blood films were prepared and stained for 10 minutes with 10% Giemsa staining solution (pH 7.2) and microscopically examined using the oil immersion objective (100X). At least 200 fields were examined before reporting a sample as negative/no malaria parasites seen. The number of parasites in thick film smears was scored using the plus system scale to indicate the parasite density in the positive cases: + (1–10 parasites per 100 fields); ++ (11–100 parasites per 100 fields); +++ (1–10 parasites per field); ++++ (>10 parasites per field). Using these scores, the parasite density was estimated in the blood samples: ± 4 to 40 parasites/µL; ++> 40 to 400 parasites/µL; +++> 400 to 4,000 parasites/µL; ++++> 4,000 parasites/µL, 50 fields were examined under oil immersion objective, and the morphology and numbers of microorganisms and cells per field were recorded.[14,15] Blood stratification and its components post-centrifugation were used in QBC for parasite detection in the blood for the presence of malarial parasites as follows. Two milliliter of venous blood was collected in a sterile heparinized tube and used for QBC, followed by fluorescent microscopic observation to identify erythrocyte clusters in layers of cells that had the parasite.[16]

Polymerase Chain Reaction

For PCR amplification, a modification of the method previously reported was employed.[17] The speciation of *Plasmodium* could be confirmed using various species-specific primers. DNA was extracted (DNeasy Blood and Tissue Kit, QIAGEN, Germany) from 200 µL of heparinized blood samples collected from each individual. To improve the sensitivity of specific oligonucleotide primer binding, the nested polymerase chain reaction (nPCR) amplifications were

Table 1 Oligonucleotide primers used in the study

Primer name	Primer sequence (5′ to 3′)	Target genus/species	Amplicon size (bp)	Annealing temperature	References
rPLU1_F	TCAAAGATTAAGCCATGCAAGTGA	*Plasmodium* genus	~1,670	54°C	[18]
rPLU5_R	CCTGTTGTTGCCTTAAACTTC				
rPLU3_F	TTTTTATAAGGATAACTACGGAAAAGCTGT	*Plasmodium* genus	240	52°C	[18]
rPLU4_R	TACCCGTCATAGCCATGTTAGGCCAATACC				
rFAL1_F	TTAAACTGGTTTGGGAAAACCAAATATATT	*P. falciparum*	205	60°C	[18]
rFAL2_R	ACACAATGAACTCAATCATGACTACCCGTC				
rVIV1_F	CGCTTCTAGCTTAATCCACATAACTGATAC	*P. vivax*	121	55°C	[18]
rVIV2_R	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA				
rMAL1_F	ATAACATAGTTGTACGTTAAGAATAACCGC	*P. malariae*	145	53.5°C	[18]
rMAL2_R	AAAATTCCCATGCATAAAAAATTATACAAA				
rOVA3_F	CGGGGAAATTTCTTAGATTGC	*P. ovale*	456	50°C	[21]
rOVA4_R	GAGAAACAGCATGAATTGCG				
Pmk8	GTTAGCGAGAGCCACAAAAAAGCGAAT	*P. knowlesi*	153	60°C	[21]
Pmkr9	ACTCAAAGTAACAAAATCTTCCGTA				
rOVA1_F	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	*P. ovale*	787	58°C	[18]
rOVA2_R	GGAAAAGGACACATTAATTGTATCCTAGTG				
PkF1140	GATTCATCTATTAAAAATTTGCTTC	*P. knowlesi*	410	50 °C	[12]
PkR1550	TCTTTTCTCTCCGGAGATTAGAACTC				

performed in a thermocycler (Eppendorf NexusGX2, Germany).[18,19] Primers binding to the conserved region in the 18S rDNA located in the small-subunit were used. These primers were employed to identify the *Plasmodium* genus, and the species identification was by nPCR using the first step product as the template. Each 30 µL reaction mixture includes 2 µL (100 ng/L) DNA template, 0.8 µL (8 8 pM) primers, 3.3 µL 10x buffer, 0.8 µL (110 mM) deoxynucleoside triphosphate, and 0.3µL (1.5 units) Taq DNA polymerase (Himedia Laboratories Pvt. Ltd., India). For quality control, nonmalaria/noninfected blood samples and nontemplate control were used as negative control and blood from malaria cases confirmed by the gold standard microscopy (TFM) and QBC was used as positive control. The reaction mix was initially denaturated at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing temperature according to the primer sets used for 30 seconds, extension at 72°C for (90 seconds for the primer pair rPLU1/rPLU5 and 60 seconds for all other primers), and final delay at 72°C for 10 minutes. For the first step reaction for the genus *Plasmodium,* the primer pair rPLU1/rPLU5 was used; for nested reaction, the primer pair rPLU3/rPLU4 was employed for PCR. Species-specific primers were used on the primary amplification product generated using genus-specific primers. ►**Table 1** presents the different primers employed. rFAL1 and rFAL2 was used for *P. falciparum*; rVIV1 and rVIV2 for *P. vivax*; rOVA1 and rOVA, rOVA 3, and rOVA4 for *P. ovale*; rMAL1 and rMAL2 for *P. malariae*; and pmk8 and pmkr9, Pk1140-Pk1150 for *P. knowlesi.*[13,20]

Sequencing Analysis

The PCR products amplified were extracted and purified using Gel extraction kit (QIAquick Gel Extraction Kit, QIAGEN, Germany); the extracted DNA was quantified by nanodrop (NanoPhotometer, IMPLEN) and both forward and reverse direction of the products were outsourced for Sanger sequencing at Eurofins Genomics India Pvt Ltd., Bangalore, Karnataka, India.

Results and Discussion

In the first case, the clinical diagnosis made by the physician as CM was based on the cardinal signs and symptoms of the condition. However, the laboratory diagnosis was based on TFM, and it was identified as *P. vivax* by observing the trophozoites and gametocyte stages. QBC was used to confirm *Plasmodium vivax* trophozoite and gametocyte. PCR was performed using primers for all plasmodial species to confirm further using the molecular method on the causative parasite. A single band with the product size for *P. vivax* was obtained. Thrombocytopenia and microcytic hypochromic anemia were also observed. It is well-known that CM caused by *P. falciparum* is a severe neurological disease caused by *P. falciparum*, where recovered individuals are prone to epilepsy, cognitive degradation, and other neurological complications and long-term brain impairments. The parasite can enter an asexual form remaining in the blood smears, causing the patient to sometimes even enter into a coma.[22] In this

Table 2 Species confirmation using different primer sets

Cases	Thick-film microscopy (TFM) with a grade of parasitemia	Quantitative buffy coat analysis	Polymerase chain reaction (PCR)	PCR reconfirmation using a different set of primers
Case 1	PVTG (+)	PVTG (CM)	PV	PV
Case 2	PVTG (++) PF (+)	PV & PF	PV +PF + PK + PO	PV + PF
Case 3	PVTG (+++)	PVTG	PV + PO + PK	PV
Case 4	PVTG (++)	PVTG	PV + PO + PK	PV

Abbreviations: CM, cerebral malaria; PF, *Plasmodium falciparum*; PK, *Plasmodium knowlesi*; PO, *Plasmodium ovale*; PV, *Plasmodium vivax*; PVTG, *Plasmodium vivax* trophozoites and gametocytes.

case, however, *P. vivax* was detected and confirmed as the cause of CM. In a previous study by Kochar et al and others[11,23–26] it was found that *P. vivax* was reported as the causative agent of CM and mechanisms, such as sequestration wherein the erythrocytes with trophozoites or schizonts attach to the endothelium of blood vessels, which is commonly associated with *P. falciparum* infection that was observed.[27] Case 2 was recorded as a mixed infection caused by several species. By TFM, trophozoites and gametocytes of *P. vivax* were observed along with *P. falciparum*. The results corroborated the observation by QBC. PCR results were contrary to the above. Test for *P. vivax* (rVIV1 and rVIV2), *P. falciparum* (rFAL1 and rFAL2), *P. ovale* (rOVA 3 and rOVA4), *P. malariae* (rMAL1 and rMAL2), and *P. knowlesi* (pmk8 and pmkr9) generated bands with all the sets of primers used except for *P. malariae*. However, it was concluded that these primers had shown cross-reaction with the primers for *P. knowlesi* and *P. ovale*. These primers were found to bind to the regions similar to the *P. vivax* primer in their binding to the target; hence, bands were generated, leading to false interpretation. To address this misidentification of *Plasmodium* species, a pair of primer sets published recently were used for *P. knowlesi*, Pk1140 and Pk1150 (►**Table 2**).[13] It is significant to note that it yielded no bands for *P. knowlesi*. Similarly, reconfirmation of *P. ovale* using a new set of primers rOVA1 and rOVA2 did not generate any PCR product; it could therefore be concluded that cross-reaction of the species-specific primers in the identification of the *Plasmodium* species was a severe issue in the identification of species. To reconfirm, the primers rPLU1 and rPLU5 were used for primary amplification, followed by nested reaction with secondary primer pair rPLU3 and rPLU4 for genus-specificity. Species-specific bands for *P. vivax* and *P. falciparum* were obtained during reconfirmation, proving that this was a mixed infection by *P. vivax* and *P. falciparum*.

In cases 3 and 4, trophozoites and gametocytes of *P. vivax* were detected by TFM and QBC in the erythrocytes. The infection was of high grade. PCR was performed using species-specific primers. The results showed positive for *P. vivax, P. ovale*, and *P. knowlesi*. On reconfirming PCR using the new primers, it revealed cross-reaction leading to false positive results for *P. ovale* and *P. knowlesi* (►**Table 2**). There was a single band for *P. vivax* in both cases, with the updated primers confirming that the infection was indeed due to *P. vivax*.

Although *P. falciparum* has been exclusively associated with the causative agent of CM, recent studies have shown the involvement of *P. vivax* with the reported complications, as recorded in case 1.[28] *P. vivax* is known to have part of the variant surface antigen known as variant interspersed repeat (*vir*) genes. These *vir* genes are extremely vital in the virulence and pathogenicity of *P. vivax* as it is involved in antigenic variation, which helps the *Plasmodium* parasite evade the host immune cells.[29] Studies have shown that the role of these genes in *P. vivax* and *P. falciparum* in a mixed infection can be deadly if they go undetected and not treated appropriately.[30] In conclusion, it is pertinent to note that the bands generated with the initial set of primers for all the species of *Plasmodium* were due to primer cross-reaction or primer cross-hybridization with shared binding regions leading to the false positive reaction following PCR.[13] This leads to positive bands suggesting a particular *Plasmodium* species when it is merely a mutation of the parasite in the samples, making them available for primer binding. The primer initially published[13,18,31] has been widely used for diagnosis, but in recent times these primers are increasingly generating bands due to false positive reactions.[32] It is hypothesized that this recent surge could be due to the increase in mixed infections and the rise of malaria in Mangalore and the Dakshina Kannada region. This, in turn, leads the newer *Plasmodium* population to mutate and evolve, causing the older primers to be less effective in correctly identifying the species.[33] As seen in this case (►**Table 2**), the *P. knowlesi* primers Pmkr8 and Pmkr9 cross-reacted with human DNA sequences, thus generating a false positive band.[7,34]

Ethics Approval

This study was approved by the ethical committee of Nitte University. The ethics approval number is NU/CEC/ICMR-05/2015, NU/CEC/2018/0183. Consent from these patients who had attended Justice K.S Hegde Charitable Hospital of Nitte University in Mangalore was obtained per the ethical guidelines.

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Conflict of Interest
None declared.

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