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Detection of the infective *Plasmodium falciparum* gametocytes by RT-qPCR assay from a malaria-endemic region of Northeastern India

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Introduction: The diagnosis of infectious reservoirs in malaria (gametocytes) is necessary, especially in low-density infections and asymptomatic malaria patients. The gametocyte stage is a surrogate marker for infection of *P. falciparum* malaria in healthy individuals. The early detection of infectious gametocytes and treatment will strengthen our efforts in curbing transmission. The nested PCR and real-time quantitative PCR (RT-qPCR) methods have been demonstrated for the diagnosis of infectious gametocyte reservoirs. In this study, RDT, blood smear microscopy, and nested-PCR were used for the detection of *P. falciparum* and *P. vivax*, and compared with RT-qPCR detection of Pfg27 gametocyte biomarker gene.

Methods: In the present cross-sectional study, 356 human blood samples were collected from endemic areas of Kokrajhar Assam (asymptomatic and symptomatic malaria patients) for malaria diagnosis.

Results: A total of 8.42%(30/356) incidence of malaria was observed. Malaria patients were observed to be both symptomatic, 80%(24/30; 13Pf+11Pv), and asymptomatic, 20%(6 (4Pf +2Pv)). More than 64%(11/17) of Pf and 92.3%(12/13) of Pv infections were observed in children and the adolescent population (age <20 years) by RDT, microscopy, nested PCR, and RT-qPCR methods. The prevalence of Pf infection was 4.77%(17/356) by RT-qPCR method. Of 16 the Pf positive samples 81.25%(13/16) were symptomatic and 18.75%(3/16) were asymptomatic. One asymptomatic individual was found positive for Pf infection by the RT-qPCR method.

Conclusion: The findings from this research study revealed that the routine microscopy and RDT methods are insufficient for detecting all asymptomatic malaria and gametocyte infectious reservoirs. The early detection of infectious *P. falciparum* gametocytes and the treatment of patients will be helpful in preventing the transmission of malaria.

KEYWORDS

***Plasmodium falciparum*, malaria, RT-qPCR, gametocyte detection, Pfg27- gametocyte biomarker gene**

Introduction

The five *Plasmodium* species- *Plasmodium falciparum* (Pf), *P. vivax* (Pv), *P. malariae* (Pm), *P. ovale* (Po), and *P. knowlesi* (Pk) are known to cause malaria in humans (1). Approximately 90% of the world's malaria mortality has been attributed to infection with *P. falciparum* and thus this strain poses a great threat to public health (2). Globally, ~ 241 million malaria cases and 6,27,000 malaria deaths were estimated, which is ~ 14 million more cases and 69,000 more deaths in 2020 compared to 2019 (3). Malaria is a major health burden in India, especially affecting the population living in rural, tribal, and forest areas. In its fight against malaria, India has already launched a national framework for malaria elimination and is aiming to eliminate malaria by 2030 (4). There are certain hurdles that threaten this ambitious malaria elimination program, among them asymptomatic carriers of the malaria parasite in the rural, tribal, and forest populations; drug resistance; and insecticide resistance hindering vector control. Detection of asymptomatic carriers of the malaria parasite requires a robust and efficient diagnostic tool capable of detecting low-density infections, something that is generally lacking in remote areas. In such areas, the only choice for *P. falciparum* malaria detection is by Pf*hrp2*/Pf*hrp3* antigen-based rapid diagnostic tests (RDTs) kits in addition to microscopy, which requires highly skilled personnel. Recently, the Pf*hrp2*/Pf*hrp3*-based RDT kits are facing challenges due to the deletion of the Pf*hrp2*/Pf*hrp3* genes in *P. falciparum* parasites (5, 6). Moreover, these RDTs are based on the target proteins that are expressed only during the asexual stages of *P. falciparum*. Detection of sexual stages, particularly early detection of gametocytes is highly desirable, but due to lack of trained microscopists, identification and differentiation of gametocyte stages (I-V) remains a challenge. There is also a dearth of highly sensitive RDT-like tools to detect the sexually infectious stages of *P. falciparum*- gametocytes. Gametocyte maturation is classically divided into five distinct morphological stages (I-V) that lasts about 8-12 days in *P. falciparum*, and only the mature and infectious stage V is released into the bloodstream. These infectious gametocytes are taken up by the mosquito vector with the feeding blood and further mature into infectious sporozoites after completing their developmental cycle in the mosquito (7). Therefore, gametocytes require timely detection in human hosts to appropriately modulate the gametocidal drug administration in an efficient manner. Due to relatively lower gametocyte densities than asexual parasites, submicroscopic infections remain undetected in malaria-endemic areas leading to continued transmission (8). The application of molecular biology tools has led to the detection of submicroscopic gametocyte carriage and examination of human infectious reservoirs (9). Even low-density gametocyte asymptomatic reservoirs are potential carriers for transmission of malaria (10–12). There are variations in the time of appearance of these gametocytes in asymptomatic and symptomatic infections (13). Gametocytes are widely known as surrogate markers for malaria transmission in a healthy population (10). The amplification of RNA transcripts of Pf gametocyte-specific Pfg27 gene by real-time-quantitative PCR (RT-qPCR) assay is a potential tool for the detection of gametocytes (10, 14, 15). The PF3D7_1302100 coding Pfg27 gene is expressed in the early stage (I-III) and plays an important role in gametocytogenesis (16). In the present study, gametocyte-specific marker Pfg27 gene was explored in an RT-qPCR assay and compared with the traditionally used tools such as RDTs, microscopy, and nested-PCR, in malaria-endemic northeastern Indian states.

Methods

Sample collection

Due to the COVID-19 pandemic situation during 2020-22 sample collection of malaria patients from the field sites were difficult. However, a mass survey was conducted in the highly malaria-endemic region of Kokrajhar, Assam, following COVID-19 appropriate protocols. The study was approved by the ICMR-National Institute of Malaria Research Institutional Ethics Committee (IEC No. ECR/NIMR/EC/2019/306). Before collecting blood samples, informed consent was taken from all adults and, in the case of minors, their respective legal parent/guardian's approval was included in the study. Blood samples were collected in RNAlater® (Thermo Fisher, USA) and transported in a cold chain to the laboratory. The thick and thin blood smears were prepared and one drop of blood was used for detection of *P. falciparum* and *P. vivax* malaria by RDT kit method. The RDT-positive patients were treated as per the national drug policy of India. Individuals having body temperature ≥ 37.5 °C were considered symptomatic/febrile, and individuals with no fever history for the previous 48 hrs. were considered asymptomatic/afebrile (17). The thick and thin blood smears were stained with Giemsa stain and examined at 100X magnification for detection of *Plasmodium* parasites. The slides were read for the presence of *P. falciparum* parasites against a background of 200 WBCs in a thick film (18–20).

Nucleic acids isolation

Three punched discs from dried blood spots were used for DNA isolation by QIAamp DNA kit, Cat. No. 51306 (Qiagen, Germany), as per manufacturer protocol. Briefly, the discs were incubated overnight in lysis buffer (ATL buffer). Buffer AL was added followed by the addition of 100% ethanol to precipitate the DNA. The mixture was loaded on a QIAmp mini spin column and centrifuged at 8000 rpm in a collection tube. The spin column was washed with buffers AW1 and AW2. The isolated DNA was eluted in 50 µl of elution buffer and stored at -20 °C, until further use. The RNA isolation was accomplished by Nucleospin RNA Blood kit, Macherey-Nagel, Germany as per manufacturer's protocol. Briefly, 200 µl of whole blood was incubated for 15 min. at room temperature (RT) in buffer DL for lysis with proteinase K. RNA was bound in a spin column and desalted, followed by DNA digestion using rDNase provided in the kit. Total RNA was washed thoroughly using wash buffers and RNA was eluted in RNase-free H_2O, and stored at -20 °C until further use.

First-strand cDNA synthesis

The extracted RNA was converted to cDNA using the prime script 1st strand cDNA synthesis kit (Takara Bio, Japan), according to the manufacturer's instructions. Briefly, template RNA (~ 1µg) was added to a reaction mix containing Oligo dT primer (50 µM) and dNTP mix (10 mM each) and incubated for 5 min. at 65 °C, followed by instant cooling on ice. Then, 10 µl of the template RNA primer mixture is added in a new reaction mix (20 µl) containing 4 µl of 5X PrimeScript buffer and 200 U/µl of PrimeScript RTase. The reaction mixture was incubated at 30 °C for 10 min followed by incubation at 42 °C for 40 min. The first strand cDNA was directly used as a template for RT-qPCR of the Pfg27 gene.

Quantitative amplification of Pfg27 gene and nested PCR for Pf and Pv

RT-qPCR was performed in a final reaction volume of 25 µl constituting 5 µl of cDNA from patient samples, 500 nM of each primers- forward 5'- CTTAGCAAGGATCCTGAGAAGTTT-3' and reverse 5'-GTTGACAATGTTATCTTGGACACGT-3', 10 µl of 2X SYBR Green master mix (Thermo Fisher Scientific, USA). The RT-qPCR conditions were as follows: 95 °C for 5 min, 40 cycles 95 °C for 15 sec, 55 °C for 1 min. The amplifications were performed in CFX-96 Connect Real-Time PCR System (BioRad, USA) (21). Further a nested PCR was performed for detection of *P. falciparum* and *P. vivax* malaria parasites using previously reported PCR primers and conditions by Snounou et al. 1993 (22).

Results

During the mass survey, blood samples of 356 malaria-suspected individuals from highly malaria-endemic districts of Kokrajhar, Assam from 2020 to 2022 were collected. There was no major difference in gender proportions among the study subjects, which comprised 172(48.31%) men/boys and 184 (51.68%) women/girls (Figure 1). The major age group in the mass survey was children and adolescents (>50%), while one-third of the total samples were from children below 10 years of age. A total of 30(8.14%) (17Pf+13Pv) malaria cases were detected by RT-qPCR (Table 1). Of the 356 individuals, 33(9.27%) were symptomatic and 323(97.73%) were asymptomatic (Table 1).

Among the 17 Pf infections, 11(67.7%) belonged to the child and adolescent population (age <20 years); whereas in the case of Pv infection, 92.3%(12/13) were observed in children (0-9 years of age) (Table 2). This was notable that among the 30 malaria positive cases 76.66%(11Pf+12Pv) infections were observed only in children and adolescent population (age <20 years) (Table 2). Of the 356 samples, RDT, microscopy and nested PCR detected (Pf- 16(4.5%) infections, whereas 17(4.77%) Pf positive cases were detected by the RT-qPCR method. The RT-qPCR method targeted for detection of infectious gametocytes could only detect one additional asymptomatic malaria case (aged 30-39), which was not detected by previously established methods (RDT, microscopy, and nested PCR). It is important to note that of the 16 Pf samples, 81.25%(13/16) were from symptomatic individuals, while 18.75%(3/16) were from asymptomatic patients, as observed by RDT and microscopy, nested PCR method. Whereas the RT-qPCR method detected 23.52% (4/17) Pf cases from asymptomatic individuals. The detection limit of the Pf gametocyte stage by RT-qPCR method is comparatively higher than RDT, microscopy, and nested PCR methods (Table 2), but not statistically significant, which may be due to the the limited sample size.

In the context of *P. vivax*, RDT, microscopy, and nested PCR methods detected 3.65%(13/356) (Pv) positive cases. As with the case of Pf infections, majority of the Pv samples 69.23%(9/13) detected, belonged to children <10 years of age. All the children of age <10 years who were Pv positive had fever as a symptom. The asymptomatic Pv cases were observed in 15.38%(2/13) individuals (Table 3). Further, no sample was observed positive for Pv in

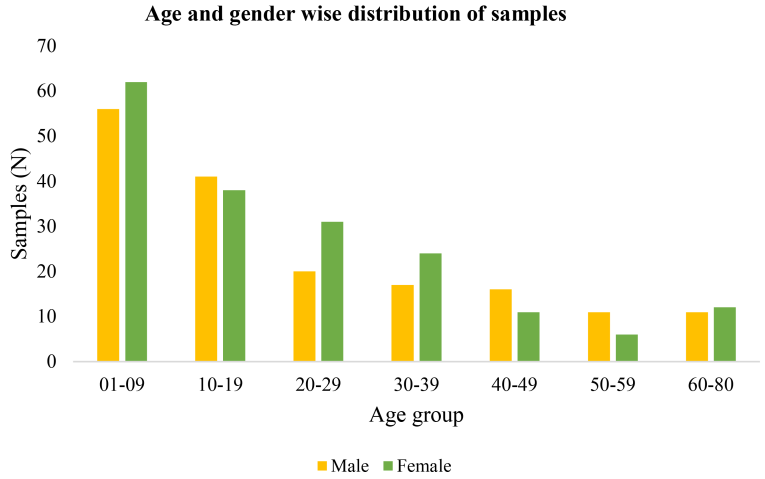

FIGURE 1
Age and gender-wise distribution of malaria-suspected samples.

TABLE 1 Age-group sorted Pf and Pv infection by nested-PCR and RT-qPCR and their prevalence (n=356).

Age Group	No of samples	Total febrile N (%) (Symptomatic)	Total afebrile N (%) (Asymptomatic)	Total (Pv & Pf) Malaria N (%)	Malaria prevalence N (%)
01-09	118	19 (5.33)	99	14 (46.66)	14 (3.93)
10-19	79	6 (1.68)	73	9 (30)	9 (2.52)
20-29	51	5 (1.4)	46	3 (10)	3 (0.842)
30-39	41	1 (0.28)	40	2 (6.66)	2 (0.56)
40-49	27	0	27	1 (3.33)	1 (0.28)
50-59	17	0	17	0	0
60-80	23	2 (0.56)	21	1 (3.33)	1 (0.28)
Total	356	33 (9.26)	323 (97.73%)	30 (8.42%)	30 (8.42)

individuals with age >30 years (Figure 2). The prevalence of Pv was observed to be 3.56%(13/356) by RDT, microscopy and nested-PCR methods in the studied subjects. There was no sensitivity difference between RDT, microscopy, and nested-PCR methods for Pv.

Discussion

Malaria in India is still a public health problem and *P. falciparum* is a predominant malaria parasite (> 50%) along with *P. vivax* (23). While malaria cases have progressively decreased in India, the endemic areas with asymptomatic and low-density transmission result in continued transmission in healthy individuals (11, 24, 25). Identification and appropriate control of infectious gametocytes is critical in curbing transmission because even at one gametocyte/µl of blood, mosquitoes can transmit the malaria parasite to another healthy individual (26). The asymptomatic and low-gametocytaemia individuals with *P. falciparum* malaria are important reservoirs and are active carriers for the transmission of malaria infection in the community. The government of India is focusing on malaria elimination by clinical case management and vector control (27). The RDTs are potential tool for surveillance of symptomatic malaria patients in the field but asymptomatic or low-density *P. falciparum* infections are often missed by RDTs (3, 28). The asymptomatic, low-density malaria parasites and low gametocytemic reservoir patients pose a significant challenge to the elimination of malaria from India by 2030 (11, 25). The PF3D7_1302100 coding Pfg27 gene is expressed in early stages (I-III) and play important role in male and female gametocytogenesis. It is important to note that the Pf gametocyte maturation from stage I to V takes up to 8-12 days; and only stage V is released into the bloodstream. Compared to other asexual stages of the parasite in humans, the infectious gametocytes are potential biomarkers for the detection of the Pf malaria parasite, as they are more stable compared to other asexual stages of *Plasmodium* in humans. Therefore, diagnosis of infectious gametocytes of *P. falciparum* and treatment is necessary for blocking of transmission in healthy individuals.

This study presents the latest malaria endemicity data from malaria-endemic areas of northeastern India. In a recent study, from different malaria-endemic states of India (Chennai, Nadiad, Rourkela) 3-8 % malaria prevalence was reported (17). The present

TABLE 2 Age-group sorted detection of *P. falciparum* infection in symptomatic and asymptomatic populations by RDT, microscopy, nested PCR, and RT-qPCR methods (n=356).

Age group	No. of Samples	RDT (Pf) N (%)		Microscopy (Pf) N (%)		Nested-PCR (Pf) N (%)		RT-qPCR (Pf) N (%)	
		Sympto matic	Asympto matic	Sympto matic	Asympto matic	Sympto matic	Asympto matic	Sympto matic	Asympto matic
01-09	118	5 (1.4)	0	5 (1.4)	0	5 (1.4)	0	5 (1.4)	0
10-19	79	4 (1.12)	2 (0.56)	4 (1.12)	2 (0.56)	4 (1.12)	2 (0.56)	4 (1.12)	2 (0.56)
20-29	51	2 (0.56)	0	2 (0.56)	0	2 (0.56)	0	2 (0.56)	0
30-39	41	1 (0.28)	0	1 (0.28)	0	1 (0.28)	0	1 (0.28)	1 (0.28)
40-49	27	0	1 (0.28)	0	1 (0.28)	0	1 (0.28)	0	1 (0.28)
50-59	17	0	0	0	0	0	0	0	0
60-80	23	1 (0.28)	0	1 (0.28)	0	1 (0.28)	0	1 (0.28)	0
Total	356	13 (3.65)	3 (0.84)	13 (3.65)	3 (3.65)	13 (3.65)	3 (0.84)	13 (3.65)	4 (1.12)

TABLE 3 Age-group sorted detection of *P. vivax* infection in symptomatic and asymptomatic patients by RDT, microscopy, and nested PCR methods (n=356).

Age group	No. of samples	RDT (Pv) N (%)		Microscopy (Pv) N (%)		Nested-PCR (Pv) N (%)	
		Symptomatic	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
01-09	118	9 (2.52)	0	9 (2.52)	0	9 (2.52)	0
10-19	79	2 (0.56)	1 (0.28)	2 (0.56)	1 (0.28)	2 (0.56)	1 (0.28)
20-29	51	0	1 (0.28)	0	1 (0.28)	0	1 (0.28)
30-39	41	0	0	0	0	0	0
40-49	27	0	0	0	0	0	0
50-59	17	0	0	0	0	0	0
60-80	23	0	0	0	0	0	0
Total	356	11 (3)	2 (0.56)	11 (3)	2 (0.56)	11 (3)	2 (0.56)

study also observed a prevelance of Pf infection 4.77%(17/356) in Assam, India, which is similar to previously reported data from different states of India [Assam (6.50%), Andhra Pradesh (9.17%), Bihar (8.23%), Chhattisgarh (2.24%), Gujarat (1.92%), Karnataka (13.14%), Madhya Pradesh (23.76%), Maharashtra (30.15%), Odisha (6.52%), Punjab (0.17%), Tamil Nadu (1.66%), and Uttar Pradesh (9.40%)] (29). A similar prevalence of Pv was observed at 3.65%(13/356) and concurred with previous findings of Pv prevalence (29). Our results suggested that the prevalence of malaria by RDT and RT-qPCR molecular approach is similar. Among the 30 malaria positive cases, symptomatic 80% (13Pf +11Pv) and asymptomatic 20%(4Pf+2Pv) infections were observed by RDT, microscopy, nested-PCR, and RT-qPCR methods; however, 30-80% of asymptomatic malaria cases have been reported in previous studies from malaria endemic areas of India (17, 30). Our study presents a lower proportion of asymptomatic malaria cases from malaria-endemic areas. It is important to note that our study found the majority of malaria-positive cases from the age groups of children and adolescents (>50%), which indicated the vulnerability of these age groups to malarial infections. Moreover, children below 10 years of age also do not have protective immunity as in the case of adults in malaria-endemic areas.

The RDT, microscopy, and conventional PCR techniques are less sensitive compared to the RT-qPCR assay to detect gametocytes (10). Therefore, RT-qPCR approaches should be routinely performed to screen Pf malaria diagnosis in addition to RDT and gold standard microscopy method (31). The Pfg27 gene-targeted RT-qPCR technique is a potential tool for early (gametocyte stage I-III) detection and treatment of malaria for curbing transmission (9, 32–35). Further studies are required with a significant number of malaria samples for statistically sound conclusions. The isolation of RNA, cDNA preparation, and RT-qPCR are labor intensive and require well-established laboratories for the detection of gametocyte reservoirs (36). This technique requires a skilled molecular biologist along with a well-curated molecular biology lab, which suggests that RT-qPCR is not applicable in the field. Therefore, we need to develop an advance gametocyte-specific serodiagnosis tool. Gametocyte-specific serodiagnosis in the field will be helpful in early diagnosis of gametocyte infectious reservoirs and will aid in curbing transmission for malaria elimination (7, 16).

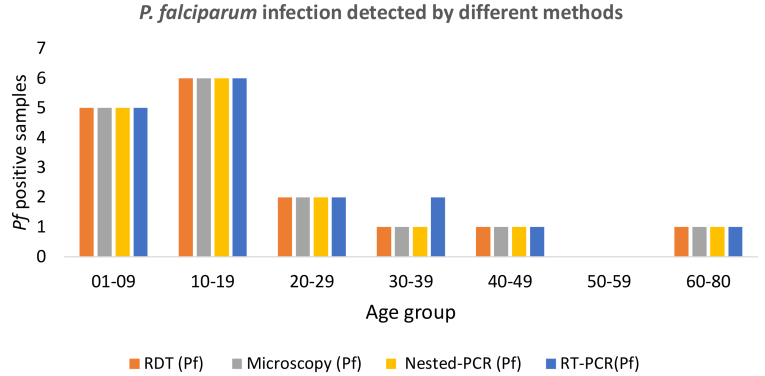

FIGURE 2
Age-group sorted *P. falciparum* infection was detected by different methods such as RDT, microscopy, nested PCR, and RT-qPCR.

Conclusions

The findings of the research study revealed that the routine gold standard microscopy and RDT method is insufficient for detecting all low-density Pf parasites and asymptomatic gametocyte infectious reservoirs. Therefore, in relation to malaria control and elimination, RT-qPCR would be a good approach for early detection of asymptomatic infectious Pf gametocyte reservoirs. The detection of infectious gametocytes will be helpful in curbing the transmission of malaria, its control, and elimination.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was approved by the ICMR-National Institute of Malaria Research Institutional Ethics Committee (IEC No. ECR/NIMR/EC/2019/306). Before collecting blood samples, informed consent was taken from all adults and, in the case of minors, their legal respective parent/guardian approval was included in the study.

Author contributions

RD: Conceptualization, Funding acquisition, Writing – original draft. KV: Data curation, Writing – review & editing. LK: Data curation, Writing – review & editing. KS: Writing – review & editing. GK: Software, Writing – review & editing. IH: Methodology, Writing – review & editing. JG: Resources, Visualization, Writing – review & editing. KP: Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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