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Assessing the Gametocyte Production in Field Isolates of *Plasmodium falciparum*

Purva Gupta, Veena Yadavendu and Vineeta Singh*

National Institute of Malaria Research (ICMR), Sector-8 Dwarka, New Delhi, India

***Corresponding author:** Vineeta Singh, National Institute of Malaria Research (ICMR), Sector-8 Dwarka, New Delhi, India, Tel: 011-25307140; Fax: 011-25307177; E-mail: vineetas_2000@yahoo.com

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Abstract

The production of gametocytes in field isolates of *Plasmodium falciparum* is not clearly understood even though the gametocytes are crucial for disease transmission. Samples collected during the malaria transmission period from two different regions of India were cultured *in vitro* for gametocyte production and analyzed by PCR and RT-PCR assay for *Pfs25* gene. A total of 20 *P. falciparum* field isolates were collected which showed varying intensity of *in vitro* gametocyte production. The isolates which produced mature gametocytes *in vitro* also had an increase in their *Pfs25* expression, indicating that the gametocyte produced is directly proportional to the *Pfs25* gene expression. The expression ranged from 0.32 to 4.56 fold in field isolates when compared to the reference strain. The *in vitro* gametocyte production in fresh field isolates directly correlated with the expression of *Pfs25* gene in these isolates as shown by ANOVA test.

Keywords: Gametocytogenesis; *Pfs25* gene; Gametocytaemia; *P. falciparum*

Background

The malaria parasite lifecycle requires the production of gametocytes and transmission of gametocytes from human host to the vector. This continuous spread of malaria causes 200-300 million clinical cases and 0.7 million deaths each year and *P. falciparum* is considered to be the deadliest of the human parasites [1]. The classical morphological gametocyte maturation is divided into five stages consisting of~10 days [2]. In acute malaria infections, the gametocytes are present at much lower concentrations compared to the asexual stages and may not be observed by microscopy [3]. Gametocytes ensure the transmission of malaria to the mosquito although they are not responsible for clinical symptoms. The common anti-malarial drugs are not able to clear all gametocytes thereby mediating transmission to mosquitoes despite clearance of other erythrocytic stages [4]. With the advent of available newer technologies for detection of gametocytes and its sub patent population several molecular detection methods are now used such as real-time quantitative nucleic acid sequence-based amplification (QT-NASBA), real-time polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP) [5-8].

The gametocytes are responsible for continuing the transmission of the parasite from an infected human host (gametocytogenesis-the development of the male and female gametocyte takes place in the asexual parasites) to a susceptible mosquito during the blood meal (gametogenesis-male and female gametocyte fertilization process) [9,10]. The mature gametocytes undergoing gametocytogenesis actively carry out protein synthesis and *Pfs25* is one such protein produced by *Pfs25* gene during early gametocytogenesis [11]. Within three hours of induction of gametocytogenesis, *Pfs25* protein appears and its expression continues during ookinete development [12]. Using this property of *Pfs25* protein we have studied the expression of *Pfs25* gene in the field isolates.

The aim of the present study was to culture the fresh clinical isolates of *P. falciparum in vitro* and to determine the sub patent population of gametocytes in these isolates. We also assessed the ability of *P. falciparum* in natural infections to produce gametocytes *in vitro* and correlated the expression of *Pfs25* gene in these isolates.

Methods

Sample collection and diagnosis

Preliminary diagnosis for identification of malaria patients was carried out by light microscopy and rapid diagnostic tests (RDT) (Bioline SD Rapid Test, USA), following which venous blood of 22 *P. falciparum* malaria patients was collected from Ranchi (East-central India) and Raipur (Central India) during the year 2013 (Table 1). The study was approved by the institutional ethics committee (IEC) and written consent was taken from the patients before samples were collected (ECR/NIMR/EC/2011/108). Blood spots were made on Whatman (number 3) filter paper for further molecular analysis. The remaining blood sample was cryopreserved and transported back to the laboratory for culture and expression studies.

Microscopic examination of field and adapted samples

All the samples collected were diagnosed by examining thick and thin peripheral blood smears under the microscope, which were stained with Giemsa to identify the presence of malaria species and asexual stages. Thick smears were analyzed for parasite density with 1000X magnification and since the fields can vary therefore the following formula was used:

Number of parasites/ number of leukocytes × 8000 = parasites/µl.

Following which percent parasitaemia was calculated after counting 200 leukocytes.

S No.	Region	Total malaria samples collected	Samples with *P. falciparum* monoinfection after 18S rRNA PCR confirmation	Isolates adapted *in vitro*	Gametocyte producing isolates
1.	Ranchi	9	8	6	5
2.	Raipur	13	12	6	5
Total		22	20	12	10

Table 1: Details of isolates collected from different regions of India.

DNA isolation and PCR analysis

Parasite genomic DNA was extracted from the filter paper spots of whole blood of patients using the QIAamp DNA Blood Mini Kit (Qiagen Inc., USA) as per the manufacturer's instructions and stored at -20°C until further use. *Plasmodium* species confirmation was carried out by the mixed infection PCR assay using 18S rRNA gene primers following the protocol and amplification conditions as published previously [13].The molecular genotyping of these monoinfections was further done by merozoite surface protein- *msp1* and *msp2* for multiclones. The amplification of *Pfs25* gene was carried out for *P. falciparum* mono-infections and the PCR amplification cycle included an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 30s, 50°C for 30s and 72°C for 30swith a final extension of 72°C for 10 min [11]. The *P. falciparum* reference strain NF54 was used as a positive control in all the laboratory experiments. Results were analyzed in electrophoresis, 2% agarose gel stained with ethidium bromide solution and visualized under UV transillumination. Nuclease free water was used as negative control.

In vitro culture

P. falciparum single infections confirmed after the PCR assay were revived using the standard method [14]. RPMI-1640 complete medium (pH 7.4) was supplemented with 25 mM HEPES, 25 mM sodium bicarbonate, 25 µg/ml gentamicin sulphate and 10% human type AB serum was used for *in vitro* culture of the isolates with 5% haematocrit. After removing the supernatant the parasitized RBCs (pRBCs) were resuspended in complete medium (CM) for culture adaptation. The whole blood sample obtained from the patients was washed twice in RPMI growth media in order to separate the RBCs from the plasma. The culture was maintained continuously and Giemsa stained thin smears were examined at 1000X magnification under the microscope every 24 hours to monitor the parasitaemia levels and the asexual stages. The isolates were sub-cultured when the parasitaemia reached 4.0% and since it is known that 1 µl of blood contains $5x10^6$ RBCs the percent parasitaemia was thus calculated [14].

Gametocytes production

The field isolates of *P. falciparum* were put to gametocyte production at 0.5% parasitaemia and 6% hematocrit under 3% O_2, 5% CO_2, 92% N_2 gas. RPMI culture medium with 25 mM HEPES, 50 mg/liter hypoxanthine, 2 g/L sodium bicarbonate, 10% human serum, no fresh erythrocytes were added to culture till 14 days. Medium was replaced every day for at least 14 days taking care to maintain all medium, pipettes, and work surfaces at 37°C [15]. Under these conditions, cultures showed progression with asexual parasitemia rising to a peak and crashing at days 4-5, gametocytes were visible from7th day following which maturation of gametocytes began.

RT-PCR

For preparation of RNA the culture samples were prepped by using the method as described previously [10]. Total RNA of culture adapted *P. falciparum* blood samples was isolated from infected RBCs (iRBCs) by QIAamp RNA Blood Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and eluted in 30µl of RNase-free water. The genomic DNA contamination was removed by treating the isolated RNA with DNase I (Thermo Scientific). First strand cDNA was reverse transcribed from 150 ng total RNA using oligo $(dT)_{18}$ primers (Thermo Scientific) according to the manufacturer's protocol. ANOVA (analysis of variance) test was used to determine the statistical significant if $p<0.05$.

Real-time PCR assay was performed using SYBR Green RT-PCR kit (Thermo Scientific) and Light Cycler 480 system (Roche Diagnostics, USA) to measure the relative expression levels of *Pfs25* gene in culture adapted *P. falciparum* isolates and NF54 reference strain. The expression signals were compared to the endogenous gene *β-tubulin*. Ten µM of each primer *Pfs25*_F – '5 - AAT GCG AAA GTT ACC GTG GA – 3', *Pfs25*_R – '5 - CAA GCG TAT GAA ACG GGA TT – 3' and 2 µl cDNA was taken for a 20 µl reaction [11]. The PCR conditions comprised of an initial denaturation of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 50°C for 25 s and 72°C for 30 s and the fluorescence acquisition temperature was 72°C. The experiment was performed in triplicates for each isolate to minimize handling errors. Melt curve analysis was carried out to further validate the amplification specificity and non-template control (NTC) was run with each reaction. Threshold cycles (C_t) were recorded for *Pfs25* and *β-tubulin* genes which were used to calculate ΔC_t values *i.e.* the difference between the C_t value of *Pfs25* and *β-tubulin* genes. $\Delta\Delta C_t$ was further calculated which was the difference between the ΔCt of *P. falciparum* isolate and NF54 reference strain. Relative expression of *Pfs25* was calculated by using $2^{-\Delta\Delta Ct}$ when compared to the reference strain [16].

Results and Discussion

Twenty-two samples showed *P. falciparum* infection by preliminary diagnosis of microscopy and RDT. Two samples (one from Ranchi and one from Raipur) were found to have mixed infection of *P. falciparum* and *P. vivax* by 18S rRNA PCR confirmation method. Molecular genotyping of 20 *P. falciparum* monoinfections by *msp1* and *msp2* genes revealed all isolates to be single clones.

Out of 20 collected isolates only 12 isolates adapted in the *in vitro* culture and the remaining eight isolates could not be adapted probably due to the fact that at the time of collection of samples, the parasites were not healthy or they lost their viability during transport from field site to laboratory. The levels of parasitaemia varied in the clinical infections and gametocytes were seen in two out of the 12 adapted samples by microscopic examination at the time of their collection (Table 2). The other erythrocytic stages of *P. falciparum* (ring stages, trophozoites and developing schizont) were identified in the peripheral smears. Out of the 12 cryopreserved samples subjected to *in vitro*

culture for revival, four samples (RNC 52, RNC 58, RNC 59 and RAP 16) revived immediately *in vitro* on transfer to the culture medium, while the remaining eight samples took a variable adaptation period extending from 4-10 weeks after the initiation of the culture. The difference in the growth pattern in different isolates may be due to their difference in commitment to differentiate into asexual or sexual stages [17,18]. After successful establishment of culture-adapted samples, the 12 isolates were put for gametocyte production *in vitro*. After 15 days of culturing in lab, 10 fields isolates viz. RNC52, RNC54, RNC55, RNC58, RNC59, RAP9, RAP10, RAP11, RAP14 and RAP16 produced gametocytes and various stages I-V were identified by microscopy. From 7th day onwards post sub-culture different stages of gametocytes were seen in smears (Figure 1). In two isolates (RNC60 and RAP4) no gametocyte production was seen inspite of their adaptation *in vitro* and the asexual stages identified in smears. The absence of complete gametocytaemia in isolates RNC60 and RAP4 may be due to genomic deletions/mutations in chromosome 9 [19]. Among the 10 clinical isolates, four isolates (RNC52, RNC58, RAP10 and RAP16) harbored a higher frequency of gametocyte production in comparison to the other isolates (Figure 2).

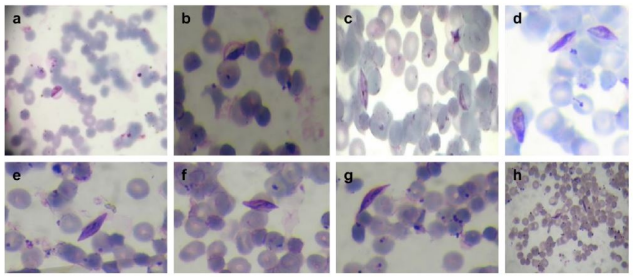

Figure 1: Different stages of gametocytes observed from days 7-14 in *P. falciparum* field isolates. a) Stage II seen on the 6th day of gametocyte production. b) Stage II seen on the 7th day of gametocyte production. c) Stage III seen on the 8th day of gametocyte production. d) Stage III and IV seen on the 9th day of gametocyte production. e) Stage IV seen on the 10th day of gametocyte production. f) Stage V seen on the 12th day of gametocyte production. g) Mature gametocyte seen on the 13th day of gametocyte production. h) Increased parasitaemia and mature gametocytes found on the 15th day of culture.

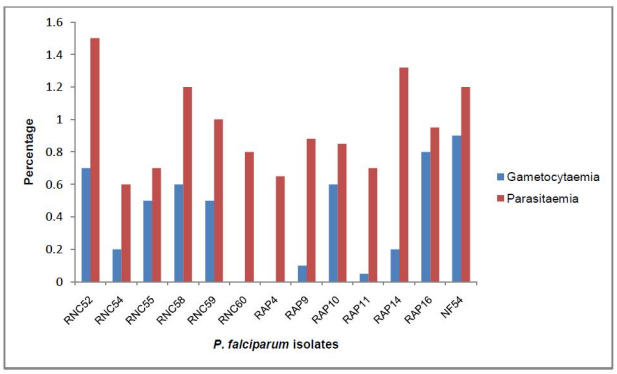

Figure 2: Comparative representation of percent gametocytemia (blue bars) and parasitaemia (red bars) in the *P. falciparum* field isolates.

The *Pfs25* gene was first amplified in *P. falciparum* isolates by PCR before the expression studies. Eleven isolates showed a band at 215bp whereasRAP4 which did not show any gametocyte production showed no amplification for *Pfs25* gene (Figure 3). We also analyzed the expression of *Pfs25* gene in the adapted field isolates *in vitro*. The expression profile of *Pfs25* gene was correlated with the efficiency of gametocyte production in the above isolates. It was observed that isolates which produce mature gametocytes in vitro also showed an increase in the *Pfs25* gene expression compared to the reference strain. The relative expression of the *Pfs25* gene in *P. falciparum* isolates ranged from 0.32 in RAP11 to 4.56 fold in RAP16 when compared to NF54 reference strain (Figure 4). RAP11, RAP9, RNC60, RNC54 and RAP14 showed lower expression of *Pfs25* compared to NF54 whereas the remaining six isolates displayed a higher expression. These results correlate with the data presented in Table 2 from which it is clear that RNC60 and RAP4 showed no or had very sub patent gametocyte production which could not be detected by these molecular methods. The absence of *Pfs25* gene expression in non-gametocyte producing isolates also substantiated the above observations. Also, the highest gametocytaemia was seen in RAP16 and RNC52 which show a 4.56 and 3.34 fold increases in expression levels of *Pfs25* respectively. Both these samples also showed high parasitaemia 0.95% and 1.50% (Table 2).

S.No.	Field isolate	Light microscopy		Gametocytaemia (%)	Gametocyte stages					PCR		RT-PCR
		Parasitaemia (%)	Gametocytes		I	II	III	IV	V	18S rRNA(*P. falciparum* monoinfections)	*Pfs25*	*Pfs25*
1.	RNC 52	1.50	+	0.7	+	+	+	+	-	+	+	+
2.	RNC 54	0.60	-	0.2	+	+	+	+	+	+	+	+
3.	RNC 55	0.70	-	0.5	+	+	+	+	+	+	+	+
4.	RNC 58	1.20	-	0.6	+	+	+	+	-	+	+	+
5.	RNC 59	1.0	-	0.5	+	+	+	+	+	+	+	+
6.	RNC 60	0.80	-	0	-	-	-	-	-	+	+	+

7.	RAP 4	0.65	-	0	-	-	-	-	-	+	-	-
8.	RAP 9	0.88	-	0.1	-	-	-	+	-	+	+	+
9.	RAP 10	0.85	-	0.6	+	+	+	+	-	+	+	+
10.	RAP 11	0.70	+	0.05	-	-	-	+	-	+	+	+
11.	RAP 14	1.32	-	0.2	-	-	-	+	+	+	+	+
12.	RAP 16	0.95	-	0.8	+	+	+	+	-	+	+	+
13.	NF54	1.2	-	0.9	+	-	+	+	+	+	+	+

Table 2: Details of the *P. falciparum* isolates studied for gametocyte production and subsequently to analyze the *Pfs25* gene expression levels.

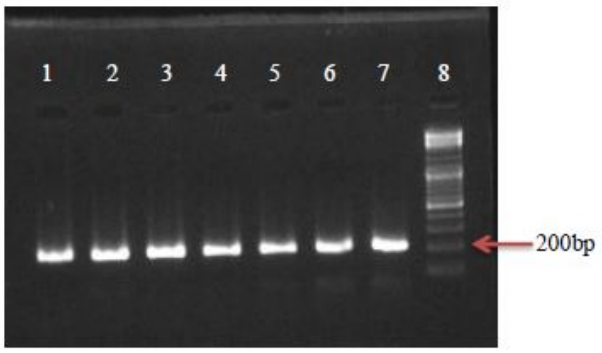

Figure 3: Gel image showing the amplification of *Pfs25* gene. Lanes 1-7 show 215bp fragment of *Pfs25* gene (lane 1-RNC52, lane 2-RNC54, lane 3-RNC58, lane 4-RNC59, lane 5-RAP9, lane 6-RAP10, lane 7-RAP14) and lane 8 shows 100 bp DNA marker.

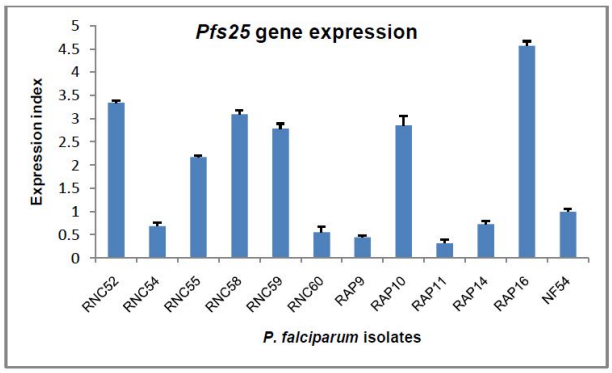

Figure 4: Gene expression pattern of *Pfs25* gene in 11 *P. falciparum* isolates when compared to the reference strain of *P. falciparum* NF54. ANOVA (analysis of variance) test was used to determine the statistical significant if $p<0.05$.

These results suggested that a correlation between *Pfs25* gene expression and the ability to produce gametocyte (mature) by the isolate exists. Our results suggest that *Pfs25* gene has a role to play in the process of gametocytogenesis in field isolates and disease transmission.

Conclusion

In summary we have been able to detect gametocytes by showing the relative expression of *Pfs25* gene by RT-PCR. It was observed that the culture adapted field isolates during gametocyte production *in vitro* also showed an increased expression of *Pfs25* gene when compared to a gametocyte producing reference strain (NF54). As observed by microscopy; RAP 4 failed to produce gametocytes *in vitro* and also did not express *Pfs25* gene whereas, the other isolate; RNC 60 expressed much lower than the reference strain. Though we could observe more field isolates which produced/expressed gametocytes by RT-PCR but this method has to be coupled with microscopic examination of smears. In this study we observed that the *Pfs25* gene was responsible for gametocyte carriers and their ability to transmit sexually, since the subpatent gametocytic stages cannot be seen by microscopy. As the sample size in this study was not very large, further studies of *P. falciparum* field isolates are required to determine the ability of Indian isolates to produce gametocytes *in vitro*.

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References

1. WHO (2010) World Malaria Report, Geneva.
2. Hawking F, Wilson ME, Gammage K (1971) Evidence for cyclic development and short-lived maturity in the gametocytes of *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 65: 549-559.
3. Bousema T, Drakeley C (2011) Epidemiology and infectivity of *Plasmodium falciparum* and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clin Microbiol Rev 24: 377-410.
4. Okell LC, Drakeley CJ, Ghani AC, Bousema T, Sutherland CJ (2008) Reduction of transmission from malaria patients by artemisinin combination therapies: a pooled analysis of six randomized trials. Malar J 7: 125.
5. Schneider P, Wolters L, Schoone G, Schallig H, Sillekens P, et al. (2005) Real-time nucleic acid sequence-based amplification is more convenient

than real-time PCR for quantification of *Plasmodium falciparum*. J Clin Microbiol 43: 402-405.

6. Maeno Y, Nakazawa S, Daole D, Yamamoto N, Giang ND, et al. (2008) A dried blood sample on filter paper is suitable for detecting *Plasmodium falciparum* gametocytes by reverse transcription polymerase chain reaction. Acta Trop 107: 121-127.
7. Buates S, Bantuchai S, Sattabongkot J, Han ET, Tsuboi T, et al. (2010) Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. Parasitol Int 59: 414-420.
8. Wampfler R, Mwingira F, Javati S, Robinson L, Betuela I, et al. (2013) Strategies for detection of *Plasmodium* species gametocytes. PLoS One 8: e76316.
9. Baker DA (2010) Malaria gametocytogenesis. Mol Biochem Parasitol 172: 57-65.
10. Kuehn A, Pradel G (2010) The coming-out of malaria gametocytes. J Biomed Biotechnol: 976827.
11. Babiker HA, Abdel-Wahab A, Ahmed S, Suleiman S, Ranford-Cartwright L, et al. (1999) Detection of low level *Plasmodium falciparum* gametocytes using reverse transcriptase polymerase chain reaction. Mol Biochem Parasitol 99: 143-148.
12. Shahabuddin M (1998) Plasmodium ookinete development in the mosquito midgut: a case of reciprocal manipulation. Parasitology 116 Suppl: S83-93.
13. Gupta B, Gupta P, Sharma A, Singh V, Dash AP, et al. (2010) High proportion of mixed-species *Plasmodium* infections in India revealed by PCR diagnostic assay. Trop Med Int Health 15: 819-824.
14. Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. Science 193: 673-675.
15. Lingnau A, Margos G, Maier WA, Seitz HM (1993) Serum-free cultivation of *Plasmodium falciparum* gametocytes in vitro. Parasitol Res 79: 378-384.
16. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.
17. Talman AM, Domarle O, McKenzie FE, Ariey F, Robert V (2004) Gametocytogenesis: the puberty of *Plasmodium falciparum*. Malar J 3: 24.
18. Dyer M, Day KP (2000) Commitment to gametocytogenesis in *Plasmodium falciparum*. Parasitol Today 16: 102-107.
19. Alano P, Roca L, Smith D, Read D, Carter R, et al. (1995) *Plasmodium falciparum*: parasites defective in early stages of gametocytogenesis. Exp Parasitol 81: 227-235.