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Plasmodium falciparum gametocytaemia with chloroquine chemotherapy in persistent malaria in an endemic area of India

P.K. Kar, V.K. Dua, N.C. Gupta, Ashish Gupta & A.P. Dash*

*National Institute of Malaria Research, Field Station, Hardwar & *National Institute of Malaria Research Delhi, India*

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***Background & objectives*: Gametocyte sex-ratio in *Plasmodium falciparum* malaria is an important determinant of transmission success and basis of disease epidemiology. Information on ratio of male to female gametocytes after an exposure of antimalarial regimens under field conditions is very limited. In this retrospective study we observed high densities of gametocytes along with high sex-ratio in *P. falciparum* cases, which may be responsible for persistent malaria transmission in this area.**

***Methods*: Laksar PHC of Hardwar district, Uttarakhand State, India was selected because it contributed 90 per cent of the total malaria cases. A total of 568 uncomplicated *P. falciparum* malaria patients were assessed to investigate prevalence of gametocytes while *339 P. falciparum* thick smears containing 5620 gametocytes were screened for measuring the gametocyte density for microgametocyte (male) and macrogametocyte (female). Homology of variance ('F' test) was checked on days 7 and 14 including the variables and risk factors namely fever, parasitaemia, gametocyte carriage in sensitive and resistant chloroquine treated *P. falciparum* cases.**

***Results*: Slide positivity rate (SPR) increased drastically from 0.23 to 11.4 per cent with the predominance in *P. falciparum* infection after 1998. All 568 cases showed gametocytes in their peripheral blood, of which 109 (19%) were infected with rings and gametocytes and 459 (81%) had gametocytes stages in their peripheral blood while 422 (74.3%) cases were infected with ring stages only. Of the 339 *P. falciparum* positive blood smears, 5620 gametocytes were screened for their sex-ratio. The mean sex-ratio was 0.31 (3.22 female per male). Prevalence of gametocytaemia was significantly higher ($P<0.05$) in chloroquine (CQ)- resistant than in CQ-sensitive patients with days 7 and 14 follow up. The homology of variance with risk factors for gametocytes on days 7 and 14 were highly significant ($P<0.001$) in the study period but during the post-exposure period of days 3 and 5, these were insignificantly correlated.**

***Interpretation & conclusion*: A high density of *P. falciparum* gametocytes was observed at the time of preparation of blood slide on day 0. Improper chloroquine treatment along with poor patient compliance for radical treatment and the presence of chloroquine resistant *P. falciparum* malaria may have enhanced the prevalence and density of *P. falciparum* gametocytes which was instrumental in signaling the persistent malaria in this area.**

Key words Chloroquine - gametocytaemia - malaria - *Plasmodium falciparum* - prevalence - sex-ratio

The infectivity of malaria parasite to mosquito vectors is determined by the availability of the gametocytes in the peripheral blood, their intrinsic capacity to infect the mosquito and the molecular composition of the blood meal which is derived largely from the blood of the gametocyte-infected host[1]. Sowunmi and Fateye[2] examined gametocyte carriage and intensities of gametocytaemia in Nigerian children suffering from uncomplicated *Plasmodium falciparum* malaria treated with antimalarial drugs. Seidlein and coworkers[3] analyzed the risk factors associated with gametocytaemia in Gambian children. Gametocyte sex-ratio in *P. falciparum* malaria is an important determinant of transmission success and basis of disease epidemiology[4].

Limited studies[4,5] have reported on the ratio of male to female gametocytes after an exposure of antimalarial regimens under field conditions. Due to a malaria outbreak in September-October, 1999 in Laksar PHC, National Institute of Malaria Research (NIMR) Field Unit at Hardwar, Uttarakhand, India, started epidemic investigation on vector control and treatment of malaria cases as per National Vector Borne Disease Control Programme's guidelines. Follow up of cases was done to find out resistant foci, if any. We observed high densities of gametocytes along with high sex-ratio in *P. falciparum* cases which may be responsible for persistent malaria transmission in this area. The results are presented here.

Material & Methods

Study area: Laksar PHC of Hardwar district (Uttarakhand State, India) was selected because it contributed 90 per cent of the total malaria cases of district Hardwar from the year 1995 to 1999. The study was carried out during the months of September and December, 1999-2001 in 12 endemic villages (total population: 14,000) covering an area of 325 km^2. Before enrollment for the study, medical history of each patient, body weight and oral or rectal temperatures were recorded.

Sample size: In all 568 patients with a median age of 24 yr (range: 1-65 yr) were selected for the study. The age groups were 1-4, >4-8, >8-14 and >14 yr. Patients with acute, symptomatic and uncomplicated *P. falciparum* malaria with fever or history of fever in 24-48 h preceding presentation with gametocytes or gametocytes with rings and with parasitaemia of >2000 asexual forms/ µl blood were considered for the study.

Patient's inclusion criteria: Patients with age >1 yr, parasitaemia (range of 1000-100,000/µl blood), fever history of previous 24 h and with auxiliary temperatures 37.5^0 C were considered.

Exclusion criteria: Presence of severe malnutrition[6], mixed infection, severe malaria, febrile illness other than malaria, pregnancy, contraindication to antimalarial regimens with history of allergy was excluded.

Chemotherapeutic schedule: *P. falciparum* cases were treated with chloroquine (Nester Pharmaceuticals Ltd, Goa, India) as per National Drug Policy[7] (25 mg/kg body weight: Day 0 - 600 mg; Day 1 - 600 mg; Day 2 - 300 mg).

Parasitological examination: Thick and thin blood smears prepared from finger-prick blood samples were left to dry for 24-48 h, dehaemoglobinised and stained with Jaswant Singh-Bhattacharji (JSB)[8] stain for parasite identification and quantification of *P. falciparum* and other parasite species (*P. vivax*) under oil-immersion microscope (objective at x 1000 magnification; Zeiss, West Germany).

Follow up of cases: Weekly active surveillance was carried out during the follow up period on day 3, 5, 7, 14 and 28 by the State Health Department and the project staff of NIMR, Field Unit, Hardwar. All blood slides were prepared before giving any treatment. After immediate completion of follow up period, all resistant cases were appropriately treated with sulphadoxine-pyrimethamine dosage schedule as per National Drug Policy[7] and also followed up for a month for any kind of severity/febrile illness if any, other than malaria.

In vivo antimalarial sensitivity (WHO 28-day extended test[9]): The antimalarial sensitivity test[9] was carried out on 339 patients with history of no re-infection during the study and also after ascertaining that no chloroquine had been taken[10]. Patients were followed for 28 days by collecting blood smears for parasite density on day (D) 0, D2, D7, D14, D21 and D28 and whenever the patients complained of fever after completion of prescribed antimalarial treatment.

End point of the study: Subjects with protocol violation, voluntary/involuntary withdrawal, treatment failure, completion of follow up with treatment failure, loss to follow up were not considered for further study.

Re-treatment of drug treatment failures: All treatment failures resistant cases were treated with three tablets of

Fansidar™ (F. Hoffman-La Roche, Basel, Switzerland) (Sulphadoxin 500 mg x 3 = 1500 mg @ 25 mg/kg body weight and pyrimethamine 25 mg x 3 = 75 mg or @ 1.25 mg/kg body weight).

Quantification of asexual and sexual parasitaemia: Parasitaemia was estimated in thick film by counting asexual parasites relative to 100 leukocytes or 500 asexual forms, whichever occurred first. Parasite density was calculated assuming a leukocyte count of 8000/µl blood. Gametocytes were also counted in thick blood films against 1000 leukocytes on day 0, 3, 5, 7, 14 and 28 of follow up period or when necessary.

Gametocyte species[11] confirmation was made on blood smears under microscope objective at x 1000 magnification. Gametocytes sex-ratio[12] was measured as the proportion of male gametocytes. The gametocyte density and infection variability were measured according to method already described[13].

Statistical analysis: Data for normal distribution of gametocytaemia were compared by Student's "t" tests in relation to chloroquine response in sensitive and resistant gametocyte carriage in the years 2000-2001. $P<0.05$ was considered significant between group means whereas homology of variance was tested by the F-test with analysis of variance (ANOVA) and the variance included risk factors for gametocytaemia on days 7 and 14 along with other variables during the time of enrollment namely fever and parasitaemia. The distribution of post-treatment gametocyte data for the 1999 was non normal so a non parametric analog to a Model 1 one-way ANOVA with Krushal-Walis test was employed to compare gametocyte densities in treated groups using a Software MTB WIN Inc, 2000, USA.

Results

The epidemiological data of Laksar PHC from 1995 to 2001 are presented in Table I. Slide positivity rate (SPR) increased drastically from 0.23 to 11.4 per cent with the predominance in *P. falciparum* infection after 1998. From 1995 to 1997 there were no *P. falciparum* cases while a single case was recorded in 1998, pointing towards total absence of surveillance system. SPR of 8.12, 11.40 and 6.19 were recorded for the year 1999, 2000 and 2001 respectively (Table I).

The chloroquine resistant cases recorded were 35.14, 33.60 and 34.78 per cent in the years 1999, 2000 and 2001 respectively and there was a trend of decreasing infectivity as gametocyte density decreased (Table II) because of the systematic deployment of chemotherapeutic schedules and vector control measures undertaken during this period. During the follow up period, microgametocytes of *P. falciparum*

Table I. Malaria incidence recorded at Laksar PHC, district Hardwar (Uttarakhand), India

Years	Blood* slides	*Plasmodium falciparum* (*Pf*%)	Other species (*P. vivax)*	Total	SPR**
1995	8542	-	27	27	0.31
1996	7689	-	40	40	0.52
1997	10982	-	95	95	0.86
1998	9353	1	21	22	0.23
1999	10316	377 (44.98)	461	838	8.12
2000	5759	372 (56.62)	285	657	11.40
2001	5848	239 (66.02)	123	362	6.19

*Blood slides were stained with Jaswant Singh-Bhattacharji (JSB)[8]

**SPR (slide positivity rate)- Number of positive out of percentage total slides examined. It is a number of prevalence among suspected population rather than among general population

Source: Epidemiological data 1995-2001 obtained from District Malaria Officer, Hardwar, Uttarakhand, India

Table II. Age-wise demographic characteristics of children (0 -1 to 9 -14 yr) with asexual and sexual stages of parasites in PHC villages, Laksar, Hardwar (Uttarakhand), India

Parameters	Year		
	1999	2000	2001
No of patients (Ratio of male : female)	129 (37 : 92)	248 (107 : 141)	191 (52 : 139)
Weight (kg)	5.8 - 33	5.7 - 37	5.2 - 38
Parasite§ density (range)			
Asexual	40 - 200	40-800	40 - 320
Sexual	11 - 660	120 - 340	11 - 275
Parasite clearance time (PCT)‡ in days	2.4 ± 0.62, 1 - 4	2.3 ± 0.8, 1 - 6	2.7 ± 0.7, 1 - 4
Antimalarial sensitivity[a]	*In vivo*[b] 37	*In vivo* 125	*In vivo* 23
	Sensitive[c] 24 (64.8)	83 (66.40)	15 (65.22)
	Resistant[d] 13 (35.14)	42 (33.60)	8 (34.78)
Cure rate[e] (%)	68.94	67.85	68.0

Values are mean ± SD

§Parasite density (as/µl blood) measured against leucocyte count of 8000/µl; ‡PCT-Time elapsing from drug administration until there was no patent parasitaemia

[a]Antimalaral sensitivity was conducted after 28 day; [b] Cases were followed for 28 days and blood smears were collected on days 0, 2, 7, 14, 21, 28 and also whenever the patients under test complained of fever after completing antimalarial treatments; [c]Sensitive to chloroquine; [d]Resistant to chloroquine; [e]Proportion of patients remained free from parasitaemia on day 14 of follow up

cases were investigated to determine the prevalence of gametocytes. All 568 cases showed gametocytes in their peripheral blood, of which 109 (19%) were infected with rings and gametocytes and 459 (81%) had gametocytes stages in their peripheral blood while 422 (74.3%) cases were infected with ring stages only. Age-wise distribution of 568 *P. falciparum* cases having gametocytes were distributed in the following age groups in years : 0-1 (62, 11%), 2-4 (108, 19%), 5-9 (97, 17%) and > 10 (301, 53%).

A total of 339 *P. falciparum* thick smears containing 5620 gametocytes were screened for measuring the gametocyte density for microgametocyte (male) and macrogametocyte (female) and the mean sex-ratio was 0.31 *i.e.*, 3.22 female per male.

Chloroquine sensitivity tests for 339 *P. falciparum* resistant patients were conducted for possible recrudescence for RI, RII and RIII grade resistance. In 1999, 2000 and 2001, sensitive cases recorded were 84, 83 and 51 respectively while resistant cases were 45, 41 and 35 respectively. All resistant cases were of RI level and responded to Fansidar treatments (Table III).

The homogeneity of variance was tested with the risk factors for gametocytaemia on days 7 and 14 which revealed that these were highly significant in 1999 [F (2,70) =13.13, $P<0.001$)], 2000 [F (2,57) = 4.67, $P<0.025$] and 2001 [F (2, 56) = 6.29, $P<0.005$] but were insignificantly correlated on days 3 and 5 gametocytaemia during 1999 [F (2,54) = 1.29, $P>0.1$, 2000: F (2,45) = 0.62, $P>0.1$ and 2001: F (2,48) = 1.48, $P>0.1$].

The sample medians for the three treatments in 1999 were calculated 13.2, 12.9 and 15.6 using Krushal-Wallis test. The Z-value (smallest absolute) for level 1 was 0.45 indicated mean rank for treatment 1 differed least from the mean rank of all values. Similarly, mean rank for treatment 2 was lower than the mean rank (Z= -2.38) of all observations and ultimately mean rank of treatment 3 was higher than the mean rank of all

Table III. Prevalence of gametocytaemia after treatment with chloroquine in sensitive and resistant cases (1999-2001) in PHC Laksar, Hardwar (Uttarakhand) India

1999 Total cases 129		2000 Total cases 124		2001 Total cases 87	
CQS (84) (Pre-treated groups)	CQR (45) (Pre-treated groups)	CQS (83) (Pre-treated groups)	CQR (41) (Pre-treated groups)	CQS (52) (Pre-treated groups)	CQR (35) (Pre-treated groups)
D0 15 (17.86 %)*	25 (55.55 %)	D0 10 (12.05 %)	22 (53.66 %)	D0 14 (26.92 %)	20 (57.14 %)
34.33**	30.08	32.5	36.27	78.78	34.80
11 - 180#	12 - 150	12 - 75	12 - 170	12 - 80	12 - 160
D3 20 (23.81 %)	18 (40.00 %)	D3 12 (14.46 %)	16 (39.02 %)	D3 18 (34.62 %)	15 (42.86 %)
32.5	35.89	38.33	52.25	37.83	50.47
12 -136	12-90	12 - 85	12 - 80	12 - 95	12 - 119
	$P < 0.5$##		$P < 0.5$		$P< 0.05$
D5 18 (21.43 %)	19 (42.22 %)	D5 5 (6.02 %)	10 (24.39 %)	D5 22 (42.31 %)	16 (45.71 %)
37.44	42.89	34.40	45.80	47.77	52.25
10 - 205	12 - 115	12 - 116	12 - 110	12 - 125	12 - 135
	$P < 0.5$		$P < 0.5$		$P< 0.5$
D7 22 (26.19%)	26 (57.78 %)	D7 8 (9.64 %)	18 (43.90 %)	D7 12 (23.08 %)	19 (54.29 %)
26.36	86.73	28.0	89.39	40.50	78.21
12 - 96	12 - 310	12 - 70	12 - 340	12 - 76	12 - 275
	$P < 0.001$		$P < 0.01$		$P< 0.01$
D14 11 (13.10%)	22 (48.89 %)	D14 6 (7.23%)	20 (48.78 %)	D14 8 (15.88 %)	20 (57.14 %)
18.73	48.91	25.33	62.35	27.62	52.35
12 - 35	12 - 92	12 - 40	12 - 210	12 - 60	12 - 89
	$P < 0.02$		$P< 0.05$		$P<0.05$

*Number of patients included with gametocytaemia on Day 0 (D0); Figures in parenthesis represent percentage inclusion of patients out of total cases registered; ** Mean of gametocyte density - Density counted on thick blood films against 1000 leucocytes on day 0, 3, 5, 7, 14 and day 28 or when necessary; #Range of gametocytaemia counted; ## *P* values (95% CI) were calculated in pretreated samples between Day 0 CQ-R *vs* Day 3, Day 5, Day 7 and Day 14. Variance included risk factors for gametocytaemia on day 7 and 14 along with fever and parasitaemia; CQS, chloroquine sensitive; CQR, chloroquine resistant

observation (Z= 2.71). Test statistic (H) had a *P* value 0.014 indicated that there was at least one difference among the treatment groups.

Discussion

A sudden increase of malaria cases after 1999 as compared to previous 4 years and the persistent cases in the following years contributed to increased *pf* per cent. Prevalence of gametocytaemia after treatment with chloroquine showed that the parasitological characteristics with or without gametocytaemia did not show any significant difference, but the duration of illness was longer in comparison to patients without gametocytaemia[2]. This may be related to longer duration of illness before being presented for treatment in clinic that permitted a sufficient time for the progression of committed asexual parasites to gametocytes. There was also a higher prevalence of pre-treatment gametocytaemia harbouring CQ-R infection.

Our results have shown reservoir-transmission status[2], which was very active in the follow up period similar to earlier results[5,14,15]. Cure rates of clearing gametocytaemia were higher in CQ sensitive than in resistant cases. It has been reported that in chloroquine resistant infections, immature gametocytes continue their growth unhindered until they reach the mature forms since chloroquine is effective against immature forms than the mature ones[16,17]. It has been observed that in post-treatment resistant groups, the dynamics of gametocyte carriage was significantly higher on days 7 and 14 showing potential implications for the use of chloroquine associated with the higher prevalence of gametocytaemia in the follow up periods because of recrudescence of CQ-resistant parasites[16,17].

Parasite stage specificity of *P. falciparum* blood smears revealed a mean gametocyte prevalence of 57 per cent, which was much higher than recorded from other parts of India. Gametocyte prevalence of 31 per cent has been reported from the State of Gujarat and Koraput, Orissa, India[18,19]. Hogh and coworkers[14] have reported 59.5 per cent gametocyte prevalence in a malaria endemic area of Mozambique.

Gametocyte density was also measured in *P. falciparum* cases to investigate the persistent malaria in Laksar area. Our data showed that 13.39 per cent cases had high gametocyte density ranging from 960-8480/µl blood while 29.1 per cent had gametocyte density with a range between 480-800/µl blood in their peripheral blood.

Strickland and coworkers[20] have reported that high density in endemic areas had an obvious influence on the intensity of mosquito infection and ultimately on malaria transmission[1,15]. Mean sex-ratio of 3.22 female per male gametocyte was recorded in the present study. It is to note that gametocyte sex-ratio is expected to vary with inoculation ratio[21]. A sex-ratio of 1:3 has also been reported[19] in an endemic area of Koraput district Orissa, India.

We observed a high density of *P. falciparum* gametocyte along with different parasite stages at the time of preparation of blood slide on day 0. Gametocytes generally appeared in the blood after the nutrient crisis and one week after treatment. This clearly suggests that most of the *P. falciparum* malaria patients took inadequate chloroquine therapy or these cases were resistant to chloroquine. *In vivo* follow up of *P. falciparum* cases after treatment with chloroquine showed that only 35 per cent cases were resistant to chloroquine. During discussion with health authorities, it was found that the time gap between presumptive and radical treatment was more than a week. Moreover, patients used to get inadequate treatment from private practitioners due to poor medical services. Sowunmi and Fataye[2] reported that following chloroquine treatment, gametocyte carriage was significantly higher and gametocyte density was also higher in children with chloroquine resistant than in those with sensitive infections. Therefore, improper chloroquine treatment along with poor patient compliance for radical treatment and the presence of chloroquine resistance *P. falciparum* malaria may have enhanced the prevalence and density of *P. falciparum* gametocytes besides their natural epidemiological factors. These may be the reasons responsible for persistent malaria in the study area.

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Reprint requests: Dr V.K. Dua, Scientist F & Officer in-Charge, National Institute of Malaria Research Field Unit
Sector III Health Centre, BHEL, Ranipur, Hardwar 249 403, India
e-mail: vkdua51@gmail.com