High prevalence and gender bias in distribution of *Plasmodium malariae* infection in central east-coast India

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Abstract. Light microscopy, the mainstay of malaria diagnosis in epidemiologic studies, exhibits limited sensitivity for detecting low level infections and often under-estimates the frequency of mixed *Plasmodium* species infections. To overcome these shortcomings we performed the PCR method for detection and identification of *Plasmodium* species in blood specimens from 242 individuals collected during the peak season of malaria incidence (July – October). Malaria prevalence was 81.4% and 43.4% by PCR and microscopy respectively. Moreover, while PCR detected *Plasmodium malariae* DNA in 108 (44.6%), microscopic examination detected only 20 (8.3%) individuals parasitized with this species. Further data analysis revealed an independent random distribution pattern of parasites irrespective of age groups (0-5 yrs, chi-square$_{7df}$ = 2.77, P > 0.95; 6-15 yrs, chi-square$_{7df}$ = 4.82, P > 0.50; >15 yrs, chi-square$_{7df}$ = 4.4, P > 0.70) and sexes (for male chi-square$_{7df}$ = 2.48, P > 0.95; for female, chi-square$_{7df}$ = 1.85, P > 0.95). However, although the parasite distribution is random irrespective of sex, females had more *P. malariae* infections (P = 0.004, OR = 2.312, 95% CI = 1.3-4.1). Our study demonstrates that the parasite distribution in Orissa is random with substantially higher prevalence of *P.malariae* than previously suspected and this may be seasonal. A study of the bionomics of vector(s) responsible for *P. malariae* transmission in Orissa is needed to provide information for the control of malaria in the state

INTRODUCTION

Malaria is a major public health problem in the tropical and sub-tropical regions, inhabited by nearly 50% of the world's population. Current estimate describes about 250 million malaria cases annually, leading to approximately one million deaths, mostly amongst children under 5 years of age. The vast majority of cases (86%) are in the African region, followed by the South-East Asia (9%) and Eastern Mediterranean (3%) regions. In the Southeast Asian region of WHO, of ~1.4 billion people living in 11 countries, 1.2 billion are exposed to the risk of malaria, most of whom (60%) live in India (WHO, 2008). In India, according to the National Vector Borne Disease Control Programme (NVBDCP), approximately 1.7 million cases of malaria were reported during 2003, of which Orissa state alone contributed 24% of total malaria cases.

Although Orissa state has about 3.6% of the total population of the country, it accounts for 47% of *Plasmodium falciparum* cases and 34% of all reported deaths due to malaria, which is the highest in India (Orissa Vision, 2010).The climate of the state is favorable for the perennial transmission of malaria in many parts and all four *Plasmodium* species of human malaria have been reported from the state (Ranjit, 2006). *P. falciparum* is the major cause of malaria in the state (>80%) followed by *Plasmodium vivax* (10-15%) which is more prevalent in coastal tract (Das *et al.*, 1989; Subramanian *et al.*, 1991). This is in contrast to the national situation, where *P. vivax* is responsible for 60-65% and *P. falciparum* for 30-35% (Sharma, 1998). *Plasmodium malariae* has been reported (up to 3%) in certain foothill areas (Das *et al.*, 1989; Yadav *et al.*, 1990) and the presence of *Plasmodium ovale* is restricted to two villages of Koraput district of the state, the

later being very rarely found outside Africa (Jambulingam *et al.*, 1989). Most of the malariometric surveys with regard to the epidemiology of malaria are based exclusively on light microscopy diagnosis (LM). Unfortunately, the differentiation of *P. malariae* and *P. ovale* from *P. falciparum* and *P. vivax* by LM can be challenging (Shute, 1988). Further, low parasitemias, which are commonly observed for *P. malariae* and *P. ovale*, require evaluation of thick-films; however, the hemolysis of erythrocytes during the preparation of slides impairs the correct identification of malaria parasites often leading to a mistaken identification of *P. malariae* as *P. vivax* (Scopel *et al.*, 2004). Therefore, in endemic regions where *P. falciparum* and/or *P. vivax* predominate, *P. malariae* and *P. ovale* are frequently overlooked. The accurate detection and the correct identification of malaria parasites are crucial, since the drug of choice for a successful treatment of malaria depends on the *Plasmodium* species involved. In recent years, the PCR method for detection and identification of the *Plasmodium* species was shown to be more sensitive and specific particularly in mixed infections (Snounou *et al.*, 1993; Coleman *et al.*, 2006). Thus, in the present study an attempt was made to find out the prevalence of malaria species by PCR diagnosis in Orissa.

MATERIALS AND METHODS

Study Area and Study Design

The study was conducted in the state of Orissa, which is considered as hyperendemic for malaria and the transmission is perennial with a seasonal peak from July to October. The total area of the state is 155,707 sq km and is divided into 30 administrative districts harbouring 3.74% of the country's population. According to the Forest Survey of India - 2001, about 30.2% of the state is covered with the forest. The average number of rainy days is 60-90 days with mean annual rainfall of 1482mm. The mean maximum temperature is between 32.8ºC and 45ºC in April-May and the minimum is between 22.8ºC and 11ºC in December (Source: Meteorology Department, Orissa). In the present study, a cross-sectional malaria survey was carried out from July to October in the year 2008 in eight districts (Mayurbhanj, Sundergarh, Keonjhar, Nayagarh, Rayagada, Kalahandi, Kandhamal and Anugul) of Orissa. Approximately 1ml of whole blood was collected (from people complaining of malaria fever and who volunteered to participate in the malaria prevalence survey) by venepuncture into EDTA vials with informed consent. Thick and thin blood smears were made before aliquoting the blood in to the collection vials and were stained with 2.5% buffered Giemsa (pH 7.2) for 35 minutes and examined by light microscopy (LM). Slides were declared negative if no parasites were seen in 100 thick film fields and a case of malaria was defined as the occurrence of fever (axillary temperature > 37.5ºC) with a positive blood slide for malaria (irrespective of parasite density). The study was approved by the Ethics Committee of the Regional Medical Research Centre, Bhubaneswar.

DNA Isolation and PCR diagnosis

The parasite genomic DNA was purified from 100µl of blood following the standard protocol (Sambrook & Russel, 2001). In brief, blood cells were lysed with lysis buffer (10 mM Tris-HCI, pH 8.0; 0.1M EDTA, pH 8.0; 0.5%SDS, and 20µg/ml pancreatic RNase) at 37ºC for 1 hour and then proteinase K (100µg/ml) was added and the lysate was incubated at 54ºC for 3 hours. DNA was obtained by Phenol-Chloroform extraction and ethanol precipitation and then resuspended in 50µl of DNase free water. The extracted DNA was used as template to amplify the parasite DNA as described by Snounou *et al.* (1993). Separate reactions were carried out using species-specific oligonucleotide primers with every sample for the detection of each species in a reaction volume of 20µl. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide (0.5µg/ml) staining.

Statistical analysis

The prevalence of individual species infections was determined by enumerating the positive samples and dividing by the total study populations. The expected number of parasite species infection assemblages (i.e., no infection, single species infection or mixed species infection) was determined by using multiple-kind lottery model (Janovy *et al.*, 1995), which assumes (i) accusation of infection is an independent event, and (ii) one possible outcome of exposure is no acquisition of infection. The $\chi 2$ test was used to assess the significance of differences in proportions.

RESULTS

Detection of *Plasmodium* species by thick blood smear and PCR

A total of 242 individuals from eight districts of Orissa had their blood evaluated for the presence of malaria parasites by both microscopic examination and species-specific PCR method of malaria diagnosis. The microscopic examination showed a malaria prevalence of 43.4% (105 positive individuals), including single infections by *P. falciparum* (26.5%), *P. vivax* (3.3%), *P. malariae* (4.5%), and (9.1%) of all the mixed infection (Table 1). On the other hand, positive PCR results, indicative of *Plasmodium* infections, were detected in 197 (81.4%) individuals. While the PCR detected *P. malariae* DNA in 108 of the samples, reaching a prevalence of 44.6% in the total population, microscopic examination detected only 20 (8.3%) individuals parasitized with this species (Table 1). Further, PCR detected high percentage of double- (26.1%) and triple-positive (2.0%) reactions involving *P. malariae* compared to microscopy which detected 3.2% and 0.4% respectively (Table 1). However, the same prevalence of single infections by *P. falciparum* (around 27%) and *P. vivax* (3.3%) was verified by both methods (Table 1). Further, 28 cases of single infection reported by microscopy were positive for more than one species of *Plasmodium* by PCR with 20 (71.4%) *P. falciparum* cases identified as *P. falciparum/P. malariae*, 5 *P. malariae* and 2 *P. vivax* (25%) as *P. vivax/P. malariae* and 1 *P. falciparum* (3.6%) as *P. falciparum/P. vivax/P. malariae*. Moreover, five cases of *P. malariae* infections were incorrectly identified by microscopic examination of thick blood smears as single infection due to *P. falciparum* (7.8%). All the *P. malariae* infections detected by microscopy were confirmed by PCR and no discrepancy in the identification of *P. vivax* as *P. falciparum* was found in the present study. These results suggest a higher sensitivity and accuracy of

Table 1. Comparison between prevalence rates of *Plasmodium* species detected by microscopic examination of thick blood smears and PCR in 242 individuals of Orissa state

PCR results	No of cases identified by microscopy								No of cases by PCR
	Pf	Pv	Pm	Pf+Pv	Pf+Pm	Pv+Pm	F+v+m	_ve	
Pf	38(15.7)	0	0	0	0	0	0	2(0.8)	66(27.3)
Pv	0	6(2.5)	0	0	0	0	0	0	8(3.3)
Pm	5(2.06)	0	6(2.5)	0	0	0	0	29(12.0)	40(16.5)
Pf+pv	0	0	0	13(5.4)	0	0	0	2(0.8)	15(6.2)
Pf+Pm	20(8.3)	0	0	0	5(2.06)	0	0	49(20.2)	54(22.3)
Pv+Pm	0	2(0.8)	5(2.06)	0	0	3(1.2)	0	7(2.9)	10(4.1)
Pf+Pv+Pm	1(0.4)	0	0	0	0	0	1(0.4)	4(1.6)	5(2.0)
Negative	0	0	0	0	0	0	0	44(18.2)	44(18.2)
Total	64(26.5)	8(3.3)	11(4.5)	13(5.5)	5(2.06)	3(1.2)	1(0.4)	137(56.6)	242

the PCR method for *Plasmodium* detection than the microscopic examination, particularly when mixed with *P. malariae* infection. None of the sample was found positive for *P. ovale* by either of the methods. Our results show that the prevalence of *P. malariae* in this endemic area can be substantially higher than previously suspected.

Age wise distribution of *Plasmodium* species infections

To evaluate whether prevalence of infection assemblages (i.e., no infection, single-species infections, or mixed-species infections) within the study population deviated from an independent random distribution pattern (null hypothesis), the multiple-kind lottery model (MKL) was applied to the microscopy and PCR diagnostic assay data. The observed and expected numbers of infections were not significantly different whether diagnosed by blood smear microscopy or PCR (microscopy, chi-square$_{7df}$ = 9.33, P > 0.2; PCR, chi-square$_{7df}$ = 3.85, P > 0.7). If interactions occurred, departures from the expected values would have been observed. The PCR diagnostic assay data was further analyzed to determine whether the random distribution of parasite species was altered when the studied population was categorized into age groups. For this analysis, subjects of the studied population were categorized into 3 age groups (0-5yrs, 6-15yrs and >15yrs). Comparison of observed and expected number of infections in all age groups (Table 2) showed no departures from the expected values (for 0-5 yrs, chi-square$_{7df}$ = 2.77, P > 0.95; for 6-15 yrs, chi-square$_{7df}$ = 4.82, P > 0.50; and for >15 yrs, chi-square$_{7df}$ = 4.4, P > 0.70), indicating an independent random distribution of *Plasmodium* species in Orissa.

Gender wise distribution of Plasmodium species infections.

A comparison of observed and expected number of infections by gender did not show any significant difference (for male chi-square$_{7df}$ = 2.48, P > 0.95; for female, chi-square$_{7df}$ = 1.85, P > 0.95), indicating no deviation from random distribution pattern of the malaria parasites (data not shown). However, the prevalence of *P. malariae* was observed to be significantly high (P = 0.004, OR = 2.312, 95% CI = 1.3-4.1) among female (61 out of 93 positive samples) compared to male (47 out of 104 positive samples). This indicates, though the parasite distribution is random irrespective of age and sex, female

Table 2. Number of *Plasmodium* infections in different age-group categories detected by PCR assay

Parasite assemblage	0-5yrs			6-15yrs			>15yrs		
	O	E	χ^2	O	E	χ^2	O	E	χ^2
Pf	7	7.18	0.004	18	22.16	0.78	41	35.96	0.71
Pv	0	1.4	1.4	3	2.57	0.07	5	4.16	0.17
Pm	2	1.4	0.26	6	7.66	0.36	32	30.6	0.06
Pf+Pv	4	2.87	0.44	8	5.83	0.81	3	4.33	0.41
Pf+Pm	2	2.87	0.26	22	17.42	1.2	30	31.86	0.11
Pv+Pm	1	0.56	0.34	2	2.0	0	6	3.68	1.46
Pf+Pv+Pm	1	1.15	0.02	2	4.58	1.45	2	3.83	0.87
NA	4	3.57	0.05	11	9.78	0.15	30	34.58	0.61

O = Observed, E = Expected, Pf = *Plasmodium falciparum*, Pv = *P. vivax*, Pm = *P. malariae*
For 0-5 yrs, chi-square$_{7df}$ = 2.77, P > 0.95
6-15 yrs, chi-square$_{7df}$ = 4.82, P > 0.50
and for >15 yrs, chi-square$_{7df}$ = 4.4, P > 0.70

Table 3. Distribution of *Plasmodium* species infection prevalence according to sex

Sex	Sample size	Total +ve cases	Pf	Pv	Pm	Mixed infections			
						Pf+Pv	Pf+Pm	Pv+Pm	Pf+Pv+Pm
Male	128	104 (81.2)	42 (32.8)	5 (3.9)	15 (11.7)	10 (7.8)	24 (18.7)	5 (3.9)	3 (2.3)
Female	114	93 (81.6)	24 (21.0)	3 (2.6)	25 (21.9)	5 (4.3)	30 (26.3)	4 (3.5)	2 (1.7)
Total	242	197 (81.4)	66 (27.3)	8 (3.3)	40 (16.5)	15 (6.2)	54 (22.3)	9 (3.7)	5 (2.0)

Pf = *Plasmodium falciparum*, Pv = *P. vivax*, Pm = *P. malariae*
Numerals in parenthesis are percent values

are more susceptible to be infected with *P. malariae* (Table 3).

DISCUSSION

Light microscopy (LM), the mainstay of malaria diagnosis in epidemiologic studies, exhibits limited sensitivity for detecting low level infections (WHO, 2000; Ohrt *et al.*, 2002; McKenzie *et al.*, 2003) and is known to under-estimate significantly the frequency of mixed *Plasmodium* species infections (Mehlotra *et al.*, 2000, 2002). To overcome this, the PCR method which is more sensitive and specific in the malaria diagnosis particularly when more than one species are involved in the same individual (Snounou *et al.*, 1993; Coleman *et al.*, 2006) was used in this study. Our results showed a high prevalence of *P. malariae* single infection (16.5%) and mixed infection with *P. falciparum* (24.4%) and *P. vivax* (5.8%) (Table 1). Although the distribution of *P. malariae* infection is reported as being patchy, it has been observed in all major malaria-endemic regions of the world (Haworth, 1988). In Asia, it is observed as an infrequent infection, with blood-smear LM prevalence rarely exceeding 1–2% (Gordon *et al.*, 1991; Ghosh & Yadav, 1995; Kawamoto *et al.*, 1999). This is consistent with previous epidemiological studies in which *P. malariae* infections have been reported as being up to 3% in certain foot hill areas of the state (Das *et al.*, 1989; Yadav *et al.*, 1990). However, in the present study 8.3% of individuals were positive for this species by light microscopy while PCR detected a prevalence of 44.6% (Table 1). The low prevalence of *P. malariae* by LM could be due to morphologic variations that may contribute to misdiagnosis or density of parasites to an undetectable level. However, the seasonal incidence of the high prevalence of *P. malariae* (by microscopy and PCR compared to earlier studies of about 3%) cannot be ruled out, as the samples were collected during the peak seasons of malaria transmission (July-October) in the state.

Most of the previous epidemiological studies involving species co-occurrence have recorded fewer mixed infections in endemic individuals than expected by chance, suggesting dominance of one species over others (Knowles & White, 1930; Rosenberg *et al.*, 1990; McKenzie & Bossert, 1999). Other mechanisms may include innate host factors that regulate parasite density (Bruce *et al.*, 2000) and acquisition of heterologous (or cross-species) immunity (Cohen, 1973; Richie, 1988). To determine whether malaria exposure influence acquisition of immunity and species distribution in this hyperendemic region, data analysis was performed using MKL model (Janovy *et al.*, 1995) which assumes that acquisition of a species infection to be an independent event. The results revealed all the three observed *Plasmodium* species (*P. falciparum*, *P. vivax* and *P. malariae*) were randomly distributed in blood-stage infections whether diagnosed by blood smear or PCR. This random pattern of parasite distribution was also observed when the studied subjects were categorized by age groups (Table 2) and gender (Table 3). These results suggest that *Plasmodium* species blood-stage infections occur independently of one another and that

age-dependent, acquired immunity against malaria may have limited influence on the distribution pattern of parasite species (Mehlotra *et al.*, 2002). Because clinical data and parasite densities were not recorded during this study, we were not able to associate parasite density in different age groups and assess the impact of mixed-species infections on clinical manifestations of malaria. Moreover, sex wise data analysis for parasite prevalence shows that, though the parasite distribution is random irrespective of sexes, females were more susceptible to get infected with *P.malariae* (Table 3). In the absence of any specific reasons for this bias prevalence of *P. malariae*, the plausible explanation may be due to the indoor biting property of the vector(s) responsible for *P. malariae* transmission in this hyperendemic region. Although the vectors responsible for *P. malariae* transmission in Orissa is not known, the higher prevalence of *P. malariae* in females may be due to higher exposure to the indoor biting vectors involved. However, many studies have suggested that human genetic polymorphisms may influence susceptibility to infection by *Plasmodium* species parasites besides disease severity. (Zimmerman *et al.*, 1999; Patel *et al.*, 2001; Hill, 2001). Because of the intimate relationship between these human polymorphisms and malaria endemicity, the influence of these polymorphisms on the distribution of *Plasmodium* species needs to be studied.

In conclusion, our study demonstrates that the parasite distribution in Orissa is random with substantially higher prevalence of *P. malariae* than previously suspected. As the study was carried out during the peak malaria transmission period, similar year-round investigation of malaria prevalence in the state should be carried out. Furthermore, the bionomics of vector(s) responsible for *P. malariae* transmission in Orissa should be studied to explain the relatively higher prevalence of the infection in females.

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