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The Prevalence of *P. malariae* in Odisha, India

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Abstract. Odisha, an eastern Indian state, is known to be highly endemic for malaria. The current national malaria control programme uses rapid diagnostic test (RDT) for early diagnosis and prompt treatment of the cases followed by microscopy for the identification of species. But as per the available literatures and from our own experience it is known that microscopy suffers with underestimation of mixed *Plasmodium* species infection, especially *P. malariae* and *P. ovale*. Since PCR has proven to have more sensitivity and specificity and able to detect multiple infection of *Plasmodium* species, we have made an attempt to find out the prevalence of species of parasites by PCR and compare the results with light microscopy. During this study 1589 individuals presenting with fever from two malaria endemic blocks namely Badampahar and Ghatgaon were investigated. Amongst them 110 cases were found to be PCR positive while 99 were microscopically positive for malaria. Out of the total PCR positive cases 42 cases (38.2%) had *P. falciparum*, 35 (31.8%) had *P. vivax* and 10 (9.1%) had *P. malariae* mono infections, while 23 (20.9 %) cases had mixed infections. Of the total 23 mixed infections 11(10%) were having *P. falciparum* plus *P. vivax* and rest 12 (10.9%) cases were having *P. malariae* mixed with either *P. falciparum* and /or *P. vivax*. However microscopically only 1(1.4%) case was found to have *P. malariae* mono infection and none of the mixed infections harbor *P. malariae*. Multiple kind lottery model analysis of PCR positive samples demonstrated that *P. malariae* is not distributed randomly in population when compared by different age groups, gender and season. The study also has shown that PCR is highly effective in screening the severe and asymptomatic malaria cases than LM. The result of this study warrants newer surveillance strategies for case detection and management by the programme.

INTRODUCTION

Elimination of malaria largely depends on accurate diagnosis of the *Plasmodium* spp. for the adequate treatment and block transmission. However, examination of Giemsa-stained thick smears (GTS) by microscope is often the only diagnostic method available to confirm the species detected by rapid diagnostic test (RDT) in the field in many endemic countries including India. GTS is sensitive, but morphological changes induced by haemolysis during processing of the sample impair the correct identification of infecting species. *Plasmodium malariae* and *Plasmodium ovale* are especially difficult to identify if the typical morphology of the infected erythrocytes is damaged. Giemsa stained thin smears, in addition to GTS, are recommended for species diagnosis, but very few peripheral laboratories can use both methods as routine because of overburden of work. As a result, currently available estimates of the global prevalence and distribution of *P. malariae* and *P. ovale* (Haworth, 1988; Molineaux, 1988) largely based on GTS-based official reports, might be a gross underestimate. Alternative diagnostic methods, such as PCR (Brown *et al.*, 1992; Snounou *et al.*, 1993a ; Li *et al.*, 1995; Arai *et al.*, 1994; Arai *et al.*, 1996) and acridine orange (AO) fluorescence microscopy (Kawamoto, 1991; Kawamoto & Billingsley, 1992) have been developed recently. Field surveys using these diagnostic methods have revealed high

proportions of mixed infections involving *P. malariae* or *P. ovale*, in Africa, Southeast Asia (Brown *et al*., 1992; Snounou *et al*., 1993b,c) and Oceania (Arai *et al*., 1994; Arai *et al*., 1996) where most of them were previously missed by GTS. Hence during the present study from July 2012 to October 2014 an attempt was made to detect prevalence of *Plasmodium malariae* and other species of malaria using PCR and conventional light microscopy (LM) among the symptomatic as well as asymptomatic malaria infections in two highly malaria endemic tribal dominated blocks of Odisha, India. The results of this investigation would help further case surveillance strategies in the region and additionally all endemic settings of India, as an essential approach towards achieving malaria elimination all over the country.

METHODS

Study area

Ghatagaon CHC of Keonjhar District and Badampahar CHC of Mayurbhanj District were selected for the study because of high endemicity of malaria and tribal population. Both the blocks are mountainous, semi forested and lies an altitude of >1000 ft with an annual rainfall of about 1100mm. The total population of Ghatagaon CHC is 102117 and Badampahar is 82188 (Census of India, 2001). Out of total population more than 60% belongs to tribal communities in both the areas and depends mainly on agriculture.

Study participants and sample collection

Patients with fever attending the OPD of the CHC hospitals of the corresponding blocks and fever treatment depots in villages of the selected sub centers were screened for malaria using bivalent ICT kit (Advantage PAN+ *Pf* Card, J Mitra & Co). The patients found to be ICT positive were selected for the study. The purpose of this research study was explained in detail to all enrolled patients in local language. Finger prick blood (100 µl) was collected aseptically in Whatman #3 filter paper as well as microscopic slide from the cases found to be ICT positive for species identification by PCR and microscopy. The study was approved by the institutional ethics committee of the centre.

Detection of malaria and species of identification

Light microscopy

Microscopic examination of thick and thin blood smears were done to diagnose the species of parasite and their density. Briefly blood smears were fixed with methanol (thin smear only) and stained with Giemsa (MERCK). Smears are initially graded for the presence/absence of *Plasmodium* and density of parasites was determined subsequently from positive smears by both percentage of parasitemia and number of asexual parasites/µl blood.

PCR diagnosis

The genomic DNA was extracted from 20µl finger prick blood collected in 3MM Whatman filter paper as described by (Bereczky *et al*., 2005). Briefly the punched filter paper were incubated at 4ºC in 1ml of 0.5% saponin in PBS and then washed for 30 minutes in PBS. After washing the filter paper were transferred to new tubes containing 25µl of stock solution (20% chelex-100) and 75µl of distilled water and vortexed for 30 seconds followed by heating at 99ºC for 15 minutes and centrifugation at 10,000 rpm for 2 minutes. The supernatant contains DNA. 15µl of it was used for PCR amplification. The extracted DNA will be 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 containing 4ng parasite DNA, 2 pmol of each primer, 1x Taq polymerase buffer containing 1.5m mol/L $MgCl_2$ and 1 unit of Taq DNA polymerase enzyme. The amplified products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide (0.5µg/ml) staining and Gel Documentation system was used to document the results.

Statistical Analysis

The statistical analysis was performed using Graph Pad Prism software. The multiple-kind lottery model (Janovy *et al.*, 1995) was used to calculate the expected distribution of parasite species assemblages in the population. Chi-square values were calculated using het-erogeneity tests (number of rows X number of columns) to compare observed versus expected values.

RESULTS

During the period under report a total number of 1589 fever cases (Ghatgaon: 974 and Badampahar: 615) suspected to be malaria were screened by bivalent RDT for malaria in selected sub-centers and patients attending malaria clinic at CHC hospitals. Out of 1589 fever cases screened for malaria 163 (10.3%) cases were found to be RDT positive. But when these samples were subjected to confirmation, PCR showed 110 out of 163 (6.9%) to be positive and microscopy 99 out of 163 (6.2%) to be positive for malaria (Table 1). On comparison of the efficacy of the three different methods used for the detection of malaria in two malaria endemic blocks, it was observed that the sensitivity and specificity of RDT was >90% and sensitivity and specificity of PCR was >99% compared to microscopy (gold standard).

Table 2 shows that out of 110 PCR-confirmed malaria cases, 87 cases (79.1%) were having mono-infection means harboring any one of the parasite species (*P. falciparum*: 29.1%; *P. vivax*: 31.8%; *P. malariae*: 9.1%), while 23 (20.9%) had mixed infections means harboring more than one parasite species (*P. falciaprum* + *P. vivax*: 10.0%, *P. falciparum* + *P. malariae*: 3.6%, *P. vivax* + *P. malariae*: 2.7% and *P. falciparum* + *P. vivax* + *P. malariae*: 4.5%). But most importantly out of 99 microscopically confirmed malaria samples only 1 (1.4%) case was found to be positive for *P. malariae* mono-infection. When compared the results between two areas no significant difference was observed in prevalence of species irrespective of methods used. The present study indicates that mixed infections were more common than it was expected. To evaluate whether the prevalence of infection within study groups deviated from an independent random distribution pattern (null hypothesis), the multiple kind lottery model was applied. The observed prevalence of parasite assemblage by LM and PCR indicates that observed prevalence by PCR had deviated significantly in heterogeneity test (χ^2=35.2, df=7, p<0.0001) from expected value which indicates that the PCR is more efficient in detecting *P. malariae* than microcopy.

Season wise distribution of malaria parasites indicates that the prevalence of *P. falciparum* and *P. vivax* were found in all seasons but *P. malariae* was more during the rainy season indicating seasonal prevalence (χ^2=26.43, df=7, p=0.0004). Further the parasites were found be more among the adult population (>15 years) during the period of investigation (χ^2=23.5, df=7, p=0.0013).

During the study period a total number of 17 cases (Male: 14, Female: 3) were found to be admitted in the hospital with severe manifestations (according to WHO criteria) from both the areas. The median age of the

Table 1. Screening of acute febrile cases for malaria by different methods

Area	No of samples collected	Microscopy No + ve (%)	RDT No +ve (%)	PCR No +ve (%)
Badampahar	615	29 (4.7)	60 (9.8)	33 (5.4)
Ghatgaon	974	70 (7.2)	103 (10.6)	77 (7.9)
Total	**1589**	**99 (6.2)**	**163 (10.3)**	**110 (6.9)**

Table 2. Species wise distribution of malaria parasites detected by Microscopy and PCR

Area	Microscopic Examination								PCR Identification							
	Total	*Pf*	*Pv*	*Pm*	*Pf*+ *Pv*	*Pf*+ *Pm*	*Pv*+ *Pm*	*Pf*+ *Pv*+*Pm*	Total	*Pf*	*Pv*	*Pm*	*Pf*+ *Pv*	*Pf*+ *Pm*	*Pv*+ *Pm*	*Pf*+ *Pv*+*Pm*
	No	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
Badampahar	29	19 (65.5)	8 (27.6)	0 (0.0)	2 (6.9)	0 (0.0)	0 (0.0)	0 (0.0)	33	11 (33.3)	17 (51.5)	1 (3.0)	3 (9.0)	0 (0.0)	0 (0.0)	1 (3.0)
Ghatgaon	70	53 (75.7)	12 (17.1)	1 (1.4)	4 (5.7)	0 (0.0)	0 (0.0)	0 (0.0)	77	31 (40.3)	18 (23.4)	9 (11.7)	8 (10.4)	4 (5.2)	3 (3.9)	4 (5.2)
Total	**99**	**72 (72.7)**	**20 (20.2)**	**1 (1.0)**	**6 (6.1)**	**0 (0.0)**	**0 (0.0)**	**0 (0.0)**	**110**	**42 (38.2)**	**35 (31.8)**	**10 (9.1)**	**11 (10.0)**	**4 (3.6)**	**3 (2.7)**	**5 (4.5)**

Pf: *P. falciparum*, *Pv*: *P. vivax*, *Pm*: *P. malariae* *Pf+Pv*: *P. falciparum mixed with P. vivax*, *Pf+Pm*: *P. falciparum mixed with P. malariae*, *Pf+Pv+Pm*: *P. falciparum mixed with P. vivax and P. malariae*

severe patients was 35.3 years (range 15-65 years). Amongst the severe cases acute renal failure (ARF) alone was present in 6 (35.3%) cases, while ARF was co-existed with cerebral malaria (CM) in 5 (29.4%) cases, with jaundice in 4 cases (23.5%), with respiratory distress in 1 case (5.9 %) and with CM and jaundice in 1 case (5.9 %). When the blood samples from these severe patients were subjected to diagnosis for malaria by PCR and Microscopy, PCR detected 6 (35.3%) cases to be positive for malaria (*P. falciparum*: 4 and *P. falciparum* + *P. malariae*: 2) while no case was found to be microscopically positive for malaria. But all cases were ICT positive and responded well to the antimalarial therapy administered by the hospital as per the NVBDCP guideline. Similarly a total of 387 asymptomatic and microscopically negative cases (Ghatgaon: 208, Badampahar: 179) were screened for malaria. Out of them 5 (1.3%) cases were found to be positive for malaria by RDT as well as PCR. Among PCR confirmed cases 2 (40%) were positive for *P. malariae* and 3 (60%) were positive for *P. falciparum*. All infected subjects were treated with standard regimen of ACT after diagnosis.

DISCUSSION

In the elimination phase of malaria control, monitoring of all cases particularly hidden parasites and accurate species identification is of major importance for institution of appropriate treatment. This highlights choosing of an acceptable detecting method, which should be, applied in all incoming asymptomatic investigation afterwards in order to detect every single carrier which continue disease transmission in the population.

Our study reveals that species specific PCR detects at least 1.2 times more active infections of malaria than LM but 1.5 times less than the RDT. The increased frequency of case detection by PCR than LM might be because an expert microscopy could theoretically detect 10 p/µL parasitemia, but average microscopy has proven to be unable in detecting lower than 100 p/µL parasitemia (Coleman *et al.*, 2002). On the other hand the high number of positivity in case of RDT during the present investigation might be due to persistence of parasite antigenaemia in the circulation after successful standardized antimalarial therapy as reported earlier (Iqbal *et al.*, 2004). While considering detection of *P. malariae* the PCR is 22 times more efficient than the LM. In our study when PCR detected around 22% of *P. malariae*, microscopy could detect 1.0%. Further use of PCR shows 2.1 fold increases in the prevalence of mixed infections than LM. Consistent with our observations, when patients have been studied using PCR-based methods in Africa, PNG and Brazil, similar large increases in *P. malariae* prevalence has been detected. In two African studies, PCR prevalence of *P. malariae* was 23.3% and 39.2. By contrast, LM detected no non-*P. falciparum* infections in the first study and 18.7% *P. malariae* in the second (Rubio *et al.*, 1999). In the Thai–Myanmar border area, up to 25% of malaria patients were observed to carry subpatent *P. malariae* infections, whereas 4% showed evidence of subpatent *P. ovale* infections (Zhou *et al.*, 1998). These observations were repeated in many other Southeast Asian settings, with an average of 16.6% and 3.5% of malaria patients harboring *P. malariae* and *P. ovale* parasites, respectively (Kawamoto *et al.*,1999). This confirmed that *P. malariae* is common across much of the region. Considering the problems with LM diagnosis, the actual burden of illness could be markedly underestimated and PCR diagnosis studies of clinical cases are thus needed.

The climate of Odisha, an eastern Indian state, seems to be favorable for the perennial transmission of malaria. All four species of human malaria have been reported from the state. Of which the *P. ovale* is the rarest and *P. falciparum* is the commonest (>80%) followed by *P. vivax* (10–15%) and *P. malariae* (4–3%) like that in Tanganyika, Africa and among the imported malaria cases in the erstwhile USSR between 1963–1973 (Ranjit, 2006). In our earlier survey carried out during July to October during

2008 we had detected 108 *P. malariae* infections by PCR (>60%) out of 212 positive cases in Mayurbhanj, Sundergarh, Keonjhar, Nayagarh, Rayagada, Kalahandi, Kandhamal and Angul district. Majority of them were found to be co-infected with *P. falciparum* or *P. vivax* (Dhangdamajhi *et al.*, 2009). Since 2009 Odisha has revised its treatment policy by adopting ACT as the first line of treatment in case of *P. falciparum* and CQ +PQ for *P. vivax* and screenings of cases are being done by using RDK in the national program. After introduction of the new strategy the incidence of malaria has come down drastically in both the study areas. In Keonjhar the API has come down from 17.08 (2008) to 10.85 (2012) and in Mayurbhanj the API has come down to 3.25 (2012) from 4.82 (2008) (unpublished report of NVBDCP, Odisha 2013). Even then during the present survey we have detected the *P. malariae* mono infections in 9.0% of cases and mixed infections in 10.9% of cases by PCR. Most importantly these infections are often found as mixed infections as reported in West Africa and PNG. The age wise distribution of species of parasites indicates that the *P. malariae* was more prevalent in older age (>15 years) like *P. vivax* but unlike *P. falciparum* which is present in all age groups in both the study areas. In West Africa, *P. malariae* prevalence has been reported to peak at ages similar to those of *P. falciparum* (i.e. in children under ten years of age) and in PNG however, *P. malariae* infection is observed predominantly in older children (7–9 years) (Mueller *et al.*, 2007). The potential interactions of *P. malariae* with *P. falciparum* and *P. vivax* might explain some basic questions of malaria epidemiology and understanding these interactions could have an important influence on the deployment of interventions such as malaria vaccines.

Although considered mild *P. malariae* can cause a chronic nephrotic syndrome that, once established, does not respond to treatment and carries a high rate of mortality (Eiam-Ong, 2003).In addition, *P. malariae* is known to cause chronic infections that can last for years (White, 2003) and might reoccur decades after initial exposure when people have long since left endemic regions (Siala, *et al.*, 2005). The health burden of such chronic or reoccurring infections in an endemic context is not clear. Therefore our findings are very important in the current scenario of screen and treat policy of the program. Under this screen and treat strategy, presence of *P. malariae* and asymptomatic cases poses a difficulty if the screening is performed by RDT alone. Further in the present study the species specific RDTs were not able to detect neither the mono infection nor the mixed infections of *P. malariae*. Therefore the molecular method can be used as a tool for surveillance to overcome such problems.

CONCLUSION

The current results emphasize the importance of using molecular methods in order to interrupt malaria transmission and institute appropriate treatment in Odisha besides microscopy and RDTs as routine methods. It should be noted that towards eliminating malaria from the country and prevention of re-introduction, all the three types of malaria detection tools (microscopy, RDT and PCR) should be used to improve sensitivity, specificity and reliability for active case surveillance in endemic settings like ours.

Conflicts of interest

The authors have no conflicts of interest connecting to the work reported in this paper.

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