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RESEARCH ARTICLE

RECURRENCE PATTERN OF P. VIVAX MALARIA FOLLOWING TREATMENT WITH CHLOROQUINE EITHER ALONE OR IN COMBINATION WITH PRIMAQUINE IN URBAN KOLKATA, INDIA

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ABSTRACT

In India, *P. vivax* malaria is treated with chloroquine (CQ) for 3 days and primaquine (PQ) for 14 days to prevent relapse. Controversies regarding the role of PQ at the dose used exist globally. Different molecular markers have been studied to differentiate between recrudescence, relapse and re-infection but not well established.

This study aims to determine the role of PQ in preventing recurrent *P. vivax* infections.

Two hundred and three *P. vivax* mono-infected patients who responded either to CQ (Group A) or CQ+PQ (Group B) were followed upto one year to determine the rate of recurrent infections. Nested PCR-RFLP method was adopted to compare the size polymorphisms of *pvmsp1* and *pvcsp* genes of paired samples.

Recurrent vivax malaria was found 23 in Group A (26.7%) and 15 (16.5%) in Group B, without statistically significant difference (p=0.1034). Among 38 paired samples, 27 were with identical genotype and 11 were different. Thirty two recurrences were within 10 weeks and 6 after 10 weeks.

Recurrence rates in both groups were similar, so role of PQ at recommended dose in preventing relapse in *P. vivax* malaria is debatable. Hence, therapeutic efficacy studies of PQ with higher dose as advised by WHO is urgently required.

INTRODUCTION

Plasmodium vivax threatens almost 40% of the world's population, causing an estimated 72–390 million clinical infections each year (Mendis *et al*. 2001; Price *et al*. 2007). *Plasmodium falciparum* and *P. vivax* coexist and are often equally prevalent (Hay *et al*. 2004) throughout the malaria endemic regions of the world except in Africa, yet the public health importance of *P. vivax* is frequently overlooked (Baird JK. 2007). Recent calls for the global elimination of malaria have brought renewed vigour to malaria control programmes, but with this a realization that the challenges in controlling and eliminating *P. vivax* are far greater than those for *P. falciparum*. In areas where intensive control measures have been implemented, the relative proportion of malaria due to *P. vivax* usually increases when compared with *P. falciparum* (Nosten *et al*. 2000). The important biological difference of *P vivax*, the development of dormant liver stages (hypnozoites) causing recurrent blood stage infections (relapses) complicated the control programme of the parasite.

Chloroquine is still in use as a schizonticidal agent in *P. vivax* malaria in most part of the world except in areas with known chloroquine resistance (WHO. 2012). Primaquine is proven to be effective and licensed to, eliminate the hypnozoites of *Plasmodium vivax* and *Plasmodium ovale* (Hill *et al*. 2006). Though effective, Primaquine is also associated with serious side effects, such as haemolysis in glucose-6-phosphate dehydrogenase (G_6PD) deficient individuals (Ramos *et al*. 2010). In India primaquine is used for radical treatment of *P. vivax* at a dose of 0.25mg/kg/day for 14 days under supervision or by detecting the (G_6PD) level. Shorter courses of primaquine (5 days therapy) and lower doses (15 mg/day) have been shown to be ineffective in preventing relapses (Dua and Sharma. 2001; Yadav and Ghosh. 2002; Carmona-Fonseca and Maestre. 2009; Galappaththy *et al*. 2007; Villalobos-Salcedo *et al*. 2000; Fernandopulle *et al*. 2003). However experimental studies with different strains of *Plasmodium* have shown that the dose of 15 mg/day for 14 days is ineffective in preventing relapses (Hill *et al*. 2006). Therefore, the current recommendation of the Center for Disease Control and Prevention, USA for use of primaquine for radical cure stands at 0.5 mg/kg/day for 14 days (maximum of 30 mg/day) (Hill *et al*. 2006).

Several experimental and clinical studies have investigated vivax malaria infections acquired in different geographic and climatic regions and have demonstrated striking differences in their patterns of relapse (Kim *et al*. 2012; White NJ. 2011; WWARN. 2013; Battle *et al*. 2014). Strains of *P. vivax* from the tropics are characterized by an early relapse infection followed by a short latent period of 5-10 weeks. In contrast *P. vivax* strains of temperate zones are characterized by a variable period of recurrent infections followed by a long latent period (5 -10 months) (Contacos *et al*. 1972; Krotoski WA. 1985).

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In a therapeutic efficacy study differentiation between re-infection and recrudescence is an important issue. This is again complicated by the phenomenon of relapse in case of *P. vivax* malaria. Molecular markers have been studied extensively to solve the problem in case of *P. falciparum* malaria by genotyping the polymorphic markers such as the *msp-1*, *msp-2* and *Glurp* (Snounou and Beck. 1998; WHO. 2007). A similar approach has been adopted for *P. vivax* but it has been less well-studied. Three polymorphic *P. vivax* genes, *pvcsp*, *pvmsp*1 and *pvmsp*3-alpha have been widely used to understand the local population structure and genetic diversity, to distinguish between distinct clones within the same infection as well as between infections (Imwong *et al*. 2005; Imwong *et al*. 2007; Koefli *et al*. 2009; Zakeri *et al*. 2010; Raza *et al*. 2013). Reports on genetic diversity of *P. vivax* population within the same and also in recurrent infections followed by therapeutic efficacy study from India, particularly from this part of the country are scarce. The present study was designed to assess the recurrence pattern of *P. vivax* malaria and the role of Primaquine in preventing relapse in urban population of Kolkata.

MATERIALS AND METHODS

Study patients

In a previous therapeutic efficacy study conducted between December 2011 to August 2012, 250 *P. vivax* mono-infected subjects were recruited and followed-up for 42 days. The study was a randomized, double-arm, open-label, interventional trial for evaluation of the clinical and parasitological responses of CQ (Group A) and CQ plus PQ (Group B) for treatment of uncomplicated *P. vivax* malaria based on the 2009 WHO therapeutic-efficacy protocol (WHO. 2009). Out of the 250 patients recruited, 203 completed the 42-day follow-up, 100 in Group A treated with chloroquine and 103 in Group B treated with both chloroquine and primaquine. The study protocol was approved by the Institutional Ethics Committee of the Calcutta School of Tropical Medicine and registered in CTRI [Clinical Trial Registry-India] of the Indian Council of Medical Research under registration no. CTRI/ 2011/ 09/002031. The schizonticidal effects of chloroquine alone or with primaquine have been published in 2013 (Ganguly *et al*. 2013).

Follow-up

Two hundred and three patients (100 in Group A and 103 in Group B) classified as ACPR (adequate clinical and parasitological response) were again followed-up up to one year to study recurrent *P. vivax* infection. These patients were advised to report to the Malaria Clinic every month for clinical and/ or parasitological assessments. Members of the study team paid home visits to patients who missed the scheduled clinic visits for clinical examination and collection of blood samples for parasitological assessment. Those patients who could not be contacted even at their residence were contacted over phone to record their clinical condition and advised to attend the clinic on next scheduled date. The one year follow-up of these patients ended in June 2013 and the resulting data on the follow-up has been submitted to CTRI.

The patients with recurrent infections were treated with CQ and PQ as per the guidelines and were not further tracked.

DNA isolation and PCR amplification

DNA was extracted from EDTA whole blood using QiAamp DNA Mini Kit (QIAGEN, USA) according to manufacturer's instructions. In this study we, analysed the highly repetitive central domain of *pvcsp*, F1 and F3 fragments of *pvmsp1* gene to compare genotype of paired samples i.e. isolates of day 0 and day of recurrent infection. A nested and semi-nested PCR method for *pvmsp1* and nested PCR-RFLP method for *pvcsp* gene were used for the purpose. All amplifications were carried out in a total volume of 50 µl as described earlier (Imwong *et al*. 2005) with minor modifications.

PCR products were analysed by gel electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized under UV transilluminator (Gel Doc, Bio-Rad,Hercules, USA).

PCR-RFLP genotyping

Amplified products of *pvcsp* were digested separately with restriction enzymes, *Alu I* and *Bst NI* in a total volume of 20 µl for 3 hours according to the manufacturer's specifications (New England Biolabs Inc., UK) to know the parasite populations carrying VK 210 and VK 247 repeat types respectively. The digested products were analysed on 2% agarose gel following ethidium bromide staining.

The size polymorphisms in *pvmsp1* (F1& F3) and *pvcsp* genes were determined using Quantity One software (Biorad, Hercules, USA). Genotypes of *pvcsp* were determined on the basis of digestion with the restriction enzymes.

Amplified products of paired samples were run side by side on agarose gel. The homology between paired samples was identified on the basis of differences/ similarities in genotypes. Similar size polymorphisms in all 3 genetic markers (*pvcsp* and F1, F3 of *pvmsp1)* of paired samples were considered as recurrent infection with identical genotypes. Difference in any of the markers in paired samples was considered as recurrent infection with different genotypes.

RESULTS

Clinical episodes of recurrent malarial infection among study patients

In study Group A (CQ arm), 11 patients were lost to follow-up, 4 were infected with *P. falciparum* and 85 completed 1 year follow-up. In study Group B (CQ+PQ arm), 6 patients were lost to follow-up, 6 were infected with *P. falciparum* and 91 completed 1 year follow-up. The rate of recurrent infections in Group A was 26.7% (23/85) and in group B was 16.9% (15/91), the difference not being statistically significant (Z = 1.6616, P = 0.09692). In both study groups, most of the recurrent infections occurred within 10 weeks (17 in group A and 12 in group B). Long latency period (>10 weeks) was noted in 6 patients in study group A and 3 in study group B (Figure: 1).

Genetic characterisation of parasite isolates of primary and recurrent infections

Recurrences with identical genotypes were noted in 13 cases in Group A and 9 in Group B with short latency period within 10 weeks. Three cases in Group A and 2 in Group B were recorded with long latency period of more than 10 weeks. All these 27 recurrences were considered as true relapse bearing identical genotype. Different genotype was noted in 4 recurrent infections with short latency in Group A and 3 in Group B whereas 2 were with long latency in Group A and 1 in Group

B (Figure 2). These 11 recurrences could not be confirmed as either relapse or re-infection. Among 38 recurrent infections all except one had VK 210 allele in *pvcsp* gene in both day 0 and day of recurrence isolates.

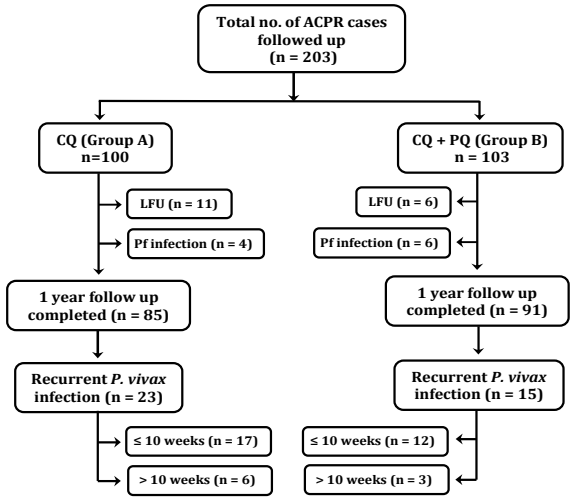

Figure 1 Consort Chart showing follow-up and pattern of recurrent *P. vivax* infection in two study groups treated with CQ and CQ plus PQ in urban Kolkata

DISCUSSION

Recurrence of malarial parasitaemia after proper treatment with schizonticidal drugs is either due to recrudescence or re-infection in case of *P. falciparum*. This is again complicated by the phenomenon of relapse due to activation of dormant hypnozoites in liver in case of *P. vivax*. Some genetically different sporozoites from the single mosquito bite or from several bites of other infected mosquitoes remain as hypnozoites in liver cells and can produce batches of merozoites which are responsible for relapse.

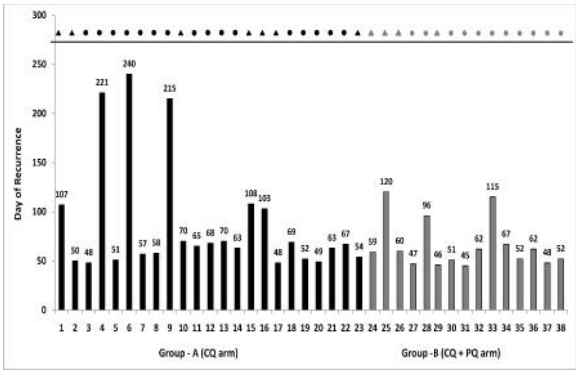

Figure 2 Summary of recurrent P vivax infection with different latency periods between first attack and day of recurrence following treatment with CQ (n=23, indicated by black bars) and with CQ+PQ (n=15, indicated by grey bars). The symbols on the top of the graph, circle and triangle denote recurrence with identical and different genotypes respectively.

In the present study no case of recurrent parasitaemia was noted during first 42 days follow-up, hence the phenomenon of recrudescence may be over-ruled. Transmission of *P. falciparum* in the study area is seasonal but *P. vivax* is perennial with low transmission intensity. So the chances of re-infection by fresh mosquito bite and relapse due to hypnozoites are equal.

In the present study, recurrences with identical genotype were noted in 27 cases -16 in Group A and 11 in Group B which could be considered as true relapse (Craig and Kain. 1996). Remaining 11 recurrent infections with different genotypes could not be classified with certainty as relapse or re-infection though scientists have shown that parasite of relapse may often bear a different genotype to that of primary infection (Imwong *et al*. 2007; Koefli *et al*. 2009).

In the present study, the rate of recurrent infections bearing either identical or different genotypes was equally recorded among patients treated with CQ alone or with both CQ and PQ. So the role of PQ in recommended dose of 0.25 mg/kg/day for two weeks to prevent relapse in the study area is debatable. Hence, effectivity studies of PQ with higher doses, as advised by WHO, is urgently needed to address the question of preventing relapse in *P. vivax* malaria.

Authors' contributions: AKM and SG conceived and designed the study protocol; SG, SKG and PS followed up the patients; SKG and SG clinically reviewed and implemented the treatment of the recruited patients; SG, PS performed PCR and RFLP analysis and interpretation of data; AKM, SG, SKG and NB drafted the manuscript. All the authors read and approved the final manuscript.

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Conflict of Interest The authors declare that there is no conflict of interest.

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