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Research paper

Clinical manifestations and molecular mechanisms in the changing paradigm of vivax malaria in India

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ABSTRACT

Background: *Plasmodium vivax* once considered benign is now being increasingly associated with complicated malaria where the spectrum of complications is vast and like *Plasmodium falciparum*. The clinical data is important with respect to the immunopathological status of the patient. Several genes like the *vir* genes and *pvcrt-o* are speculated to be attributing to the severity of *P. vivax* malaria.
Methods: In the present study we carried out the transcription analysis of five *vir* genes (*vir 14-related*, *vir 12*, *vir 17-like*, putative *vir 14* and *vir 10-related*) and *pvcrt-o* gene in severe (n=12) and non-severe (n=7) *P. vivax* clinical infections and studied the correlation of these genes with clinical disease severity.
Results: This study revealed multiorgan involvement in severe vivax cases with severe thrombocytopenia and anemia, the predominantly occurring symptoms. Four out of five *vir* genes and *pvcrt-o* showed a significant increase in expression levels of severe infections compared to the non-severe infections indicating their possible role in the changing pathogenesis of *P. vivax*.
Conclusions: The increased virulence in vivax malaria seems to be the result of multifactorial parameters changing it phenotypically as well as genotypically. However more studies are needed to understand the still nascent severity of *P. vivax* malaria.

1. Introduction

Plasmodium vivax is more widely distributed than *P. falciparum* and is a potential cause of morbidity and mortality among the 2.85 billion people living at risk of infection across the world (Guerra et al., 2010). Historically, India is highly endemic to *P. vivax* but recent reports suggest that malaria due to *P. falciparum* and *P. vivax* are in equal proportions (Singh et al., 2009). Although *P. falciparum* is responsible for majority of severe manifestations of malaria and mortality associated with it worldwide, *P. vivax* is no more considered benign and could also result in severe infections (Kochar et al., 2009). It has been known to induce *P. falciparum* like severe complications including cerebral malaria, renal failure, circulatory collapse, acute respiratory distress syndrome (ARDS), jaundice, severe anemia, thrombocytopenia, multiorgan dysfunction (MOD) and potentially leading to life-threatening episodes (Kochar et al., 2009; Lacerda et al., 2012). Clinical and pathological aspects of many severe *P. vivax* cases have been studied previously across the world as well as in India (Bhattacharjee et al., 2013; Sharma et al., 2012). The molecular mechanisms of the changing paradigm of *P. vivax* disease have not been dealt with chiefly due to the lack of a continuous *in vitro* culture system of asexual stages and low parasitaemias associated with natural infections.

P. falciparum virulence is primarily caused by sequestration — the phenomenon of withdrawal of infected erythrocytes (Pf-iEs) from the peripheral circulation to the internal organs. Sequestration of the parasite is mediated in the capillaries and sinuses of the inner organs to escape the spleen clearance of mature asexual stages. Cytoadhesion of Pf-iEs to the endothelial cells has been known to be mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) encoded by the *var* multigene family (Kyes et al., 2001; Scherf et al., 2008). There are many genes that are speculated to be involved in the complicated *P. vivax* malaria as there is very limited data available till date. The *P. vivax* multigene family variant interspersed repeats (*vir*) belong to the variant surface antigen (VSA) family and as they are highly polymorphic, their role in the antigenic variation in the severity of the disease is speculated.

Recent studies have shown cytoadhesion of *P. vivax*-infected erythrocytes (Pv-iEs) to the endothelial cells partly mediated by VIR proteins encoded by the *vir* genes signifying their role in the severity of *P. vivax*

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(Carvalho et al., 2010). Like severe *P. falciparum*, rosetting was also observed in *P. vivax* infections from Thailand and Brazil suggesting that this phenomenon might be contributing to anemia, one of the most common manifestations of severe *P. vivax* malaria (Chotivanich et al., 2012; Marin-Menendez et al., 2013; Mayor et al., 2011; Russell et al., 2011; Udomsanpetch et al., 1995).

Besides the emergence of severe pathology in *P. vivax* malaria, the reports of chloroquine (CQ) resistance have further questioned the portrayal of *P. vivax* as benign. Clinical studies show a high prevalence of *P. vivax* CQ resistance across Indonesia with a rising prevalence throughout south and south-east Asia and more recently in South America (Baird, 2004; Goncalves et al., 2014; Price et al., 2007; Santos-Ciminera et al., 2007).

Unlike *P. falciparum* where polymorphism in *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene confers CQ resistance, its orthologue in *P. vivax*, *pvcrt-o* gene cannot yet be called a genetic marker for *P. vivax* CQ resistance (Fidock et al., 2000; Suwanarusk et al., 2007). Even though no association has been found between polymorphisms in *pvcrt-o* gene and the clinical response of *P. vivax* to CQ, this transporter gene has been speculated to have a possible role in the CQ resistance of vivax malaria (Nomura et al., 2001; Sa et al., 2006). CQ resistance has been reported to be associated to the increasing severe clinical cases associated with *P. vivax* infections (Tjitra et al., 2008; Carlton et al., 2008). Further, it has been demonstrated that clinical severity could be associated with increased expression levels of parasite gene likely to be involved in chloroquine resistance *i.e. pvcrt-o* gene (Fernandez-Becerra et al., 2009; Melo et al., 2014).

In this study, we characterized *P. vivax* monoinfections as severe and non-severe by their clinical pathophysiology using World Health Organization (WHO) criteria originally validated for *P. falciparum* disease (Lanca et al., 2012; WHO, 2010). The present study was undertaken to study the molecular mechanisms possibly having a role in the severe vivax infections. The transcript levels of *vir* genes and *pvcrt-o* were quantified and compared between severe and non-severe infections. Relative transcript levels of these genes would help us gain a better understanding of the role these functional genes probably have in the changing *P. vivax* pathogenesis.

2. Materials and methods

2.1. Study site and design

This study was conducted on admitted patients <18 years of age showing symptoms of malaria at Kalawati Saran Children's Hospital, New Delhi, India, from July to October 2013. After the diagnosis of malaria, detailed clinical and biochemical examinations were done on each patient suffering from *P. vivax* malaria. The classification of severe malaria was done according to the WHO guidelines (WHO, 2010). The non-severe malaria patients were classified according to their symptoms to be used in the current study to understand the expression of the *vir* and drug resistance gene in severe and non-severe infections.

2.2. Ethical clearance

Ethical clearance for the study was taken by the "Institutional Ethics Committee, National Institute of Malaria Research" which reviewed and approved the study and all the patients provided a written informed consent prior to the collection of the blood samples.

2.3. Sample selection criteria

The study group consisted of three categories *viz.* non-infected (n = 50), non-severe (n = 12) and severe (n = 12). Blood was collected from the patients by the finger prick method and diagnosis of *P. vivax* malaria was done by microscopy and rapid diagnostic tests (RDT) (FalciVax Zephyr Biomedical systems). Thick and thin blood smears were made for *P. vivax* infections and parasitaemia was counted for each sample along with differential counting of the asexual stages (rings, trophozoites and schizonts) to rule out any difference between the severe and the non-severe groups (Tables S1 and S2). The patients were excluded from the study if their blood culture or serological tests showed the presence of infections, other than *Plasmodium* on physician's advice. Blood samples were taken from the selected patients prior to any treatment with anti-malarial drugs.

Samples positive for *P. vivax* were chosen for the study and 2 ml venous blood was collected from them. Whatman filter paper (number 3) was used to make blood spots for further diagnosis and species identification by PCR. Genomic DNA was extracted from the blood spots by QIAamp DNA Blood Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and the analysis of mixed infections of *P. falciparum* and *P. vivax* was done by PCR assay using 18S rRNA primers (Gupta et al., 2010). The *P. vivax* monoinfections were further genotyped with merozoite surface protein 3α (*msp3α*) gene for confirming the presence of single clone infections by the PCR-RFLP method previously described with slight modifications (Bruce et al., 1999). Briefly, nested-PCR amplification was carried out for *msp3α* gene in severe and non-severe *P. vivax* groups. The amplified products were visualized on 1.5% agarose gels containing ethidium bromide after electrophoresis. The PCR products were digested individually with restriction enzymes *Alu I* and *Hha I* and DNA fragments visualized after electrophoresis under UV illumination.

2.4. RNA isolation and cDNA synthesis

The infected blood sample was diluted five-fold with incomplete RPMI-1640 media containing 4.2% sodium bicarbonate and was passed through a CF-11 column to eliminate the presence of leucocytes and to ensure that no human RNA contamination takes place. Total RNA of *P. vivax* was isolated from the infected red blood cells (RBCs) of severe and non-severe *P. vivax* malaria patients by the QIAamp RNA Blood Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. To minimize the risk of DNA contamination, RNA was treated with DNase I (Thermo Scientific). First strand cDNA synthesis was reverse-transcribed from 150 ng of total RNA of both severe and non-severe samples using oligo $(dT)_{18}$ primers (Thermo Scientific) according to the manufacturer's protocol. Fresh uninfected human blood was used as a negative control and was processed along with other isolates.

2.5. Gene selection and primer design

Five *vir* genes were chosen for the current study according to their *in silico* data (Bernabeu et al., 2012; Neafsey et al., 2012). *Vir 14-related* (PVX_113230) belonging to subfamily C was chosen for its high invariance and highest similarity out of all the *vir* genes to the homologous *kir* gene family in *P. knowlesi*. This gene locus also shows conserved synteny with other distantly related rodent malaria parasites. It has been suggested that *vir 14-related* could be the founder of the *vir* gene family of *P. vivax* owing to its ultraconserved nature and could also have an ancestral role (Neafsey et al., 2012). It is said to be functionally related to erythrocyte invasion as suggested by its distinct expression profile relative to other *vir* genes (Bozdech et al., 2008). The *vir 12* gene (PVX_097525) belonging to subfamily E was selected for its very high diversity and a number of repeat regions found in our population genetic studies from India previously (Gupta et al., 2012). The remaining three *vir* genes were chosen on the basis of their encoded proteins with different subcellular localizations as described in previous studies (Bernabeu et al., 2012). *Vir 17-like* (PVX_112645) from subfamily A has sequence similarities to *P. falciparum* SURFIN proteins situated on the surface of infected erythrocytes and merozoites (Winter et al., 2005). Putative *vir 14* (PVX_108770) from subfamily C contains a J-domain, which is said to transport proteins through the cytosol independent of Maurer's cleft