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PROFILE OF DRUG RESISTANCE GENES IN PATIENTS SHOWING SEVERE MANIFESTATIONS IN MALARIA

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ABSTRACT:

Background:Plasmodium falciparum and Vivax, one of the four species of Plasmodium that cause human malaria, is well known for producing the disease in varied degrees of severity, with the most difficult form of the illness sometimes referred to as Severe Malaria.

Methods: People who lack immunity to the disease or whose immunity has declined are most likely to get severe malaria. 25–35 patients' serum samples were examined using an enzyme-linked immunosorbent test (ELISA). Genebank received the finished drug resistance gene sequences from a variety of representative isolates (displaying both mutant and wild genotype). Result: Data interpretation depicts a region I and region II of non-repeated amino and carboxyl sequences with highly conserved stretches flanking a central repeat domain. 20 severe samples were tested in all, and 13 samples (or 65 percent) revealed the wild type allele at codons 51, 58, and 117. In 3 samples (Patient Nos. 12, 13, and 18), Serine was substituted by Asparagine at codon 117. Patient Nos. 1, 2, 8, and 9 all had 4 samples that displayed the double mutation S117N + C58R. Furthermore, four samples (Patient Nos. 4, 5, 6, and 19) with the wild type genotype had a Histidine instead of a Serine at codon 93. Similar to the Pvdhfr gene, the repeat region in the Pvdhps gene displayed a variable amount of repeats. The examination of codons 72 to 76 revealed that all of our isolates had the CVMNK haplotype. In the instance of P. falciparum, this haplotype indicates the chloroquine-sensitive halpotype.

Conclusion: Before any conclusions can be made, more samples from other sites need to be evaluated. Unfortunately, there is no effective way to address drug resistance in vitro in these parasites because P. vivax culture conditions have not yet been standardised.

KEYWORDS: *Drug resistance, Malaria, Gene, DNA sequencing*

Introduction

Malaria, the most common and dangerous parasite disease of humans, is thought to claim the lives of between one and two million people annually, mostly children. Except for artemisinins, resistance has developed to all kinds of antimalarial medications, which is to blame for the recent rise in malaria-related mortality, especially in Africa. Antimalarial medication combinations can be used to stop the de novo emergence of resistance. Combinations of artemisinin derivatives are particularly useful because they operate quickly, are well tolerated, and are very effective. The widespread use of these medications may help malaria decline (Marsh, 2008).

Plasmodium falciparum, one of the four species of Plasmodium that cause human malaria, is well known for producing the disease in varied degrees of severity, with the most difficult form of the illness sometimes referred to as Severe Malaria. People who lack immunity to the disease or whose immunity has declined are most likely to get severe malaria. All locals in regions with minimal or no malaria transmission, as well as young children and expectant women in regions with high transmission, fall under this category (Nosten et al., 2000). Cerebral malaria, involving abnormal behaviour, impairment of consciousness, seizures, coma, or other neurologic abnormalities, is one of the symptoms of severe malaria, according to WHO (2004). severe anaemia as a result of hemolysis (red blood cell destruction), related to hemolysis, hemoglobinuria (haemoglobin in the urine), Acute respiratory distress syndrome (ARDS), which can happen even after parasite numbers have dropped as a result of treatment, or pulmonary edoema (fluid accumulation in the lungs), cardiovascular collapse and shock, abnormalities in blood coagulation and thrombocytopenia (reduction in blood platelets), The following symptoms should also cause concern: acute renal failure Malaria parasites infect more than 5% of the red blood cells in a condition known as hyperparasitemia. Hyperacidity in the blood and tissue fluids, or metabolic acidosis, is frequently linked to hypoglycemia (low blood glucose). Additionally, hypoglycemia can happen in expectant women with simple malaria or following quinine therapy (Korenromp et al., 2003).

For a long time, it was believed that P. vivax was not a cause of severe malaria but only P. falciparum. The infection was assumed to be mixed even if a P. vivax patient was found to have severe malaria. However, new findings from Bikaner and other parts of the world have unequivocally demonstrated the presence of severe P. vivax malaria symptoms. Clinical evidence from these strongly suggested that P. vivax can cause severe malaria complications related to sequestration as well as those unrelated to sequestration, such as cerebral malaria, renal failure, circulatory collapse, severe anaemia, hemoglobinuria, abnormal bleeding, ARDS, and jaundice, all of which are frequently linked to P. falciparum infections (Trape et al.,2008). Therefore, the purpose of this study is to analyse the predicted molecular markers in cases of severe malaria.

MATERIAL & METHODS

Collection of Malaria infected blood samples

Between the years of 2015 to 2020, skilled clinicians in the North of India collected blood samples from cases of malaria that had been established by clinical evidence. This region, which is located in India's northwest, experiences erratic outbreaks of Plasmodium vivax and Plasmodium falciparum malaria, particularly following the rainy season. A sample of 2 to 5 ml of infected blood was taken with the patients' informed agreement, placed in a 16 percent acid citrate dextrose solution, and shipped in a cold chain (4°C) to our lab. Before DNA extraction, collected blood samples were kept at -20°C. The Institutional Human Ethics Committee gave its approval to the protocol. In this investigation, P. vivax and P. falciparum samples totaled sixty each.

Sera collection and separation (For ELISA)

The north of India was the area selected for the study and sera collection. Approximately 1 to 3 ml of P. vivax-infected blood sample was randomly collected at various times from various locations in this region, where seasonal episodes of P. vivax malaria were present from 2015 to 2020. Patients between the ages of 20 and 50 had their blood samples taken with their informed consent. Following blood collection, the blood was overnighted at 4°C to allow the cells to settle. After that, the serum and clot were separated by centrifuging at 10,000 rpm for 10 minutes at 4°C. The serum was kept at -70°C until further examination(Plowe et al.,2003) .

ELISA (Enzyme Linked Immunosorbent Assay)

Material Used: 96 well Microtitre plates (Nunc, Denmark), peptides (a gift from Dr. Udhaya Venkatachalam of the CDC in Atlanta), and one volume of PBS were employed (Phosphate Buffered Saline), Wash buffer: 1X PBS - Tween 20; Coating buffer: 1 X Carbonate-Bi carbonate buffer (pH 9.6) 5 percent casein in 1X PBS serves as a blocking buffer. Goat anti-human IgG peroxidase (Bangalore Genei) and TMB/H2O2 are the conjugates (Bangalore Genei), Solution to halt: 2N H2SO4

Enzyme-linked immunosorbent assay (ELISA) was used to assess the total IgG titres against CSP peptides based on the recurrent B cell epitope regions in serum samples from 25–35 patients (White, 2009). Using the appropriate antigen (150 ng of peptide diluted in 100 l of 1X coating buffer per well), microtiter plates were coated before being incubated at 4°C overnight. The wells were blocked using the blocking buffer, rinsed three times with 1X PBS - Tween 20 (0.2 percent), and then incubated at 37°C for two hours. Human serum diluted (1/100) in blocking solution was added (100 l/well) and washed three times with the wash buffer before being incubated at 37°C for two hours. 100 l of secondary antibody diluted (1/1000) in blocking buffer was added to each well after three rounds of washing with wash buffer, and each well was then incubated at 37°C for two hours. The substrate solution was added once more after washing, and 100 l was poured in the dark and left at room temperature for 15 to 30 minutes so the colour could develop. Using an ELISA Reader (STATFAX 2100), 100 l of stop solution was added, and the absorbance was read at 450 nm (Hasting et al.,2000).

DNA Sequencing

Many commercial service providers, including Labmate Pvt. Ltd. in Gurgaon and ShankaraNetralaya in Chennai (Rathod et al.,2007) , sequenced the clones and amplicons. Genebank received the finished drug resistance gene sequences from a variety of representative isolates (displaying both mutant and wild genotype). The sequences that were submitted had the following Genbank Accession Numbers: EU682753-EU682757 (Pfmdr-1), EU682758 (Pvcg10), EU682759-EU682760 (Pfdhfr), EU717689-EU717695 (Pfdhps), EU478857-EU478864 (Pvdhfr), EU478865-EU478871 (Pvdhps).

Results and Observation:

Patients from the North region of India who were exhibiting severe P. vivax symptoms had blood samples taken. These patients had jaundice, acute respiratory distress syndrome, severe anaemia, hemoglobinuria, renal failure, circulatory collapse, cerebral malaria, and other symptoms (Table 1.1). The patients were admitted to a designated malaria critical care unit and ranged in age and sex. All of the patients underwent a thorough diagnostic evaluation, which included PBF testing, a malaria rapid diagnostic test (OptiMAL test, DiaMed AG, Switzerland, which is based on detecting specific Plasmodium LDH antigen by using monoclonal antibody directed against isoforms of the enzyme), and a multiplex PCR based on the 18S ribosomal gene of the malarial).

Table 1.1: Clinical characteristics of severe *P. vivax* malaria patients

Patient No.	Age(y) / sex	Clinical presentation/ other relevant information	Diagnostic tests for malaria			*Outcome*
			PBF	RMDT OPTIMAL	PCR	
1	53, M	Jaundice, haemoglobinurea, Epistaxis	+	Positive	+	*Recovered*
2	20, F	Cerebral (GCS - 3) Severe anemia, ARDS, PCF, CSF – N, BP < 70mm Hg	+	Positive	+	*Died within 5 days of admission*
3	45, M	Renal failure, Jaundice	+	Positive	+	*Recovered*
4	18, F	Cerebral (GCS - 5), anemia, Primigravida, CSF – N, CT scan head - N	+	Positive	+	*Recovered, PMNS-Psychosis Premature delivery, baby survived*
5	28, F	Renal failure, ARDS, PCF	+	Positive	+	*Recovered*
6	25, F	Jaundice, Haemoglobinurea, Second gravida	+	Positive	+	*Recovered, pregnancy continued*
7	50, M	Jaundice	+	Positive	+	*Recovered*
8	38, M	Jaundice	+	Positive	+	*Recovered*
9	20, F	Jaundice, epistaxis, abnormal bleeding, blood transfusion twice	+	Positive	+	*Recovered, pregnancy continued*
10	18, M	Jaundice, anemia, blood transfusion thrice	+	Positive	+	*Recovered*
11	25, F	Cerebral (GCS – 8), anemia	+	Positive	+	*Recovered*

12	56, M	Jaundice	+	Positive	+	*Recovered*
13	37, F	Jaundice	+	Positive	+	*Recovered*
14	20, M	Jaundice	+	Positive	+	*Recovered*
15	15, M	Cerebral (GCS – 9)	+	Positive	+	*Recovered*
16	17, F	Jaundice, Renal Failure, ARDS	+	Positive	+	*Recovered*
17	20, M	Jaundice	+	-	+	*Recovered*
18	36, M	Severe anemia	Negative	Positive	+	*Recovered*
19	30, M	Jaundice	+	-	+	*Recovered*
20	*23, M*	*Jaundice*	+	*Positive*	+	*Recovered*

(**ARDS – Acute Respiratory Distress Syndrome; BP – Blood Pressure; CT – Computerized Tomography; CSF – Cerebrospinal Fluid; GCS – Glasgow coma scale; N – Normal; PCF – Peripheral Circulatory Failure; PMNS – Post Malarial Neurological Syndrome**)

The circumsporozoite protein's repeat areas were examined using an ELISA based on these repeat regions as well as CSP gene sequencing to determine the type of vivax infecting these people and generating severe symptoms. The most prevalent polypeptide on the surface of sporozoites, circum sporozoite protein is a crucial component of the parasite and has a role in the formation of infectious sporozoites in mosquitoes as well as the invasion of liver cells. It has a core repeat domain bordered by sections I and II, which are non-repeated amino and carboxyl sequences with highly conserved lengths (Fig. 1.1).

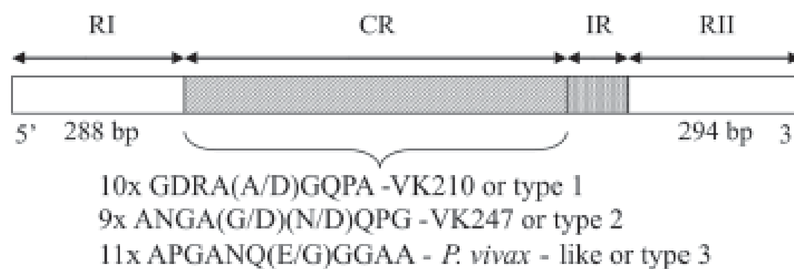

Figure 1.2: Structureof the *Plasmodium vivax CSP* gene, with two highlyconservedterminal non-repeat regions (RI and RII); a central repetitive(CR) domain, with a variable number of tandem repeats, and a short IR(insertion region).

P. vivax Type 1 peptides revealed good peaks in the ELISA of all the serum samples from clinically confirmed severe and non-severe malaria isolates (Fig. 1.3) Based on the mean plus two standard deviations of the reactivity of sera from a group of negative controls, the

threshold of positivity was an OD value of 0.6892. Standard error was used to display the results. The peptides based on the repetition regions of Type 2 and P. vivax-like isolates were also used in ELISA tests. Both P. vivax Type 2 and P. vivax-like peptide readings were significantly below the threshold limit. This demonstrated that all P. vivax infections, whether severe or not, were Type 1.

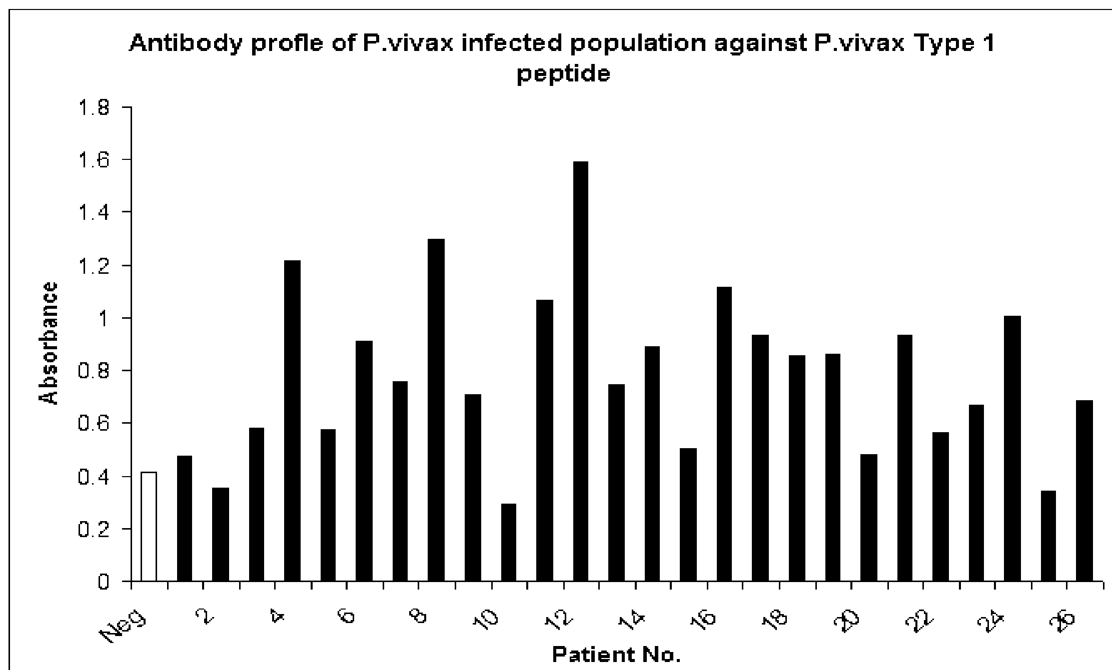

Figure 1.3: ELISA of serum samples from clinically proven severe and non – severe malaria isolates for *P. vivax* Type 1 peptides

To further confirm the presence of Type 1 *vivax* and to see if any change is seen from the reported CSP of *P.vivax* type 1, the CSP gene was amplified from these severe cases. A few non severe cases were also used for the study. The 1.2 kb Circumsporozoite Protein (CSP) encoding gene amplification was performed from *P. vivax* field isolates using primers AL60 and AL61 .The obtained CSP gene sequences from Indian *P. vivax* isolates were

approximately 1136bp in length. A thorough analysis of translated CS protein sequence showed presence of total 17*P. vivax* type 1 (VK210) sequence repeats (GDRA(D/A)GQPA) in all Indian isolates

. **Analysis of drug resistant genes in *P.vivax* isolates showing severe manifestations:**

***Pvdhfr*gene**

The *dhfr* gene was amplified from the samples collected from the patients showing severe manifestations (Table 1.1) using the conditions described in Chapter 5. The amplified product was gel purified and was sent for sequencing. On alignment of the obtained sequences with the standard wild type sequence of *dhfr-ts* (GenBank Accession No. X98123), it was observed that the severe samples showed wild type, single mutant as well as double mutant genotypes (GenBank Acc. No. EU478857 – EU478864). Out of total 20 severe samples analyzed, 13 samples (65%) showed wild type allele at codon 51, 58 and 117. 3 samples (Patient Nos.12, 13, 18) were single mutant at codon 117, where Serine was replaced by Asparagine. 4 samples (Patient Nos.1, 2. 8, and 9) showed double mutations i.e. S117N + C58R. In addition four samples (Patient Nos. 4, 5, 6 and 19) with wild type genotype showed a Histidine at codon 93 in place of Serine.

Apart from these non synonymous mutations, at codon 69, a silent mutation coding for Tyrosine was observed (TAT → TAC) in 6 isolates (Patient Nos. 3, 11, 12, 15, 17 and 20). A lot of polymorphism was observed in the repeat region as well. Different number of repeats ranging from 2 to 4 was seen. Most of the samples showed three GGDN repeats, while Patient no. 19 showed a deletion of GGDN resulting in only 2 repeats. An extra GGDN repeat was seen in Patient No. 13. The connector region was different in all our isolates as compared to standard *P.vivax* DHFR sequence (GenBank Acc. No. X98123). The connector region was same as other isolates from Bikaner and the sequence was found to be -

DSASENCNALNCNAPKCSAPNCRSPNGGTAQQGEWGKGPACPWQKNNAEAEE DDLVYFSFNNKVGEKNPEHLQDFKIYNSLKIK

The TS domain showed a N368K change in the conserved residue as seen in all other isolates from this region.In addition to this,one sample(Patient No. 16) showed a silent mutation in the TS region at the codon 574; a change from AGC to AGT (both codes for Serine).

***Pvdhps* Gene**

The same 20 samples that displayed severe signs of the condition had their pppk-dhps gene amplified and evaluated (GenBank Acc. No. EU478865 – EU478871). At the codons 383 and 553 that are thought to confer resistance to sulfadoxine, all the genes had the wild type alleles. In the severe samples, a few new mutations were also found. In one strain (Patient No. 9), the codon 365 was changed from Phenylalanine to Leucine, and in another isolate (Patient No. 12), the codon 459 was changed from Asparagine to Alanine. Four samples (Patient Nos. 11, 13, 19 and 20) revealed a change from Glutamic Acid to Aspartic Acid at codon 618, while two samples (Patient Nos. 8, 11) revealed a Methionine at codon 601 replaced by Isoleucine. Patient No. 11 was the only isolate to exhibit double mutations at codons 601 and 618. Additionally, one synonymous mutation at codon 617 was found in 6 severe samples, replacing GGG with GGT (both codes for Glycine).

Similar to the Pvdhfr gene, the repeat region in the Pvdhps gene displayed a variable amount of repeats. The number of repeats in the severe and non-severe samples ranged from 5 to 9. The maximum number, or 50%, of the 20 severe samples revealed 7 repeats. Four samples (Patient Nos. 4, 9, 12, and 14) showed six repeats, whereas six repeats were seen in six samples (Patient Nos. 7, 10, 15, 16, 19 and 20). There were no 9 duplicates in any of the severe samples. Both the severe and non-severe forms of the pppk gene displayed a change at codons 207 and 217. All of our isolates had Threonine in place of Proline at codon 217 and Aspartic Acid in place of Alanine at codon 207.

***Pvcrt - o*gene:**

The initial 1kb region carrying the codon 76 was amplified from blood samples of 20 severe cases(GenBank Acc. No. EU682758). No change was observed between the non severe and the severe cases. All the isolates showed no change from the cg10 sequence of Salvador. The

analysis of the codon 72 – 76 showed the presence of CVMNK haplotype in all our isolates.This haplotype denotes the chloroquine sensitive halpotype in case of *P. falciparum*.

Discussion

Analysis of the dhfr gene from complicated and uncomplicated P. vivax malaria patients revealed that, other from the reported alterations, no difference was seen between the severe and non-severe cases in the dhfr-ts gene (White et al., 2003). The majority of the isolates exhibited the genotype of the wild type, indicating the parasites' susceptibility to the medication pyrimethamine. The majority of the mutant isolates, or S117N + S58R, were discovered to be double mutants.

It was discovered that the Pvdhps gene in the severe cases displayed a number of new mutations when compared to the uncomplicated and complicated cases. The repeat region is affected by the mutations at codons 601 and 618. Korsinczky thinks that it is unlikely that the mutations in this region will influence drug binding (Day et al.,2006). By creating the structure of the mutant form and carrying out molecular docking with the medicinal molecule sulfadoxine, the other two mutations at codons 365 and 459 were examined. These alterations had no impact on the drug binding in our investigation. Comparatively, the pvcrt-o gene showed no difference between the severe and non-severe cases in the early region.

In India, the morbidity and fatality rates from P. vivax malaria have increased during the past ten years. It has been hypothesised that the growth in the prevalence of parasite resistance to different antimalarial medications is to blame for the rise in morbidity and mortality in P. falciparum (Simpson et al.,2002). However, our study's sequence analysis of the numerous marker genes known to be involved in conferring resistance against various antimalarial medications seems to rule out the presence of a drug-resistant condition in the severe P. vivax cases examined (Fig. 1.3). Thus, there is a discrepancy between the parasite's virulence and the mutations that give rise to its medication resistance. Similar reports have been found from Eastern Sudan, where the wild variety of the parasite predisposed people to severe P. falciparum malaria (White et al.,2007). Before any conclusions can be made, more samples from other sites need to be evaluated. Unfortunately, there is no effective way to address

medication resistance in vitro in these parasites since P. vivax culture conditions have not yet been established.

CONCLUSION

Plasmodium , one of the four species of Plasmodium*falciparum* and *vivax* that cause human malaria, is well-known for causing varied degrees of malaria severity, with the most severe form of the disease commonly referred to as Severe Malaria. Before any judgement can be made, additional samples from many sites must be evaluated. Due to the lack of standardisation of P.vivax growing conditions, there is no in vitro method for addressing medication resistance in these parasites.

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