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Allelic Diversity of Vaccine Candidate Antigen, PfMSP6 (Merosoite Surface Protein 6) of Malaria Plasmodium Falciparumin South-East of Iran

Adel Ebrahimzadeh PhD and associated professor, Department of Medical Parasitology and Mycology and membership in InfectiousDiseases and Tropical MedicineResearch Cente,Zahedan University of Medical Sciences, Zahedan, Iran Corresponding Author

Taregh Bamedi MSc student, Department of Medical Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran

Khadijeh Saryazdi BSc in Nursing, Department of Medical Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran

ABSTRACT

Plasmodium falciparummerozoite Surface Protein-6 (PfMSP6) is a potential vaccine candidate at an early stage in development. Different subtypes of Plasmodium falciparum including 3D7 and K1 had different diversity and then each had a potentially capable for vaccine generation. Thus this study assessed prevalence of 3D7 and K1 in southeast of Iran. This study was conducted on 65 infected patients with Plasmodium falciparum in different parts of Sistan and Baluchistan, southeast of Iran. Blood was collected from each individuals and genomic DNA was extracted. Initially DNA was amplified by external primers and then obtained products were amplified using nested PCR to determined prevalence of K1 and 3D7 in this part of Iran. Eventually obtained data was analyzed by SPSS software. The mean age of affected individuals was 39 years and 51.5 percent of them were male and 48.5 percent was female.. Our study revealed that 3D7 (52.4%) frequency was higher than K1 (37.7%). 9.9% of patients had the mix infection of 3D7 and K1. Most patients infected by both alleles of 3D7 and K1were residues of Chahbahar city. respectively.3D7 subtype of Plasmodium falciparum was common in southeast of Iran and based on low diversity of use of vaccination in this part of Iran in successful.

KEYWORDS Plasmodium falciparum, Merozoite Surface Protein-6, South-East, Iran

Introduction

Malaria is a life-threatening parasitic infection that affects individuals from all race and regions. The exact prevalence of this parasitic infection was not determined, but the incidence of disease in tropical area is high [1].

More than half of the world's population in approximately 100 countries is exposed to malaria. Iran is situated in the Eastern Mediterranean region, where about 45% of the population live with the risk of both *falciparum* and *vivax*malaria mixed infection [2]. Countries of this region are situated in either Afrotropical (such as Somalia, Sudan), Oriental (such as Pakistan, south-eastern Iran, part of Afghanistan) or Palearctic (such as Turkmenistan, Uzbekistan, Tajikistan) eco-epidemiological zones regarding malaria [3, 4].

The highest prevalence of malarial is in tropical and sub-tropical regions of the thalassemia belt including Mediterranean countries, parts of North and West Africa, the Middle East, the Indian subcontinent, southern and southeastern Asia, a wide area that Iran is located in [1, 5].

Plasmodium falciparum Merozoite Surface Protein-6 (*PfMSP6*) is a potential vaccine candidate at an early stage in development, which still lacks critical field data to inform the go/no-go decisions necessary to either advance it down the pipeline or remove it from consideration. PfMSP6 is a secreted antigen that is proteolytically processed by PfSUB1 into a 36 kDa fragment that associates with fragments of *PfMSP1* and *PfMSP7* to form a multi-subunit complex on the merozoite surface [3-7].

PfMSP6 is encoded by one gene in a multi-gene family arranged in close proximity along chromosome 10 [8]. All members of this multi-gene family appear to encode merozoite surface antigens, one of which, *PfMSP3*, has already advanced to several Phase I vaccine trials [9-13].

Although the function of *PfMSP6* remains unknown, it has been postulated to participate in erythrocyte recognition and binding, as have many other merozoite surface proteins of unknown functions. *PfMSP6* is, therefore, in the right place to be a theoretical vaccine candidate, and its potential is supported by field studies that have observed anti-*PfMSP6* antibody responses in serum from *P. falciparum*-infected individuals, which inhibit *P. falciparum* growth in vitro [14, 15] Differences between the alleles are largely restricted to a series of indels in the Nterminal domain, but also include single nucleotide polymorphisms (SNPs) within each allele class that are found in both the N-terminal domain preceding the PfSUB1 cleavage site as well as the generally more conserved C.terminal domain (16, 17). A recent study of 89 *PfMSP6* gene sequences from around the world identified 7 K1-like and 11 3D7-like haplotypes [18]. The aim of this study was determination of allele frequencies in southeast of Iran by nested PCR.

Material and Methods

Data Collection

This study was conducted on 25 patients with malaria infection during 2013. Data was collected from health center of Sistan and Baluchistan. Total of 25 patients was diagnosed as malaria infection. The diagnosis of infection was made based on patients' clinical manifestations; Complete Blood Cell Counts (CBC) and blood smear examination. Clinical manifestations of patients were assessed by a physician and then patients were referred to laboratory for more investigations. In the laboratory patient's blood was collected in an anticoagulant tube and technician make two thick and thins smears for assessment and direct observation of parasite. Each smear was assessed by two exerted technicians for present of malaria parasite. Only smear which not show any parasite and this

was confirmed with both technicians was consider negative. In case of positive malaria parasite, this should be confirmed by both technicians.

Blood Collection and DNA Extraction
The sample collection process, involving both passive and active case detection, has been detailed previously (14, 15).

Passive case detection occurs when symptomatic individuals seek care at the community health outpost, where confirmation of malaria is made by microscopy. In contrast, active case detection occurs through routine community visits and identifies asymptomatic individuals. This study design increases the likelihood of sampling both symptomatic and asymptomatic P. falciparum infections. All patients submit a 0.5 ml blood sample and, upon malaria diagnosis are re-evaluated, submit another blood sample, and are cleared of parasites by co-administration of mefloquine and artesunate. Samples are separated by centrifugation into serum and packed erythrocyte fractions. Plasmodium DNA is extracted from the erythrocyte fraction using a BloodDNA kit (Qiagen), and the species is identified by PCR using species-specific primers. All samples are catalogued and stored at -80°C until needed. For this study, *P. falciparum* isolates collected during 2013 were selected at random, excluding only subsequent infections in the same individual that occurred within 60 days of the initial infection in order to reduce the risk of duplication due to parasite recrudescence.

PCR and Nested PCR
The region of PfMSP6 where all detected inter- and intraallele genetic diversity has been shown to occur was amplified using a nested PCR protocol with the amplified fragment corresponds to nucleotides 221-784 of the reference 3D7 PfMSP6 sequence, excluding primer sequences. 1.0 µl of genomic DNA, extracted from P. falciparum-infected patients, was amplified using ChoiceTaq (Denville) in 35 cycles of 95°C for 30 seconds, 51.1C for 30 seconds, 65C for 1 minute, and 65°C for 5 minutes.

For the nested PCR reaction, all conditions remained the same except that 1 µl of the primary PCR reaction was used as the template. Multiple negative controls were included in each PCR experiment to monitor for contamination. Allele-typing of *PfMSP6* was performed using ethidium bromide-stained agarose gel electrophoresis. *PfMSP6* amplified from the *P. falciparum*strains HB3 and Dd2 were used as controls and run on all agarose gels to aid classification of infections as either 3D7-like or K1-like allele type.

Statistical Analysis
Results were reported as mean ± standard deviation (SD) for quantitative variables and percentages for categorical variables. Statistical significance was based on two-sided design-based tests evaluated at the 0.05 level of significance. All statistical tests were two-tailed and performed using a 5% significance level in SPSS software.

Results
Prevalence of Plasmodium Falciparum
Among our 68 patients, most were residues of Chahbahar city (27 persons). The prevalence of Plasmodium falciparum among other cities was 18, 12 and 11 patients in Iranshahr, Nikshahr and Sarbaz respectively. The precise prevalence of *Plasmodium falciparum* was show in Figure 1.

Figure 1. Prevalence of Plasmodium falciparum in different cities of Sistan and Baluchistan..

Prevalence of K1 and 3D7 Alleles of Plasmodium Falciparum

Our results revealed that 3D7 was the most common subtypes of *Plasmodium falciparum* in Sistan and Baluchistan Provinces (52.4%). Prevalence of K1 was 37.7% in all over the Province (Table 1).

Table 1.Prevalence of 3D7 and K1 subtypes of Plasmodium falciparum in different cities of Sistan and Baluchistan province.

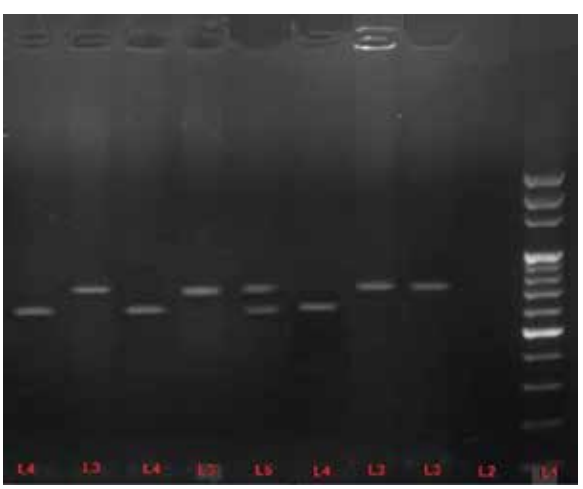
	3D7	K1	3D7/K1	Negative	Total
Chahbahar	12	9	3	3	27
Iranshahr	9	6	1	2	18
Nikshahr	6	4	1	1	12
Sarbaz	5	4	1	1	11
Total	32	23	6	7	68

Distribution of Plasmodium Falciparum
K1 subtype of *Plasmodium falciparum was observed in 12 females and 11 males. Prevalence of Plasmodium falciparum K1 and 3D7 subtypes infection among male and femalewere significantly different (P value:0.03) (Table 2 and Figure 2).*

Table 2.Prevalence of different subtypes of falciparum in study population.

Index	3D7-Class	K1-Class	P-value
Male	17	11	0.03
Female	15	12	0.07

Figure 2: Electrophorse Representation of K1 and 3D7 alleles of Merozoite Surface Protein, PfMSP6 on 2% Agarose gel



Line 1 100bp ladder Marker , Line 2 Negative control, L3 and L4 Ki 780 bp and L5 3D7 612 bp

Discussion
Malaria infection is a life-threatening parasitic infection with high rate of morbidity and mortality. The precise incidence of disorder is not determined but estimated incidence of disease revealed a high prevalence of disorder in malaria belt, a wide area ranges from Africa, the Middle East, southern Europe and South Asia that Iran located in. The prevalence of disorder is variable in different area of Iran with a high rate in south, southeast and north [1, 3].

While a vaccine targeting a sporozoite stage antigen is currently undergoing Phase III trials [3,4]. vaccines targeting asexual stage antigens, which in theory would have the clinical advantage of limiting symptoms even if they were not completely effective in eliminating parasites, have lagged somewhat in development. Genetic diversity is clearly a major hurdle for many asexual antigen vaccines (5), and is presumably

responsible for the disappointing results from recent field trials of a *PfMSP1*- based vaccine [6,7]. To avoid similar disappointment in the future, it is essential that all potential vaccine candidates undergo rigorous go/no-go analysis in the pre-clinical phase, with candidates being eliminated from consideration if they do not meet certain criteria. Several approaches can be used to inform these go/no-go decisions, including experimental genetic manipulation and detailed field studies investigating both natural genetic diversity and immunoepidemiology. *PfMSP6* is a merozoite candidate antigen at an early stage of pre-clinical development, lacking significant field data that supports its potential role as aviable vaccine candidate. To help inform go/no-go decisions for *PfMSP6*-based vaccine development, PfMSP6 diversity was followed over multiple transmission seasons in a hypoendemic transmission environment in Peru. At a sequence level, *PfMSP6* diversity was very limited in this setting. No intra-allele sequence variants were found in over 500 distinct P. falciparum infections spanning four transmission seasons at the MIGIA cohort study site near Iquitos, Peru. While P. falciparum genetic diversity is, in general, much lower in South America than other regions [8-11] SNPs were detected in two other vaccine antigens, *PfMSP119* and *PfMSP3,* at the same site over the same period [7,11-14]. Genetic stability in low transmission is a generally low bar for vaccine candidate antigens, but the relative stability of the *PfMSP6* gene compared to other vaccine antigens even in this setting certainly supports its further investigation as a vaccine candidate [15-18].

Conclusion

In this study we assessed allele frequency of K1- and 3D7 in southeast of Iran to determine which subtype is more frequent. We found that 37.7% of individuals affected by K1 and 52.4% were affected by 3D7. Most of infected individuals with 3D7 were male while in patients affected with K1 most were female. The difference between males was statistically significant (P:0.03).

The results of our study revealed that frequency of 3D7 allele was higher and based of previously studies K1 had a low variability and thus is completely suitable as a vaccinate candidate.

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