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Allelic Diversity of Polymorphic AMA-1 (Apical Membrane Antigen 1) Vaccine Candidate Antigen of *Plasmodium falciparum* in Two Population of Imported and Indigenous Cases in South-East of Iran using Nested-PCR and RFLP

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Abstract

The *Plasmodium falciparum* Apical Membrane Antigen 1 (AMA1) is a leading Malaria Vaccine Candidate Antigen. Antigenic variation is one of the main obstacles in the development of a universal effective malaria vaccine. Antibodies against AMA1 have been shown to block parasite invasion of human erythrocytes. Therefore, detailed studies on the molecular polymorphism PFAMA1 a Geographic area before each experiment or design the vaccine is administered. This study was designed to determine the distribution of AMA1 allele class of *Plasmodium falciparum* in two population of imported cases and indigenous cases in South-East of Iran .We used the Nested PCR and RFLPs methods with specific primers and restriction enzymes, which improves the three AMA1 allele class(K1-3D7-HB3).Overall the 94 confirmed P. falciparum samples obtained from four different districts, in two population of imported cases(46) and indigenous cases(48) in the south East of Iran. There are three classes of allelic AMA1 were compared in two populations of Indigenous and Imported, 3D7 in Indigenous Population was the higher prevalence than Imported Population. Considering on these results, 3D7 alleles suitable candidates for malaria vaccine design can be. The data reported here will be valuable for the development of AMA1 based malaria vaccine.

Keywords: *Plasmodium falciparum*; Apical Membrane Antigen 1 (AMA1); Imported and indigenous cases; South-east of Iran

Introduction

Malaria is an important tropical disease with an estimated global burden of 300 to 660 million cases every year, of which around 90% occur in sub Saharan Africa where mortality due to malaria is also reported to be higher than elsewhere [1,2]. The extensive genetic diversity of the malaria parasite constitutes major drawbacks to the development of a successful malaria vaccine [3,4]. Such extensive antigenic polymorphism greatly enhances the parasites ability to evade immune recognition, making it difficult to elicit adequate responses against the full range of variants circulating in the parasite population [5]. Several malaria vaccines have undergone field trials but these have shown low efficacy during the field trials [6]. One of the reasons for the low efficacy could be the antigenic polymorphism in the vaccine candidate antigens [1].

Apical membrane antigen is an 83-kD type I integral membrane protein with a 55-amino acid cytoplasmic segment and a 550-amino acid extracellular region that can be divided into three domains on the basis of intra domain disulfide bonds. Although its function is still not well characterized, it is expressed in the late schizont stage of the parasite and is required for merozoite invasion of erythrocytes and sporozoite invasion of hepatocytes. Antibodies against AMA1 have been shown to block parasite invasion of human erythrocytes [7].

Plasmodium flaciparum AMA1is highly polymorphic [8,9] and the Domain-I has been shown to contain most of the polymorphism [8,10]. Based on restriction fragment length polymorphism (RFLP) analysis, Domain-I can be subdivided into four groups termed I, II, III, and IV [9].

Protective responses induced by this antigen are strain specific, suggesting that these polymorphisms have arisen by diversity selection [11].

The Plasmodium proteins expressed on the parasite's surface are more exposed to human immune system and thus have been found to exhibit high antigenic diversity [12]. Natural immune responses (both humoral and cellular) against the AMA1 antigen have been observed in populations exposed to *P. falciparum* malaria [13] .Thus, AMA1 has been included as one of the potential components of an asexual stage multivalent malaria vaccine [14]. Therefore, there is an urgent need for the development of effective malaria vaccine [15]. AMA1 is a strong vaccine candidate with limited epidemiologic data that are needed to support its continued development along the proposed malaria vaccine roadmap.

Iran is located in the Eastern Mediterranean Region, and grouped as low moderate endemic region [16]. Sistan and Baluchistan Province, South-east of Iran, is the endemic area of falciparum malaria and is considered as the oriental eco-epidemiological region of malaria [17]. It is bordered by Pakistan and Afghanistan. The analysis of genetic variation among the isolates of *P. falciparum* prevalent in a region is

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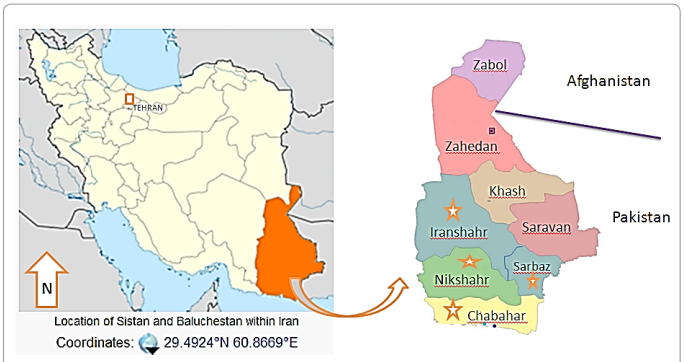

Figure 1: Map of study area, Districts of Sistan and Baluchistan, South-East of Iran.

important before the development or field trial of a malaria vaccine in that geographical region.

This study investigates genetic variation in the *P. falciparum* AMA1 three allelic classes (3D7-HB3-K1) in samples collected from four different endemic regions in South-East of Iran. To date, no study has yet looked at the extent of *P.falciparum* AMA1 three allelic class (3D7-HB3-K1) variations in this geographic region in two Population of Imported cases (from Pakistan and Afghanistan) and Indigenous cases by RFLP method.

Experimental

The study enrolled residents from four different districts of the Sistan and Baluchistan province: Chabahar, Sarbaz, Iranshahr and Nikshahr in two Population of Imported cases (from Pakistan and Afghanistan) and Indigenous cases. All these four districts are located in South-East of Iran. Sistan and Baluchistan Province in the south-eastern part of Iran is an area with substantial human migration originating mainly from Afghanistan and Pakistan (Figure 1). Malaria cases are reported during the whole year with two peaks, the first with predominantly *P. vivax* in April through September and the second peak with 45% to 50% *P. falciparum* infections after September [18].

A total of 94 *P. falciparum* infected blood samples used in this study were collected from patients attending the clinics and hospitals in the four study districts in two Population of Imported cases (from Pakistan and Afghanistan) and Indigenous cases from March 2011 to September 2012. Residence in the regions for over 6 months, no history of anti-malarial treatment for the last month, and written informed consent were required for inclusion in this study. The presence of *P.falciparum* infections in the samples were confirmed microscopically using thick and thin Giemsa-stained slides in Department of Parasitology, Zahedan University of Medical Sciences. Venous blood (2 ml) was collected from each consenting patient into tubes containing Ethylene Diamine Tetracetic Acid (EDTA) as an anticoagulant. The samples were stored at -20°C until using for DNA extraction. DNA was extracted from the blood sample using Fermentas Genomic DNA Purification Kit (Thermo Fisher Scientific Inc.). All DNA samples were stored at –20°C before genotyping with a polymerase chain reaction.

The AMA1 haplotypes were analyzed by using PCR-RFLP Oligonucleotide primer sequences for the primary and secondary amplifications are listed (Table 1).

Purified DNA from *P. falciparum* 3D7 (MRA-102G), HB3 (MRA-149G), K1 (MRA-159), strains was provided by the Malaria Research and Reference Reagent Resource Center, American Type Culture Collection (Manassas, VA) and used as positive controls during the amplification reactions. The second AMA1amplification product was digested by using three restriction enzymes (MseI, SspI, and BfCUI), which generated digestion fragments specific for the 3D7, K1, and HB3/7G8 AMA1allele classes, respectively. Table 1 shows a list of primers.

Positive and Negative controls and a 1000 bp Ladder Marker (Bioneer, Korea Rep) were used to interpret the fragments sizes. The AMA1K1, 3D7, and HB3/7G8 alleles were identified as single fragments of 285, 400, and 335 basepairs, respectively, when subjected to digestion with MseI, SspI, and BfuCI, respectively.

Results and Discussion

The main goal of this study was to analyze the polymorphic antigen AMA1gene across South-East of Iran among four different districts in two population of imported cases and indigenous cases to identify differences in allele frequency and genetic diversity. All of the 94 confirmed *P. falciparum* samples obtained from the four districts, 74 samples were successfully scored for AMA1. In the present study, 94 samples were collected from south eastern in Imported cases (46) samples and (48) samples were obtained from Indigenous cases.

Nested PCR was conducted to amplify and genotype *P.falciparum* AMA1 *P. falciparum* infected individuals. The primers amplified the *P.falciparum* AMA1, where the majority of genetic diversity has been shown to occur and the allele class identified using agarose gel electrophoresis. The size differences confirmed that three allele classes of *P.falciparum* AMA1 are present in the region of study (Figure 2).

The AMA1 allele classes (k1-3D7-HB3) showed comparable prevalence in two populations of imported and indigenous cases (Table 2).

Three classes allelic antigen of AMA1 in both populations studied

Primer name	Sequence length 5'-----3'
External primers	
AMA1 (VM785/3) F	CCGGATCCCCTTTGAGTTTACATATATG
AMA1 (VM990) R	AAATTCTTTCTAGGGCAAAC
Internal primers	
AMA1 (VM815) F	GGAACTCAATATAGACTTCC
AMA1 (VM990) R	AAATTCTTTCTAGGGCAAAC

Table 1: List of Primers and sequences.

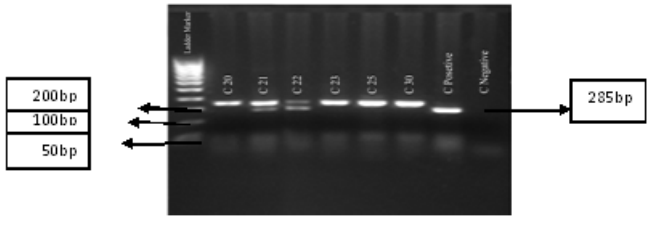

Figure 2: Gel electrophoresis image of the product RFLP (alleles K1).

Imported cases	Indigenous cases	AMA1 alleles
17	20	K1
24	21	HB3
19	15	3D7

Table 2: AMA1 allele prevalence in two population of imported cases and indigenous cases in South-East of Iran.

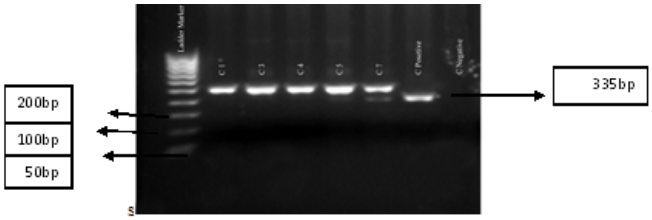

Figure 3: Gel electrophoresis image of the product RFLP (alleles 3D7).

were observed. The K1 allele populations of indigenous cases are more than of its frequency among population of imported cases from Pakistan. The allele frequencies HB3-3D7 obtained in the patient population of imported cases from Pakistan more than of its frequency among population of indigenous cases. In comparison AMA1 alleles in populations of its Geographic Region that naturally contain. chi-square test on allele frequencies of K1, HB3, 3D7 showed no significant difference (P>0.05). In addition, the chi-square test was found, significant relationship between time of sampling and transmission type (P<.001).

This survey reported for the first time in the country. The RFLP gel electrophoresis image of the products (Figure 3).

For the first time with comparative views with Nested PCR and RFLP in this study were analyzed. Due to sensitivity and specificity of Nested PCR over 94% demonstrated in some studies [19]. Patients, according to local of malaria transmission by Anopheles bites, were divided into two populations (imported and indigenous cases) not based on nationality.

In a study by Issiaka Soulama partners in West and Central Africa was conducted in 2010 revealed that PfAMA1 heterozygosity more than MSP3 and EBA 175 [20,21] and it turns out that parasites geographic maps can be different by antigenic variation. The significant relationship between allele frequency distribution (K1 and HB3) obtained in agreement with our study. But, significant correlation between the frequency of the allele 3D7 in different geographic areas were conducted. However, in the present study the 3D7 allele exhibited a no significant relationship between the frequency of geographic areas. Other results showed that the 3D7 allele frequency is lower in East Africa than in West and Central Africa, which may be due to natural selection region to humoral responses [22].

Naturally diverse AMA1 in areas of very high selection that can make it difficult to produce a vaccine based on this antigen. The AMA1 antigen demonstrated four allelic classes that are highly variable. Three allelic classes was observed in Sub-Saharan Africa and the fourth has not been observed and assumed that there can be no in sub-Saharan regions [22].

AMA1 polymorphism analysis in the area of Brazil and Peru, three classes of four classes allelic AMA1 [23] and another analysis in India, only two classes of AMA1 allelic classes have been observed [24]. In the present study examined three allelic classes and is reported for the first time in study areas.

Vaccine based on AMA1 (3D7 and FVO) is already high efficiency of the human being, despite clinical evidence for the efficacy of the vaccine, however, is now more than 60 sites polymorphic protein AMA1 recognition is that the results of this study it can be said that the 3D7 as a better candidate malaria vaccine is designed [25] and are in agreement with our study. According to the results of this study can be inferred that (3D7), given that its frequency changed during transmission, it is apparent that the more frequently. A study in 2009, by Dr. Mardani and Keshavarz et al., [26] 48 samples from Iran as a Nested- PCR has been studied. This survey in summary, exhibited the high level of variation in nucleotide and haplotype of (DOMAIN-I) PfAMA-1. The results also demonstrated that natural selection has played a major role in maintaining the genetic diversity of AMA1. The genetic variation in the observed variation of AMA1 may participate the comparative approach and confirmed the presence of allelic classes, depending on the patient population with a larger sample size to accurately determine the location of the transmission was done using RFLP.

Conclusion

Interpreted results of regional geography that alleles (3D7), lower prevalence in parts of East Africa, which could be due to the immune response to individual [27,28]. In this study of lower prevalence in Iran than Pakistan, perhaps this is the reason why. So in general, AMA1 is a high variation, comparing different regions, regional differences in the allelic distribution of the gene showed that it is possible to design a vaccine based on AMA1 have a problems. Although protected areas (AMA1) in previous studies, which have been shown [9,24]. However, it is important that we know the extent of genetic diversity in AMA1 know to succeed in the design of effective vaccines. Allele frequency of 3D7 at various time intervals varied so in March - April and the period August - October with a higher frequency among isolates were isolated, but no statistically significant association between allele frequency with peak transmission was observed (P> O.O5) It is interesting to note that in the period of transmission of malaria cases than in other months of the year. And it results in a more effective vaccine design and proper drugs, and finally getting around achieving the elimination of local transmission program brought into falciparum malaria. Further studies may help to determine the relationship between the allele frequency distribution of the different timescales discussed at malaria transmission. Perhaps natural selection occurs. Considering on these results, 3D7 alleles suitable candidates for malaria vaccine design can be. The data reported here will be valuable for the development of AMA1 based malaria vaccine.

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