

Genotyping of erythrocyte binding antigen 175 KD (eba-175) alleles of *plasmodium falciparum*-malaria using nested-PCR in south-east of Iran

Ebrahimzadeh Adel^{1, *}, Jamshidi Ali²

¹Department of Medical Parasitology and Mycology and Membership of Infectious Diseases and Tropical Medicine Research Center, Zahedan University of Medical Sciences, Zahedan, Iran

²Department of Medical Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran

Email address:

adel1336@yahoo.com (Ebrahimzadeh A.), Ebrahimzadeh@zaums.ac.ir (Ebrahimzadeh A.), jamshidi@zaums.ac.ir, (Jamshidi A.), jamshidiali91@yahoo.com (Jamshidi A.)

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Abstract: The Erythrocyte Binding Antigen-175 (EBA-175) on *Plasmodium falciparum* (*P.falciparum*) merozoites mediates sialic acid dependent binding to glycophorin a on host erythrocytes and, therefore, plays a crucial role in cell invasion. Dimorphic allele segments have been found in its encoding gene with in FCR-3 strains (F-segment) and CAMP strains (C-segment). This study was designed to determine the distribution of EBA-175 alleles of *P.falciparum* in the South-East of Iran. Nested Polymerase Chain Reaction method used with specific primers, which improves the two fragments of the EBA-175 gene. Ninety-four microscopically positive blood samples were collected from the infected *P.falciparum* malaria patients from four different districts. The 88 out of 94 confirmed *P.falciparum* samples were successfully scored for EBA-175. The allelic genotyping exhibited CAMP strains (714 bp) and FCR-3 strains (795 bp) in 31(32.97%) and 49(52.12%) cases, respectively and 8 cases (8.51%) showed mixed allelic CAMP/FCR-3 infections. The two fragments of dimorphic EBA-175 gene were observed and the FCR-3 allele was more prevalent in South-East of Iran. This distributional pattern should be considered in designing to control *P. falciparum* malaria in the region.

Keywords: *Plasmodium falciparum*, Erythrocyte Binding Antigen-175, South-East of Iran

1. Introduction

Malaria causes an estimated 300–500 million clinical cases and 1–3 million deaths annually. The disease is caused by parasites of the genus *Plasmodium*, of which *Plasmodium falciparum* is the most prevalent and is responsible for almost all the associated mortality [1, 2]. Despite enormous efforts to control and prevent malaria, multiple factors, including insecticide resistance in the mosquito vectors, the lack of effective vaccines, and the emergence and rapid spread of drug-resistant strains, are contributing to the global worsening of the malaria situation [3].

Therefore, there is an urgent need for the development of effective malaria vaccines [4]. However, extensive genetic diversity in natural parasite populations is a major blockage for the development of an effective vaccine

against the human malaria parasite, since antigenic diversity limits the efficacy of acquired protective immunity to malaria [5]. Such extensive antigenic polymorphism intensely improves the parasites ability to invade host's immune defense, making it difficult to evoke adequate responses against all of the antigenic variants circulating in the parasite population [6]. A true understanding about the frequency and alterations of vaccine candidate antigens in natural parasite populations is crucial to design a successful and effective malaria vaccine, as well as providing useful facts for interpretation of responses to the vaccine. A handful of *P. falciparum* stage-specific antigens have been characterized as vaccine candidates by means of molecular techniques. Determining the distribution of malaria parasite antigen genotypes in a wide geographic area could be useful to provide valuable genetic information to design malaria vaccine.

This survey aimed to analyze genetic diversity of

Erythrocyte Binding Antigen 175 KD (EBA-175) of *Plasmodium falciparum* antigen as a potential vaccine candidate. The erythrocyte binding antigen-175 gene, located on chromosome seven. The EBA-175 gene is including three cysteine-rich regions (F1, F2 and C) and binding regions F1 and F2 positioned at the N-terminus of the molecule, exhibit low polymorphism. Conversely, region III, which is centrally located, is characterized by two dimorphic segments termed FCR3 and CAMP. This dimorphism results from different sized insertions located at slightly different positions in the region II [6, 7]. EBA-175 binds to sialic acid and the peptide backbone of glycophorin A on the erythrocyte surface [8, 9].

The South-east of Iran is a main center of the endemic falciparum malaria area that is bordered by Pakistan and Afghanistan. Malaria transmission occurs almost in a year with two peaks, May-June and October-November. Both *P. falciparum* and *P. vivax* are found in the country. National

Malaria Control Program reported 15,909 malaria cases in the year 2006 of which more than 83% were autochthonous. *P. falciparum* malaria cases are included nearly 11% of total malaria in Iran [10, 11, 12].

2. Materials and Methods

2.1. Study Area

This study was designed to determine the distribution of EBA-175 alleles in the south-East of Iran, and to analyze some potential protective association of the EBA-175 alleles fragments.

Peripheral blood samples were collected from the 94 *P. falciparum* infected individuals participating in a descriptive cross sectional survey in from four district in the Sistan and Baluchistan south-East of Iran (Chabahar, Nikshahr, Iranshahr and Sarbaz) in 2012 (Figure 1).

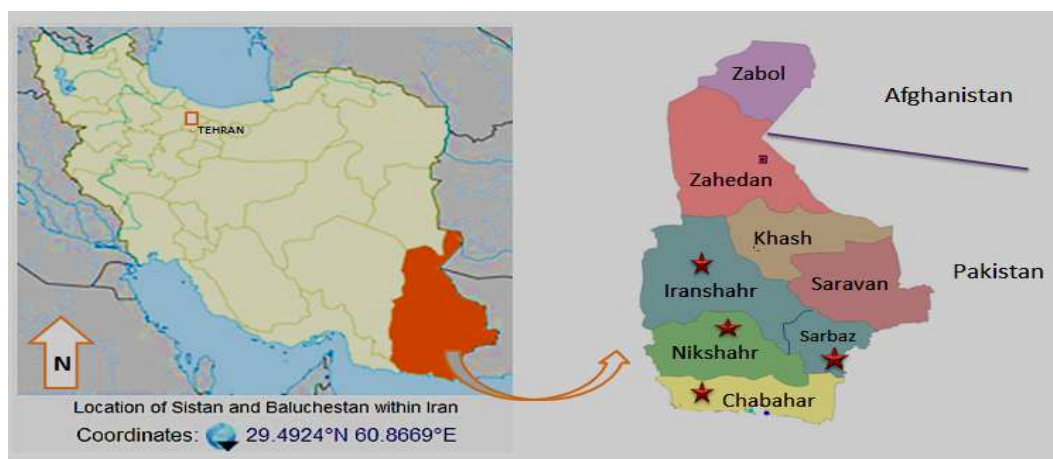


Figure 1. Map of study area, Districts of Sistan and Baluchistan, South-East of Iran.

2.2. Samples Collections

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 from March 2011 to September 2012. 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.

2.3. DNA Extraction and PCR Amplification

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.

Nested PCR was used to amplify on region III of *P. falciparum* EBA-175 gene using the sequence of primers consisted of EBA-175 (Table 1). Purified DNA from *P. falciparum* CAMP (714 bp) and FCR-3 (795 bp), strains

was provided by the Malaria Research and Reference Reagent Resource Center, American Type Culture Collection (Manassas, VA) and used as a positive control during the amplification reactions. The second amplification products were directly separated by electrophoresis on a 2.0% ethidium bromide agarose gel and visualized on a Trans-illumination Imaging System.

Table 1. List of Primers and sequences.

Primer name	Sequence length 5'→3'
External primers	
EBA1(F)	CAAGAAGCAGTTCCTGAGGAA
EBA2 (R)	TCTCAACATTATATTAACAATTC
Internal primers	
EBA3(F)	GAGGAAAACTAGAAATAGCACAC
EBA4(R)	CAATTCCTCCAGACTGTTGAACAT

3. Results and Discussion

The main goal of this study was to analyze the polymorphic antigen EBA-175 region III genes across South-East of Iran among four different districts to identify differences in allele frequency and genetic diversity. Of the

94 confirmed *P.falciparum* samples obtained from the four districts, 88 samples were successfully scored for EBA-175. The allele classes were identified using agarose gel electrophoresis. Size differences confirmed that both FCR-3 and CAMP allele classes of *P.falciparum* EBA-175 are present in the region of study (Figure 2). The EBA-175 allele classes (FCR-3 and CAMP types) showed comparable prevalence in all districts (Table 2). Overall frequencies of FCR-3 and CAMP allele classes were 93.61 % for both. The frequency of FCR-3 alleles (795bp) in Chabahar, Iranshahr, Nikshahr, and Sarbaz were

16(51.61%), 13(54.1%), 11(47.82%), and 9(56.25%) respectively. Furthermore, the frequency of CAMP alleles (714bp) in mentioned districts were respectively 10(32.25%), 8(33.33%), 9(39.13%) and 4(25%) (Table 2). The Positive and Negative controls and a 1000bp Ladder Marker (Bioneer, Korea Rep) were used to interpret the fragments sizes. The EBA-175, FCR-3 allele was identified as a single 795 bp and CAMP allele with a single 714 bp fragment. The Mixed infection were defined by the presence of FCR-3 and CAMP alleles simultaneously (Figure 2).

Table 2. EBA-175 region III alleles prevalence in South-East of Iran.

District Allele	Chabahar No. (%)	Iranshahr No. (%)	Nikshahr No. (%)	Sarbaz No. (%)	Total
samples	31(32.97)	24(25.53)	23(24.46)	16(17.02)	94(100%)
FCR-3=F loop (795bp)	16(51.61)	13(54.1)	11(47.82)	9(56.25)	49(52.12)
CAMP=C loop (714bp)	10(32.25)	8(33.33)	9(39.13)	4(25)	31(32.97)
Mix (C+F)	2(6.45)	1(4.16)	3(13.04)	2(12.5)	8(8.51)
Negative for presence of Allelic classes	3(9.67)	2(8.33)	0(0.00)	1(6.25)	6(6.38)

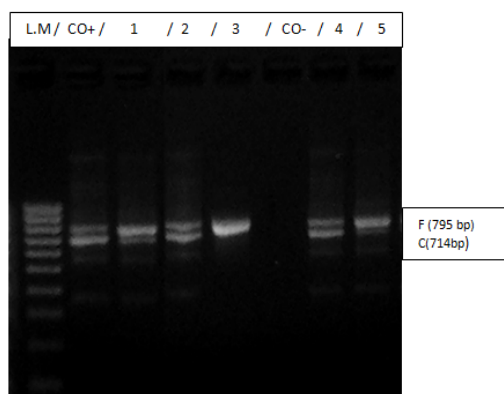


Figure 2. Agarose gel of nested PCR amplicons from *P. falciparum* DNA extracted from four districts in South-East of Iran showing that both *P.falciparum* EBA-175 F loop (top arrow) and C loop (bottom arrow) allele classes are present. L.M: Ladder Marker; co+: Positive Control (F+C); co-; Negative Control.

In this study, the Nested PCR used to screen allelic diversity within the malaria vaccine candidate *P.falciparum* EBA-175, region III. The allelic genotyping exhibited, 88 individual *P.falciparum* infections containing, both CAMP and FCR-3 allele classes. The *P.falciparum* EBA-175 region III domain, where the majority of genetic diversity has been shown to occur in many different studies [5, 6, 13]. Also, minor cases of co-infections (C loop + F loop) of EBA-175 gene were observed to some extent in studied districts. This study was accomplished ignoring seasonal frequencies of each allele classes. The FCR-3 genotype was more prevalent (52.12%) which is in agreement with the results obtained in the Lao PDR in Asia [14], Sarbaz in South-East of Iran [13], Western and Central Africa [5], Gabon in Africa [15] and in Ghanaian children [16].

These data differs from results reported in Sudan [17] and Brazilian endemic area [2]. While there are different populations of *P.falciparum* in various parts of the world, the distribution of alleles of the EBA-175 gene should be different according to the geographical region studied [18].

The possible cause of the observed changes in allelic frequency among geographical regions might be extraneous factors such as genetic drift, which is a major contributor to allele variation in small populations. Another hypothesis would be that differences in the genetic background among study population may select for different EBA-175 alleles. In this case, allele frequencies might be predicted to be shifted to FCR-3 genotype in reality while allele frequencies change during seasonal transmission peaks, a consequence of natural selection [19]. This study showed that the prevalence of coinfection is lower in South-East of Iran malaria infected patients than other geographic regions in the world [14, 15] and this findings are equal to Heidari *et al.*, [20] findings in Sarbaz district. However, the presence of either the F loop or C loop allelic forms merely does not exclude the possibility of multiple infections [13].

This findings are not consistent with the study performed in Lao PDR in which the distribution of EBA-175 alleles were different between north and south provinces [14] and are different from the results of Soulama study in malaria endemic area of Burkina Faso [5, 6]. This findings confirmed the presence of different polymorphic allele classes of Erythrocyte Binding Antigen 175 in this region, while the FCR-3 genotype (F loop) was the dominant allelic class in this study area.

4. Conclusions

Since, no study has yet looked at the extent of *P.falciparum* EBA-175 in this geographic region including the four different districts of malaria transmission, these data are needed to support development of a vaccine based on EBA-175 antigen along the malaria vaccine roadmap. Also, the results showed the remarkable predominance of FCR-3 allele in the studied area. There should be a comparative analysis in different seasonal peaks to indicate the allelic polymorphisms of the EBA-175 over a period of time.

Recent studies have revealed the EBA-175 region III of *P.falciparum* as a highly more immunogenic domain than other regions [6], supporting our recommendation that the region III should be reassessed for future vaccine developments. These data supports the hypothesis of a biologically important role for EBA-175 in parasite development and highlight the importance of evaluating the distribution of EBA-175 allelic forms in different geographical regions to provide valuable genetic information to design an effective malaria vaccine despite the extensive present genetic diversity.

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