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PCR-RFLP analysis of *Plasmodium vivax* reticulocyte binding protein2c gene in field isolates of Iran

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Abstract. A family of reticulocyte-binding proteins of *Plasmodium vivax* (PvRBP) is localised at the apical pole of the merozoites and appears to bind to reticulocytes specifically and has also been involved in identifying host cells. Protein component produced by the *Pvrbp2c* gene is highly antigenic. The aim of this study was to detect the genetic diversity in the *Pvrbp2c* gene of Iranian *P. vivax* field isolates using the polymerase chain reaction- restricted fragment length polymorphism (PCR-RFLP) technique. A total of 79 *P. vivax* malaria patients with fever participated in the study. Alu1 and Apo1 restriction enzymes were independently used to identify allelic variants of the *Pvrbp2c* gene. All of the samples exhibited a single band of about 2 Kb in nested PCR. Among 79 *P. vivax* field isolates in the RFLP with Apo1 and Alu1 restriction enzymes, 15 and nine patterns were observed, respectively. In total, 24 various patterns were detected from the combined findings of both Alu1 and Apo1 fragments in RFLP. This study revealed that *Pvrbp2c* has genetic diversity in southeast Iran. Genotyping of *Pvrbp2c* not only shows the heterogeneity of *P. vivax* but also provides important information that could be used to control vivax malaria.

INTRODUCTION

Malaria continues to be an important infectious disease in many countries of the world (Coronado *et al.*, 2016). Among five *Plasmodium* species that cause malaria in humans, *Plasmodium vivax* is the main species that induces malaria infection in people of Asia, Oceania, Central and South America and some parts of Africa (WHO, 2015). It accounted for about 14 million malaria cases worldwide in 2015 (WHO, 2015). In Iran, *P. vivax* is the dominant malaria species and contributes to 90% of the malaria cases (Ehtesham *et al.*, 2015). Despite WHO reports indicate a dramatic decrease in malaria cases in Iran, however during the past decade, *P. vivax* is still being transmitted locally especially in the south-east of the country (Heidari *et al.*, 2012; Sheikhzadeh *et al.*, 2016).

The asexual form of *Plasmodium* species invades host RBC via a complex process (Aikawa *et al.*, 1978; Hadley *et al.*, 1986; Li *et al.*, 2012). *Plasmodium falciparum* is able to invade both mature erythrocytes and reticulocytes using different ligands and receptors, while *P. vivax* merozoites only invades reticulocytes (Gunalan *et al.*, 2013; Malleret *et al.*, 2016; Hietaen *et al.*, 2016). It is noted that such differences in host RBC selection are concerned with the use of various ligands and molecules for RBC recognition (Hietanen *et al.*, 2016). A family of reticulocyte-binding proteins of *Plasmodium vivax* (PvRBP) is

localised at the apical pole of the merozoites and appears to bind to reticulocytes specifically and has also been involved in identifying host cells (Malleret *et al.*, 2015; Gunalan *et al.*, 2013; Hietanen *et al.*, 2016). At first, only two members of this family named PvRBP1 and PvRBP2 that were involved in RBC recognition and selection have been found in the Belem strain of *P. vivax* (Galinsky *et al.*, 1992).

Sequencing of *P. vivax* Salvador strain indicated that the *Plasmodium vivax* reticulocyte-binding protein genes family is composed of 8- protein coding genes (*Pvrbp1a*, *Pvrbp1b*, *Pvrbp2a*, *Pvrbp2b*, *Pvrbp2c*, *Pvrbp1p*, *Pvrbp2p1 and Pvrbp2p2*) and 3-pseudo genes (*Pvrbp2d*, *Pvrbp2e and Pvrbp3*) (Carlton *et al.*, 2008; Gunalan *et al.*, 2013; Hietanen *et al.*, 2016). The main *Pvrbp2* gene of the Belem strain was matched to the *Pvrbp2c* gene of the Sal-1 strain and is now named *Pvrbp2c* (Hietanen *et al.*, 2016). Generally, except *Pvrbp2c*, the other members of *Pvrbp* family reveal limited diversity. Protein component produced by the *Pvrbp2c* gene is highly antigenic, so it could be considered as a potential candidate in vaccine development (Lie *et al.*, 2012; Prajapti *et al.*, 2012; Hietanen *et al.*, 2016). Understanding genetic diversity of *Plasmodium* species genes is not only essential for detecting the population structure of malaria parasites but is also important for designing control tools, including malaria vaccines and assessments of the effects of malaria control measurements (Mzilahowa *et al.*, 2007; Khan *et al.*, 2014).

Genetic diversity is a main concern in potential malaria vaccine candidates because produced antibodies against an allelic form of an antigen may not protect erythrocytes against invasion of *plasmodium vivax* parasites that exhibit in other allelic forms of that vaccine candidate thus, the diversity can restrict the efficacy and universally apply of a unique malaria vaccine (Rayner, 2005). Therefore, study of genetic variation at the *Pvrbp2c* from geographical field isolates is an important phase for identifying the structure of the *Plasmodium* population that exists in the region endemic for malaria and essential for development of efficient malaria vaccine.

Even though genetic variation in *Pvrbp2c* has been investigated in a few malaria endemic areas of the world, the current study is the first investigation of this kind conducted in Iran.

The aim of this study was to detect the genetic diversity in the *Pvrbp2c* gene of Iranian *P. vivax* field isolates using the PCR-RFLP technique.

MATERIALS AND METHODS

Specimen collection

Venous blood samples were taken from symptomatic malaria patients attending malaria centers in the Sarbaz and Chabahar districts in Sistan & Baluchistan province from May to December 2014. Giemsa stained blood slides were screened to determine *P. vivax* mono infection with a microscope at 1000x magnification. A total of 79 *P. vivax* malaria patients with fever participated in the study. Whole blood samples were poured into EDTA coating tubes and stored at –20ºC.

Nested PCR

DNA was extracted from 200 µL blood samples using a QIAquick PCR purification kit (Qiagen, Germany), according to the manufacturer's instruction. Nearly 100 ng of extracted DNA was applied in initial PCR amplification. Nested PCR was performed using the 1 µL from primary PCR product Primers and nested PCR amplifying procedures have been clarified previously (Prajapati *et al.*, 2012). Both positive and negative controls were used for nested PCR. The PCR products were run in 2% agarose gel electrophoresis that was stained by DNA Safe Stain and visualised under ultraviolet transillumination.

RFLP

Alu1 and Apo1 restriction enzymes were independently used to identify allelic variants of the *Pvrbp2c* gene. So Alu1 and Apo1 identified sequences of 5AG CT3 and

5R AATTY3, respectively. Enzyme digestion was carried out in a total volume of 25 µL, including 6 µL PCR product, 2.5 µL buffers, 1 µL enzyme and 15.5 µL distilled water. After that, the digestion reactions consisting of Alu1 and Apo1 were incubated at 56ºC (18 h) and 37ºC (24 h), respectively. Finally, *Pvrbp2c* of the digestion products was run at 2% agarose gel electrophoresis in order to visualise the separated fragments.

For better access to the genetic structure of *Pvrbp2c*, 10 samples were sequenced. Sequencing was carried out in both forward and reverse strands. For sequencing, we applied three primers to amplify several overlapping fragments to access greater length of the gene.

Ethical considerations

Informed, written consent was obtained from each patient. The use of human blood samples in this study was approved by the Research Ethical Review Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran with approval number1392.711.

RESULTS

Blood samples were collected from 79 *P. vivax* symptomatic malaria patients. All of the samples exhibited a single band of about 2 Kb in nested PCR. There was no variation in the PCR products.

The intron and exon regions both consisted of the amplified PCR fragments. RFLP digestion with Alu1 and Apo1 restriction enzymes depicted various fragment sizes in the *Pvrbp2c* gene (Figs. 1 and 2). Among 79 *P. vivax* field isolates in the RFLP with Apo1 and Alu1 restriction enzymes, 15 and nine patterns were observed, respectively (Tables 1 and 2). Additionally, in RFLP three samples exhibited a size greater than 2000 Bp.

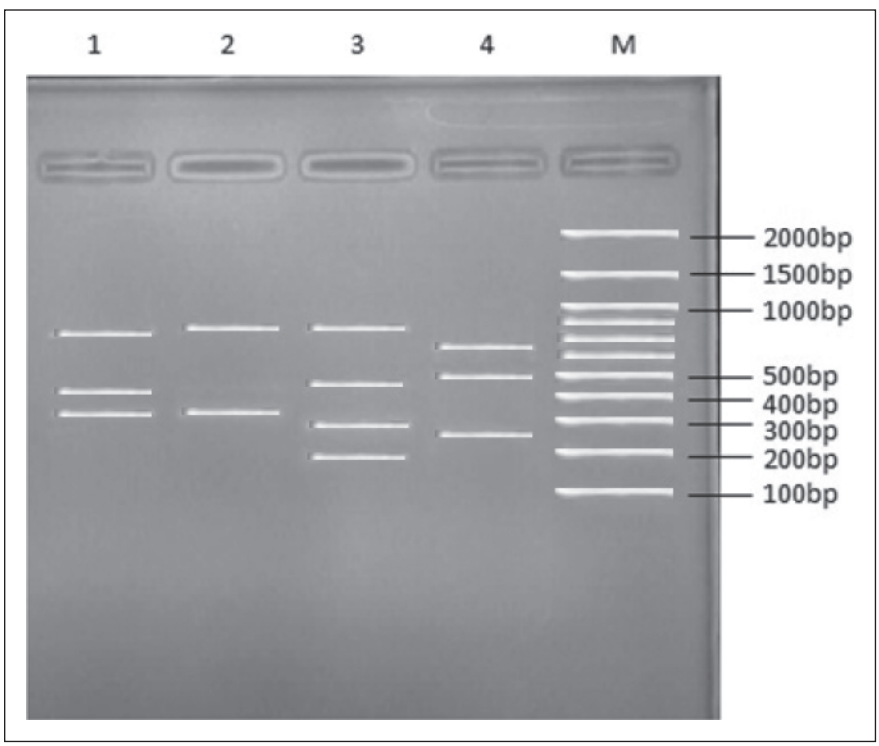

Fig. 1. RFLP fragments of the *Pvrbp2c* Iranian isolates using Alu1 restriction enzyme. Lanes 1-4 illustrate digestion pattern of field isolates. M shows 2000bp ladder that used as DNA marker (M).

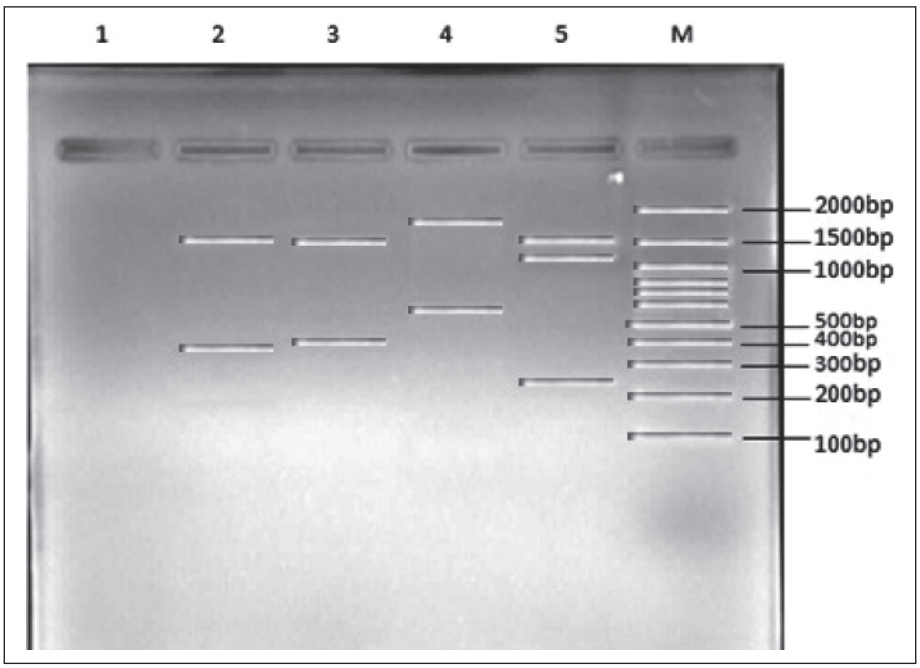

Fig. 2. RFLP fragments of the *Pvrbp2c* Iranian isolates using Apo1 restriction enzyme. Lanes 1-4 illustrate digestion pattern of field isolates. M shows 2000bp ladder that used as DNA marker (M).

Table 1. PCR -RFLP allelic Patterns of *Pvrbp2c* gene in Iranian *Plasmodium vivax* field isolates using Alu1 enzyme. P=pattern F=fragments

P	F1	F2	F3	F4	F5
1	850	550	400	–	–
2	850	650	380	–	–
3	650	500	400	350	–
4	650	500	450	400	–
5	800	650	280	–	–
6	800	500	380	–	–
7	800	600	480	250	–
8	800	600	500	280	–
9	800	600	480	300	–
10	800	600	500	300	–
11	800	650	500	350	–
12	800	650	500	300	–
13	700	600	550	280	–
14	800	480	300	–	
15	900	480	300	200	–
16	800	600	300	–	–
17	800	600	300	280	
18	800	600	300	300	280
19	700	600	280	200	–
20	700	500	380	200	–
21	900	700	500	–	–
22	800	600	500	100	–
23	800	500	300	200	–
24	800	650	500	300	–
25	700	550	300	–	–
26	800	500	300	100	–

Table 2. PCR -RFLP allelic Patterns of *Pvrbp2c* gene in Iranian *Plasmodium vivax* field isolates using Apo1 enzyme. P=pattern F=fragments

P	F1	F2	F3	F4	F5
1	1200	480	300	–	–
2	1000	430	250	–	–
3	1500	400	300	–	–
4	1500	1200	250	–	–
5	1500	380	–	–	–
6	1200	380	280	–	–
7	1500	480	–	–	–
8	1500	500	80	–	–
9	1500	380	200	–	–
10	1600	1200	480	300	–
11	1200	430	280	–	–
12	1500	400	–	–	–
13	1500	1200	430	–	–
14	1600	400	–	–	–
15	1600	600	–	–	–

In total, 24 various patterns were observed from the combined findings of both Alu1 and Apo1 fragments in RFLP. Sequence of the one isolates was deposited in the Gene Bank with accession number KU052616. Intron, consisted 201 nucleotides, is located between 505 and 706 nucleotide

positions in this isolate. At the nucleotide level, the *Pvrbp2c* Iranian isolate is 98% and 93% identical with the Belem and Sal1 strains, respectively, using Blast. The Iranian isolate in the Restriction Site Analysis software exhibited nine digestion fragments using the Apo1 enzyme (788, 410, 244, 232, 110, 58, 37, 28, and 24). While using the Alu1 enzyme, the isolate was illustrated in a 3-digestion fragments (1519, 369, 33).

DISCUSSION

Elimination of malaria in the world is one of the main goals of WHO and in this context the making of an efficient malaria vaccine and effective treatment have been highlighted (WHO, 2015). Genetic diversity of *Plasmodium* genes is an obstacle to the elimination of malaria and to the making of a vaccine to control the disease. Thus, one of the important steps to achieve this goal is detection of the genetic variation of the protein coding genes that could be used in the malaria vaccine. In the *Pvrbp* family gene, *Pvrbp2c* has been considered a candidate for malaria vaccine (Prajapati *et al*., 2012; Kosaisavee *et al*., 2012). It has also been applied as a marker in genetic diversity studies in the world. Since *Plasmodium vivax* is a dominant species in the malaria regions of Iran and there are reports indicating the existence of different allelic variants of *P.vivax* in these areas, the current study was conducted to understand the genetic diversity of the *Pvrbp2c* gene using PCR-RFLP in the field isolates.

This study, demonstrated genetic diversity in *Pvrbp2c* among the field isolates. The results are in agreement with studies that have been conducted in India and Thailand which have also reported high genetic variation of *Pvrbp2c* (Prajapati *et al*., 2012; Kosaisavee *et al*., 2012). Previous studies on several *Pvrbp* gene sequences have indicated a remarkably high degree of genetic diversity in the *Pvrbp2c* gene and have revealed evidence of positive diversifying selection (Rayner *et al*., 2005; Kosaisavee *et al*., 2012; Hietanen *et al*., 2016). It seems the extent of genetic variation is concerned with immune pressure natural selection and transmission rate of malaria (Escalante *et al*., 2004). Diverse variants however, are considered a threat to public health (Peyerl-Hoffman *et al*., 2001). Hietanen *et al*. (2016) found that *P. vivax* malaria adult patients in western Thailand produced IgG against PvRBP2c protein and they also found the level of this antibody correlated to *Pvrbp2c* high genetic diversity. Another recent study by Han *et al*. (2015) revealed that the N-terminal fragment of PvRBP2c protein is highly immunogenic and has potential immunodominant B-cell epitopes. It is possible that the high level IgG antibody may be switched *Pvrbp2c* sequence diversity to escape from host immune pressure (Ferreira *et al*., 2014).

Although Iran has been defined as a hypo-endemic region for malaria, *Pvrbp2c* has exhibited higher genetic diversity consistent with the other studies that have been conducted on *P. vivax msp1* and *P. vivax ama1* genes in the country (Miahipour *et al*., 2012; Heidari *et al*., 2013). It appears that shared borders with Pakistan and Afghanistan and consequently migration of people and movement in the border lines, as well as host immune-selective pressure, could be attributed in the genetic variation of *Pvrbp2c*. RFLP-PCR patterns of *P. vivax* in the Iranian field isolates have exhibited patterns of *P. vivax* Sal-1, Belem and a combination of both strains. The existence of these strains according *Pvrbp2c* is consistent with findings of the study by Zakeri *et al*. (2006) on *Pvmsp1* in Iran that reported Belem and Sal-1 strains. The Apo1 enzyme demonstrated more genetic diversity at *Pvrbp2c* than the Alu1 enzyme in RFLP-PCR and that is consistent with a study by Prajapati *et al*. (2012) that was conducted in India on field *P. vivax* isolates. Therefore, Apo1 enzymes alone could be used for detecting rapid *Pvrbp2c* genetic variation in field isolates. In the RFLP digestion pattern, three samples exhibited size greater than 2000 bp; it seems infection by two clones could be explained in these patterns. Overall, in this study Belem-like isolates were found to exhibit more frequency.

The Iranian isolate deposited at Gene Bank has shown differences with other isolate sequences recorded at that Gene Bank.

This study revealed that *Pvrbp2c* has genetic diversity in southeast Iran. Genotyping of *Pvrbp2c* not only shows the heterogeneity of *P. vivax* but also provides important information that could be used to control vivax malaria. Otherwise, RFLP-PCR could be applied for detecting the genetic diversity of field *Pvrbp2c* isolates

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