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Antibody Responses and Avidity of Naturally Acquired Anti-*Plasmodium vivax* Duffy Binding Protein (PvDBP) Antibodies in Individuals from an Area with Unstable Malaria Transmission

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Abstract. *Plasmodium vivax* remains an important cause of morbidity outside Africa, and no effective vaccine is available against this parasite. The *P. vivax* Duffy binding protein (PvDBP) is essential during merozoite invasion into erythrocytes, and it is a target for protective immunity against malaria. This investigation was designed to evaluate naturally acquired antibodies to two variant forms of PvDBP-II antigen (DBP-I and -VI) in malaria individuals ($N = 85$; median = 22 years) who were living in hypoendemic areas in Iran. The two PvDBP-II variants were expressed in *Escherichia coli*, and immunoglobulin G (IgG) isotype composition and avidity of naturally acquired antibodies to these antigens were measured using enzyme-linked immunosorbent assay (ELISA). Results showed that almost 32% of the studied individuals had positive antibody responses to the two PvDBP-II variants, and the prevalence of responders did not differ significantly ($P > 0.05$; χ^2 test). The IgG-positive samples exhibited 37.03% and 40.8% high-avidity antibodies for PvDBP-I and PvDBP-VI variants, respectively. Furthermore, high-avidity IgG1 antibody was found in 39.1% of positive sera for each examined variant antigen. The avidity of antibodies for both PvDBP variant antigens and the prevalence of responders with high- and intermediate-avidity IgG, IgG1, and IgG3 antibodies were similar in patients ($P > 0.05$; χ^2 test). Moreover, the prevalence of IgG antibody responses to the two variants significantly increased with exposure and host age. To sum up, the results provided additional data in our understanding of blood-stage immunity to PvDBP, supporting the rational development of an effective blood-stage vaccine based on this antigen.

INTRODUCTION

Plasmodium vivax is the second most prevalent human malaria parasite worldwide and accounts for approximately 80% to 90% of cases in Asia, Oceania, and Latin America.[1] The emergence of drug-resistant *P. vivax* isolates[1] is associated with severe and fatal malaria.[2,3] Also, the limitation of our understanding of *P. vivax* epidemiology has caused problems in elimination programs, particularly in countries where this parasite is prevalent. Therefore, all these factors highlight the need for considering *P. vivax* in control measures to reduce vivax malaria burden.

P. vivax initiates erythrocyte invasion through expression of several surface and apical organelles on the merozoite that binds to erythrocyte surface proteins.[4,5] One of the well-characterized ligand–receptor interactions involves the Duffy binding protein (DBP), which is required for junction formation during merozoite invasion to the host cell.[5–7] Duffy-negative individuals are naturally protected against clinical *P. vivax* malaria,[7] because invasion by the parasite is dependent on binding to the Duffy antigen receptor for chemokines (DARC).[8] However, recent reports have described the transmission of *P. vivax* to a Duffy-negative population in Kenya, suggesting that *P. vivax* could have alternative invasion pathways, although it is rare and no other means of invasion have been identified.[9,10] Thus, the *P. vivax* DBP (PvDBP) represents one of the most promising subunit vaccine candidate antigens against the asexual stages of the parasite for reducing or eliminating the blood stages from malaria parasites and their pathological outcomes.

The PvDBP is a type I membrane protein (140 kDa) that belongs to a family of homologous Duffy binding-like erythrocyte binding proteins (DBL–EBP) located within the micronemes of merozoite[4–11] and is characterized by a functionally conserved cysteine-rich region.[5–11] This cysteine-rich region in region II (PvDBP-II) was identified as the domain binding to DARC on the erythrocyte surface[12,13] that includes cysteines 5 to 8.[14,15] Critical binding motifs in PvDBP-II have been mapped to a region between amino acids 291 and 460.[14] Although the cysteine and some other hydrophobic amino acid residues are conserved in the binding motif, other amino acid residues are highly polymorphic,[16–18] and this diversity varies geographically from region to region.[16,18–20]

After natural exposure to *P. vivax* infection, individuals residing in malaria-endemic areas develop antibodies that block binding of DBP to DARC-positive erythrocytes.[21] It has been hypothesized that polymorphisms in PvDBP-II arose from immune selection[16,18–22] so that the frequency of non-synonymous mutations exceeds that of synonymous mutations in PvDBP-II. These polymorphic regions represent B- and/or T-cell epitopes recognized by the host immune response that might inhibit protective immunity against DBP. Therefore, assessment of the level of genetic diversity of *Pvdbp-II* between and within populations from distinct geographic regions and also its effect on naturally acquired immunity must be considered for vaccine development.

Antibody responses to PvDBP have been shown in endemic populations of *P. vivax* infection in Papua New Guinea,[23–25] Brazil,[26–28] and Colombia.[29] In addition, different studies suggested that stronger humoral and cellular immune responses to PvDBP-II develop progressively with increasing age,[22,24,29,30] showing a boosting effect that was likely because of repeated exposures to the infection.[28] Also, the anti–PvDBP-II antibodies in populations living in areas endemic for *P. vivax* could block PvDBP-II ligand DARC-positive erythrocytes[21,31] and inhibit erythrocyte invasion *in vitro*.[28,32]

As shown by several studies, selection of an antigen for vaccination requires a detailed understanding of natural immune responses elicited by the protein from different malaria-endemic regions with various epidemiology, and the result of similar studies from one malaria-endemic region cannot be extrapolated to other areas of malaria-endemic regions in the world.[33] Therefore, the current investigation was designed,

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for the first time, to evaluate naturally acquired antibodies to the two variant forms of PvDBP-II antigen (DBP-I and -VI) in malaria-infected individuals living in hypoendemic areas in Iran in the Middle East region. Moreover, the association between level and avidity of anti–PvDBP-II immunoglobulin G (IgG) with host age and exposure was evaluated. The development of vaccine and other novel interventions strategies for controls requires a better understanding of naturally acquired immune response; hence, the result of this study could possibility contribute to current efforts on vaccine development against *P. vivax* for elimination of malaria in vivax-endemic regions.

MATERIALS AND METHODS

Study area and subjects. This study was performed in the tropical southeastern region of Chabahar district in Sistan and Baluchistan province, Iran.[34] In this area, malaria is hypoendemic to mesoendemic, and transmission is unstable, with seasonal fluctuations occurring throughout the year with two peaks: first peak from May to August with *P. vivax* as the predominant species and second peak from October to November when both *P. falciparum* and *P. vivax* infections are recorded. The burden of malaria declined gradually over the last few years from 15,712 total cases in 2007 to 6,122 in 2009 because of applying different malaria control tools in these areas. In this region, most of the patients are adults and may experience several infections by *P. falciparum* and/or *P. vivax* with clinical symptoms. There is no record of severe malaria or death because of malaria in Iran.

In this investigation, 85 subjects (male = 64, female = 21; mean age = 26.14 years) with patent *P. vivax* infection were enrolled from Chabahar district during cross-sectional surveys carried out from May 2005 to October 2006. A questionnaire was given to all study participants to obtain demographic and clinical information and also, to assess their exposure to malaria. The blood samples ($N = 35$) were obtained from the residents in Tehran (Iran) with no known pervious exposure to malaria as negative controls for all assays. Before blood collection, informed consent was obtained from adults or parents/legal guardians of children who were participants in this study. This study was approved by the Ethical Review Committee of Research of Pasteur Institute of Iran. The diagnosis of vivax malaria was made by microscopic examination of blood smears stained with Giemsa staining in the study areas. From all subjects, 5 mL venous blood were collected for both *P. vivax* DNA detection and serum collection. After transferring to the main laboratory in Tehran, all blood samples were again analyzed for *P. vivax* DNA by nested polymerase chain reaction (PCR) amplification as described previously,[35] and the results were used to confirm the microscopy detection.

Expression and purification of recombinant PvDBP-II. In this study, the most prevalent (PvDBP-VI; 41.8%) and least prevalent (PvDBP-I; 7.3%) types of PvDBP-II strains were considered as antigens (Table 1).[20] The PvDBP-II variants (PvDBP-I and -VI), corresponding to 594–1,764 bp Sal-I sequences with GenBank accession numbers EU860428.1 and EU860433.1, were cloned and expressed in *Escherichia coli* M15 as His-tag fusion using pQE30 vector (Qiagen, Hilden, Germany).

Briefly, overnight culture of *E. coli* M15 pQE30–PvDBP-II was grown in Luria broth (pH 7.2) containing ampicillin (100 μg/mL) and kanamycine (25 μg/mL) with shaking (150 rpm) at 37°C. Expression of PvDBP-VI was induced with 1 mM Isopropyl beta thiogalactopyranoside (IPTG) (Sigma-Aldrich Co., USA) when cell density of the culture reached an optical density (OD at 600 nm) of 0.6–0.7. The culture was further grown for 4 hours, and the *E. coli* cells were harvested by centrifugation and kept at –80°C until use. Both PvDBP-II variants were expressed in inclusion bodies; therefore, the cell pellets were dissolved in denaturation buffer containing 6 M guanidine thiocyanate, 20 mM Tris·HCl, 500 mM NaCl, 20 mM immidazol, and 1 mM phenylmethanesulfonylfluoride (PMSF), pH 7.9. The cells were lysed on ice by sonication (Ultraschallprozessor, Deutschland, Germany) with 10 cycles, each consisting of 20-second pulses with 20-second intervals. The bacterial lysate was centrifuged at $14{,}000 \times g$ at 4°C for 30 minutes. The supernatant was incubated with Ni^{2+}–nitrilotriacetic acid agarose resin (Ni–NTA Agarose; Qiagen) at 4°C for 2 hours, and the resin was packed into a column and washed with a 10-column volume of wash buffer (6 M urea, 20 mM Tris·HCl, 500 mM NaCl, and 30 mM immidazol, pH 7.9). The bound protein was eluted with elution buffer containing 8 M urea, 50 mM Na_2HPO_4, 300 mM

TABLE 1
Frequency distribution of *P. vivax* DBP-II variant antigens identified in malaria-endemic areas of Iran[20]

Position (antigen; %)	306	308	371	384	385	386	390	398	404	417	419	424	437	Insertion 475	503
Sal-I	F (TTT)	R (AGG)	K (AAA)	D (GAT)	E (GAA)	K (AAG)	R (CGT)	S (TCT)	T (ACA)	N (AAT)	I (ATA)	L (TTA)	W (TGG)	–	I (ATA)
DBP-I (7.3%)	L (TTG)	S (AGT)		G (GGT)	K (AAA)	N (AAT)	H (CAT)	T (ACT)	R (AGA)			I (ATA)		L (CTA)	
DBP-II (1.8%)		S (AGT)		G (GGT)	K (AAA)	N (AAT)	H (CAT)	T (ACT)	R (AGA)					L (CTA)	
DBP-III (16.4%)			E (GAA)	G (GGT)						K (AAA)	M (ATG)	I (ATA)	R (CGG)		
DBP-IV (7.3%)			E (GAA)	G (GGT)						K (AAA)		I (ATA)	R (CGG)		K (AAA)
DBP-V (5.4%)										K (AAA)		I (ATA)	R (CGG)		K (AAA)
DBP-VI (41.8%)				G (GGT)			H (CAT)								K (AAA)
DBP-VII (3.6%)				G (GGT)											K (AAA)
DBP-VIII (1.8%)													R (CGG)		K (AAA)
DBP-VIII (14.5%)															

NaCl, and 250 mM immidazol (pH 7.9). The fractions containing PvDBP-II variants were desalted with Econo-Pac 10DG columns (BioRad, USA) according to the manufacturer's manual and then concentrated with a concentrator (Eppendorf, Hamburg, Germany). The elutes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gels under reducing conditions) to check purity and then detected by Western blotting using anti-His antibody (Penta His Antibody; Qiagen) as well as the *P. vivax*-infected human sera. Afterward, the fractions containing a clear single-protein band were pooled, and the concentration of the protein was determined using Bradford assay with a spectrophotometer (Eppendorf, Hamburg, Germany).

Enzyme-linked immunosorbent assay. IgG and subclasses antibody responses to recombinant PvDBP-II antigen (represents DBP-I and -VI variants) were evaluated by an enzyme-linked immunosorbent assay (ELISA). Briefly, Maxisorp flat-bottomed, 96-well microplates (Grainer, Labortechnic, Germany) were coated with 100 ng affinity-purified PvDBP-II (based on checkerboard titrations) in 0.06 M carbonate–bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), microplates were blocked with 100 µL PBS containing 1% bovine serum albumin (BSA) (pH 7.4) at room temperature for 1 hour. Then, the duplicate antigen-coated wells were incubated with 100 µL test sera at 1:100 dilution. After washing with PBS-T, the plates were incubated with enzyme-labeled secondary antibody (horseradish peroxidase-labeled anti-human immunoglobulin; IgG, 1:20,000; Sigma-Aldrich Co., USA). Then, the plates were washed again with PBS-T, and the enzyme reaction was developed with o-phenylediaminedihydrochloride–H_2O_2 (OPD; Sigma-Aldrich Co.). The reaction was stopped with 2N H_2SO_4, and the OD was recorded at 490 nm by the use of a microplate reader (Biotech, USA). To detect subclasses of human IgG among anti–PvDBP-II antibodies, an ELISA was performed as described above, but secondary antibodies were added at a dilution of 1:2,000 using biotin-conjugated isotype-specific anti-human IgG subclass antibodies (Sigma-Aldrich Co., USA) at room temperature for 1 hour. After washing, streptavidin–peroxidase conjugate (Sigma-Aldrich Co., USA) was added at a dilution of 1:2,500 and incubated at room temperature for 1 hour. The enzyme reaction was developed with OPD–H_2O_2 (Sigma-Aldrich Co., USA) and stopped with 2N sulfuric acid. The ELISA cut-offs were obtained from the average of the negative sera ($N = 35$) plus 3 standard deviation (SD). The serum of one of our patients, who had one of the highest OD values of IgG, was selected and added to duplicate wells of all the tested plates as a positive control.

Avidity evaluation. In this study, the positive sera that react to PvDBP-II were used for the definition of functional affinity of IgG and cytophilic antibodies IgG1 and IgG3. The avidity of anti–PvDBP-II antibodies was assessed with an urea elution-based ELISA.[36] Briefly, the microplates were coated with recombinant proteins as described above, and after the washing step, sera (1:100) were incubated in duplicates for 1 hour. In the washing step, one of the duplicates was washed three times with PBS-T, and the other, incubated with the same serum, was washed with dissociation buffer, including PBS-T–urea (8 M) with vigorous shaking. Then, the plates were washed one time, with an additional wash with PBS-T buffer. Incubation with secondary antibody, washing steps, and developing enzyme reactions were performed as mentioned above for ELISA. Avidity index (AI) was calculated by the ratio of OD value of urea-treated samples by that of non-treated samples multiplied by 100. All AI values less than 30% were considered as low-avidity antibodies, values between 30% and 50% were intermediate-avidity antibodies, and values greater than 50% were considered as high-avidity antibodies.[37]

Statistical analysis. A database was created with SPSS 16.0 for Windows (SPSS Inc., USA). The Spearman's correlation test was used to correlate antibody levels in PvDBP-I and -VI variants and also, antibody level and AI with age and exposure. In addition, differences in the proportions of positive sera for IgG and different subclasses were assessed using the χ^2 test. In all tests, P values < 0.05 were considered significant.

RESULTS

Antibody response to PvDBP-II antigen. The PvDBP-II variants (DBP-I and -VI) were expressed in *E. coli* in a soluble form, and the purified proteins were analyzed by SDS–PAGE and had a molecular mass of ~45 kDa. Total IgG antibody responses to these recombinant proteins were analyzed in 85 individuals (aged 5 to 75 years; median = 22 years) with patent *P. vivax* infection.

In this study, the prevalence of anti–PvDPB-II IgG responses in our population was not statistically different for both PvDBP-I and -VI variant antigens (31.76% each, cut-off = 0.5 each, mean OD_{490} = 0.94 and OD_{490} = 0.92, respectively, $P > 0.05$; χ^2 test) (Figure 1 and Table 2). This prevalence was also unrelated to variant frequencies (PvDBP-I, 7.3%; PvDBP-VI, 41.8%) in the examined population (Table 2). Moreover, analysis of the antibody responses to PvDBP-I and -VI showed high (8.23% and 7%), medium (16.47% and 15.3%), and low (7.05% and 9.41%) positive as well as negative (68.2% each) responses, respectively (Figure 2). None of the sera from healthy individuals (control group) contained IgG antibodies to PvDBP-II, which confirms the specificity of the present results. However, a significant correlation was found for

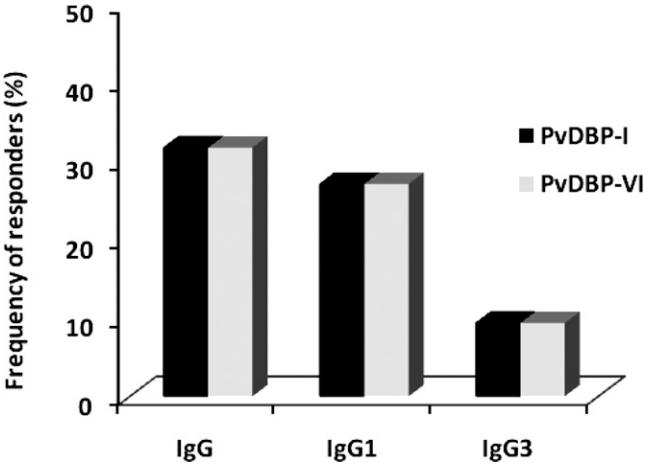

FIGURE 1. Prevalence of IgG and its subclasses responses to two variant forms of PvDBP-II antigens among individuals ($N = 85$) with *P. vivax* patent infection from Iran. Cut-off values are 0.5 for IgG, 0.28 and 0.26 for IgG1, and 0.24 and 0.23 for IgG3 responses to PvDBP-I and -VI variants, respectively. Difference in the prevalence of anti–PvDBP-II IgG responses in our population was not statistically significant in the two variants ($P > 0.05$; χ^2 test).

TABLE 2

Prevalence (percent positive) and OD mean values of IgG and subclass-specific responses to PvDBP-II variants

Haplotypes	Mean OD_{490} ± SD (%)		
	IgG	IgG1	IgG3
PvDBP-I	0.940 ± 0.206 (31.8)	0.987 ± 0.483 (27)	0.451 ± 0.256 (9.4)
Cut-off	0.5	0.28	0.24
PvDBP-VI	0.92 ± 0.170 (31.8)	0.99 ± 0.4 (27)	0.432 ± 0.288 (9.4)
Cut-off	0.5	0.26	0.23

SD = standard deviation. The cut-off values are determined as mean plus 3 SD of the antibody levels in control groups. No significant difference was observed in the proportion of IgG and IgG subclasses responses to PvDBP-I and -VI ($P > 0.05$; χ^2 test). There was a significant correlation of the mean ODs of IgG, IgG1, and IgG3 with PvDBP-I and -VI variant antigens (IgG, $r = 0.957$, $P < 0.0001$; IgG1, $r = 0.788$, $P < 0.0001$; IgG3, $r = 0.351$, $P = 0.001$; Spearman's correlation test).

the levels of IgG to PvDBP-I and -VI variants ($r = 0.957$, $P < 0.0001$; Spearman's correlation test).

Compositions of anti–PvDBP-II IgG subclasses. Serum samples that were positive for total anti–PvDBP IgG were characterized for IgG subclass responses to PvDBP-I and -VI antigens. In individuals who were infected with *P. vivax*, the IgG1 (27.1%, $OD_{490} = 0.98$ for PvDBP-I; 27.1%, $OD_{490} = 0.99$ for PvDBP-VI) was the predominant subclass, whereas IgG3 (9.4%, $OD_{490} = 0.45$ for PvDBP-I; 9.4%, $OD_{490} = 0.43$ for PvDBP-VI) was the second most prevalent subclass that recognized PvDBP-II antigen (Table 2 and Figure 1). The results also indicate that IgG1 and IgG3 are predominant over IgG2 and IgG4 antibodies, and both tested antigens were equally recognized by IgG1 and IgG3 antibodies in individuals from hypoendemic malaria regions in Iran ($P > 0.05$; χ^2 test). In addition, a significant correlation was found for antibody level of both IgG1 and IgG3 with the two variant antigens ($r = 0.788$, $P < 0.0001$ and $r = 0.351$, $P < 0.0001$, respectively; Spearman's correlation test).

Avidity of IgG, IgG1, and IgG3 anti–PvDBP-II. The avidity maturation of IgG antibody to PvDBP-I and -VI was examined in positive sera ($N = 27$) using 8 M urea as a dissociation agent. High-avidity IgG was found in 37.03%, whereas 44.4% and 18.5% had intermediate- and low-avidity antibodies for PvDBP-I, respectively (Table 3). Also, for PvDBP-VI variant, 40.8% high-, 40.8% intermediate-, and 18.5% low-avidity antibodies were identified (Table 3). Furthermore, high-avidity IgG1 was found in 39.1% of positive sera, whereas 52.2% and 8.7% showed intermediate- and low-avidity antibodies for each tested variant antigen, respectively (Table 3). Regarding IgG3, high- or intermediate-avidity antibodies were mostly detected against both antigens (Table 3). The avidity of antibodies for both PvDBP variant antigens and the prevalence of responders with high- and intermediate-avidity IgG, IgG1, and IgG3 antibodies were similar in patients ($P > 0.05$; χ^2 test) (Table 3).

Exposure and age-dependent IgG1 and IgG3 response. The proportions of anti–PvDBP-II IgG-positive subjects increased with exposure to malaria transmission (Figure 2). The highest IgG seroreactivity against PvDBP-I and -VI was found among 14 of 32 (43.75%) with long-term exposure (more than two *P. vivax* infections) to malaria (Figure 2). The group who had experienced a single *P. vivax* infection (first exposure) had very low response to both variants (24.5%). Significant correlations were found in the level of IgG ($r = 0.362$, $P = 0.001$; $r = 0.354$, $P = 0.001$) and IgG1 ($r = 0.411$, $P < 0.0001$; $r = 0.312$, $P = 0.004$) antibodies to PvDBP-I and -VI with exposure to the disease, respectively. Moreover, no correlation was found in the level of IgG3 antibody to PvDBP-I and -VI with exposure to the disease ($P > 0.05$; Spearman's correlation test) (Table 4).

Furthermore, a significant correlation was found in the level of IgG antibody response to PvDBP-I and -VI with age ($r = 0.289$, $P = 0.007$; $r = 0.331$, $P = 0.002$, respectively; Spearman's correlation test). However, in the case of IgG1 and IgG3, no significant correlation was found between the antibody response to PvDBP-I and -VI with age ($P > 0.05$; Spearman's correlation test) (Table 4).

DISCUSSION

The goal of developing PvDBP-II vaccine against blood stages of *P. vivax* is to elicit an antibody response that inhibits the adhesion of this parasite ligand to its erythrocyte receptor and thereby, abrogate merozoite invasion. However, the presence of a highly polymorphic sequence[38–40] in this antigen interferes with its use in vaccine development. Therefore, understanding the nature of this polymorphism present in PvDBP-II among *P. vivax* isolates as well as an immunoepidemiological study on various populations with a different genetic background are key features for vaccine development. In the present investigation, after our previous study on the level of PvDBP-II polymorphisms among Iranian *P. vivax* populations,[20] naturally acquired immune responses to the two PvDBP-II variants (DBP-I and -VI) were analyzed in the individuals living in the same study areas.

In this study, about 32% of the individuals from a low malaria-endemic region displayed specific antibodies to PvDBP-II, which is consistent with an unstable malaria transmission region such as Colombia (40%).[29] Also, the prevalence of responders to high (PvDBP-VI, 41.8%) and low (PvDBP-I, 7.3%) prevalent variant antigens did not differ significantly ($P > 0.05$; χ^2 test). Although the PvDBP-I variant differed from PvDBP-VI by 9 amino acids at various residues (Table 1), these amino acid substitutions did not alter the protein's

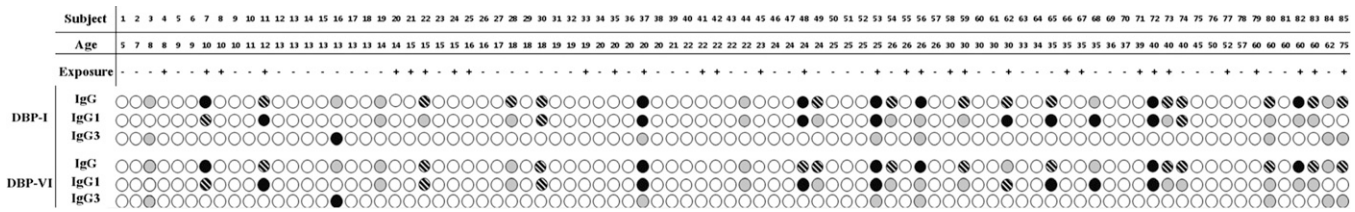

FIGURE 2. Patterns of total IgG, IgG1, and IgG3 responses to two PvDBP-II variant antigens in Iranian individuals who were infected with *P. vivax* isolate. Ages are given in years. Exposures to malaria are shown as single exposure (–) and more than one exposure (+). Antibody reactivity of each sample is measured using ELISA. Cut-off values are 0.5 for IgG, 0.28 and 0.26 for IgG1, and 0.24 and 0.23 for IgG3 responses to PvDBP-I and -VI variants, respectively. The OD mean values for IgG, IgG1, and IgG3 responses have been divided into four groups: ● high positive responses (OD > 1), ◙ medium positive responses ($0.8 \le OD \le 1$), ◍ low positive responses (cut-off < OD < 0.8), and ○ negative responses (OD < cut-off).

TABLE 3

Prevalence of the high, intermediate, and low avidity of IgG, IgG1, and IgG3 antibodies to PvDBP-II variants in individuals with patent *P. vivax* infection

	Avidity								
	Total IgG (%)			IgG1 (%)			IgG3 (%)		
Antigens	HAI	IAI	LAI	HAI	IAI	LAI	HAI	IAI	LAI
PvDBP-I	10/27 (37.03)	12/27 (44.4)	5/27 (18.5)	9/23 (39.1)	12/23 (52.2)	2/23 (8.7)	3/8 (37.5)	5/8 (62.5)	–
AI ± SD	57.7 ± 5.81	44.76 ± 2.9	23.08 ± 5.86	59.5 ± 8.8	41.8 ± 6.25	16.45 ± 1.2	71.3 ± 11.7	45.1 ± 6.1	–
PvDBP-VI	11/27 (40.8)	11/27 (40.8)	5/27 (18.5)	9/23 (39.1)	12/23 (52.2)	2/23 (8.7)	3/8 (37.5)	4/8 (50)	1/8 (12.5)
AI ± SD	57.8 ± 7.89	40.81 ± 4.39	26 ± 5.39	59.47 ± 9.65	42.9 ± 5.6	23.65 ± 8.85	71.6 ± 10.56	44.1 ± 5.8	27.6

Avidity index (AI) was calculated by the ratio of the OD value of urea-treated samples to untreated samples multiplied by 100. An AI value less than 30% was considered as low-avidity antibodies, between 30% and 50% was intermediate-avidity antibodies, and greater than 50% was high-avidity antibodies. HAI = high-avidity index; IAI = intermediate-avidity index; LAI = low-avidity index; SD = standard deviation. A significant correlation was observed between AI for IgG and IgG1 and the two variant antigens ($r = 0.717$, $P < 0.0001$ and $r = 0.567$, $P = 0.005$, respectively; Spearman's correlation test).

antibody binding capacity. Therefore, it seems that the majority of our individuals might have antibodies against both PvDBP-II variants. Nevertheless, recognition of both variant types by examined individuals might not be surprising, because both strains that exist in the population and individuals are likely to have been exposed to both variants. Therefore, additional study is needed to clarify the protective nature of these antibodies to different variants of PvDBP-II.

Furthermore, in the present investigation, the significant percentage of non-responders in all age groups suggests that the PvDBP is a poor immunogen. One explanation could be the presence of cysteine-rich content[41,42] in PvDBP-II, and as a consequence, the antigen is poorly processed, which may account for the reduced frequency of antibody in individuals. In addition, poorly immunogenic PvDBP-II might be because of the fact that some conformational epitopes could not be properly represented in the recombinant PvDBP-II produced in this study.

To understand the immune responses to malaria, both patterns of the subclasses antibodies and evaluation of the avidity of antibody (functional affinity) are important.[43] In the present investigation, the prevalence of IgG1 but not IgG3 antibodies seems to predominate in the patent *P. vivax* infection. No IgG2 and IgG4 antibodies to PvDBP-II variant antigens were detected among examined samples. Also, there was heterogeneity in IgG1 and IgG3 recognition that might be related to either different epitopes in PvDBP-II antigen, recognized by these two IgG subclasses, or short half-life of IgG3 antibody in the serum sample. Moreover, the investigation of the quality of anti–PvDBP-II IgG in studied individuals showed that a significant proportion of individuals (81%) had high- and intermediate-avidity antibodies. In fact, high-quality antibody is one of the important factors involved in preventing and protecting against infections as well as preventing severe disease in malaria infections. This is the first study with qualitative features of antibody responses to the PvDBP-II variant antigen, and the data support the rational development of an effective blood-stage vaccine based on this antigen.

TABLE 4

Correlation analysis of IgG, IgG1, and IgG3 antibody responses in two PvDBP-II variants with host age and exposure

	Age		Exposure	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
IgG (I)	$r = 0.289$	0.007	$r = 0.362$	0.001
IgG (VI)	$r = 0.331$	0.002	$r = 0.354$	0.001
IgG1 (I)	$r = 0.148$	0.177	$r = 0.411$	< 0.0001
IgG1 (VI)	$r = 0.164$	0.135	$r = 0.312$	0.004
IgG3 (I)	$r = 0.068$	0.537	$r = 0.037$	0.739
IgG3 (VI)	$r = 0.051$	0.645	$r = 0.009$	0.932

The *P* value denotes the Spearman's correlation coefficient test. *P* value < 0.05 was considered significant. I = PvDBP-I; VI = PvDBP-VI; *r* = correlation coefficient.

In malaria-endemic regions, individuals have experienced a variable number of previous episodes of clinical malaria; therefore, individuals who had been infected multiple times with malaria should exhibit higher frequency of antibodies against PvDBP-II than those who had been infected one time.[28,33] However, the serologic responses of residents in vivax malaria-endemic regions did show a positive correlation with exposure and host age, as shown in the previous studies.[23,28,30,44] This result suggests a possible boosting of the PvDBP-II antibody response because of accumulated age-related exposure. In addition, our present results showed that exposure and age represent key determinants of the quantitative nature of the IgG response to PvDBP-II. One explanation could be that PvDBP-II is expressed in small quantities for a limited time in late schizogeny, and it is localized intracellularly in micronemes[45] but is not released until erythrocyte attachment.[11] Therefore, the host immune system seems to have little opportunity to recognize and produce an efficient antibody response, because the invasion process may take less than 1 minute to be completed.[46] However, to efficiently block the binding of *P. vivax* merozoites to its ligand on erythrocytes, antibodies directed against PvDBP would need to be in abundant amounts and also have high binding rates and affinities. Therefore, there is a possibility that exposure to very low parasitemias, which occur in low-endemic malaria areas such as those areas in the present study, may be poorly effective in inducing anti-PvDBP antibody. This may explain why, in the studied population, a long-term exposure to *P. vivax* parasite with a certain level of parasitemia has occurred for the subjects to acquire high-level anti-PvDBP antibodies.

In conclusion, our results add additional information to the available data on the characteristics of naturally acquired antibodies to PvDBP-II antigen in populations who were exposed to *P. vivax* in an area of unstable and hypoendemic malaria transmission. Affinity maturation and age-dependent responses to PvDBP-II implicate this molecule as being partially involved in acquired immunity to *P. vivax* in an unstable malaria region; this region could be challenging to address with vaccine development strategies against *P. vivax* parasites. Further studies are needed to clarify whether naturally acquired antibodies to PvDBP-II variant antigens in Iranian malaria patients have the ability to block erythrocyte cytoadherence to DBP.

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