Original Article

Evaluation of Naturally Acquired Antibody Responses to Two Variant Forms of *Plasmodium vivax* Apical Membrane Antigen-1 in Individuals Living in Areas of Low and Unstable Malaria Transmission of Iran

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Abstract

Background: Acquired antibody responses following natural infection provide valuable information for selection of candidate antigens for malaria vaccines. Apical membrane antigen-1 of *Plasmodium vivax* (PvAMA-1) has potential as a component of a subunit vaccine for vivax malaria. In addition, genetic diversity in this antigen is responsible for challenges in the development of an effective PvAMA-1 based vaccine. Therefore, the main aim of this study was to determine whether allelic polymorphisms in *pvama-1* influence the recognition of naturally occurring antibodies. Also, the profile of IgG isotypes to two sequence types of PvAMA-1 antigen was evaluated among subjects exposed to *P. vivax* in areas of low and unstable transmission.

Methods: For this purpose, the two variant forms of PvAMA-1 (PvAMA-1A and B) were expressed in *Escherichia coli* M15-pQE30 system using genomic DNA from Iranian individuals with patent *P. vivax* infection. Anti-AMA-1 response and isotype composition to two variant forms were measured in target *P. vivax*-infected individuals (n = 110, 2 to 65 years old) using Enzyme-linked immunosorbent assay.

Results: The results showed that 65.5% of the studied individuals had positive IgG responses to two PvAMA-1 variants, and the prevalence of responders did not differ significantly ($P = 0.32$). Also, a marked isotype switching to cytophilic (IgG1 /IgG3) antibodies was evident with increasing age, and adults responded more frequently to these antigens than did younger children.

Conclusion: The presence of mature, protective isotype antibodies and equal immune responses to two genetically distinct variant forms of antigens in individuals from low transmission areas implicates that one of these forms could be used in a universal blood-stage vaccine based on PvAMA-1 antigen.

Keywords: Apical membrane antigen-1, immuno-epidemiology, malaria, naturally acquired antibody, *Plasmodium vivax*

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Introduction

Malaria is one of the most devastating parasitic human diseases in the world, accounting for the death of about one million people annually.[1] *Plasmodium vivax* is the second most common agent of human malaria that is responsible for significant morbidity in Latin America, Papua New Guinea, some parts of Africa, the Middle East, and Asia.[2,3] This parasite also has a potential of fatal infection[4–6] that causes periodic relapse with the emergence of chloroquine-resistant strains and lower sensitivity to primaquine, which cause great difficulty in achieving malaria eradication. Since there is no effective tool for prevention of this parasite species, its control, elimination, and eradication are more difficult than *Plasmodium falciparum*.[7,8] Therefore, traditional control measures such as insecticides and anti-malarial drugs are insufficient to combat vivax malaria, and the development of a vaccine can be considered a priority.[9–12] However, development of such a vaccine requires the understanding of natural immune responses from different malaria endemic regions.[13–16]

Asexual blood stages of *Plasmodium* life cycle are responsible for the clinical symptoms associated with the infection, and a vaccine against these stages would reduce clinical burden and parasite load. Therefore, asexual blood-stage antigens (expressed in the merozoite parasite form with critical roles during the invasion of red blood cells) are attractive targets for development of an effective vaccine.[17–20] Apical membrane antigen-1 (AMA-1) is a leading asexual blood-stage vaccine candidate, and it is a type I integral membrane protein present in all *Plasmodium* species. It is a well-characterized antigen that is synthesized and localized in the rhoptries of the apical complexes of both merozoites[20–22] and sporozoites.[23] AMA-1 is expressed as an 83-kDa precursor form.[23–25] Before translocation to the merozoite or sporozoite surface, prior to invasion, this precursor form is proteolytically processed to a 66-kDa and then to 48- and 44-kDa products.[21–24,26–28] Moreover, this protein contains three putative ectodomain regions (I, II, and III) defined by eight intra-molecular disulfide bonds.[29]

The data available from rodent and non-human primate studies have shown that PvAMA-1 is a target for protective immune responses.[30–32] There is strong evidence that antibodies against this molecule can inhibit the invasion of parasite into hepatocytes[23] and red blood cells of human hosts.[33,34] Furthermore, immune-epidemiological studies performed in different areas of Brazil,[35–38]

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India,[39] and Sri Lanka[40] have demonstrated naturally acquired human immune responses to PvAMA-1 even in individuals with a limited exposure to the disease. Also, several studies on naturally acquired immunity to PvAMA-1 have illustrated that cytophilic immunoglobulins (IgG1 and IgG3) against this protein are correlated with protection.[13,24,40] Altogether, these data indicate that PvAMA-1 can be considered among the most promising blood-stage antigens to be used as a subunit malaria vaccine.[34]

In spite of the predominance of *P. vivax* malaria in Iran, as well as in the Indian subcontinent, relatively few studies have been performed to assess the specificity of naturally acquired immune responses in individuals exposed to *P. vivax* infection.[39,41–44] In addition, genetic diversity in protective antigens, a common phenomenon in a complex pathogen such as malaria parasite, is responsible for challenges in development of an effective malaria vaccine, and this phenomenon will increase the parasite ability to evade immune responses. Besides, limited genetic polymorphisms have been reported in *pvama-1* gene from field isolates[24,45–51]; consequently, these polymorphisms could alter the antigenic character of the protein,[51,52] which could possibly hinder the development of PvAMA-1-based vaccine by strain-specific response. Therefore, development of a malaria vaccine needs the evaluation of antigenic diversity as well as naturally acquired antibodies to different variant forms of vaccine candidate antigens in various populations.

Therefore, given the importance of understanding naturally acquired immunity to *P. vivax* antigens in vaccine development, and in continuation of our immuno-epidemiological studies in the malaria-endemic region of Iran, the purpose of this study was to determine whether allelic polymorphisms described for *pvama-1*[51] influence the recognition of naturally occurring antibodies. Also, the profile of IgG isotypes to two sequence types of PvAMA-1 antigen as well as the association between naturally acquired anti-PvAMA-1 isotype responses and host age was evaluated among subjects exposed to *P. vivax* in areas of low and unstable transmission.

Materials and Methods

Study area and subjects

This study was carried out in tropical southeastern region of Iran (Chabahar district in Sistan and Baluchistan Province). In this region, malaria is hypoendemic, and more than 80% of cases have been reported as *P. vivax* species. Over the last few years, the burden of malaria was reduced gently from 15,712 total cases in 2007 to about 1373 in 2013 (Iranian CDMC, surveillance report, unpublished). In this area, the majority of patients have experienced several infections by *P. falciparum* and *P. vivax*; however, no reports of deaths or serious infections caused by malaria have been reported. In this investigation, 110 subjects (male = 94, female = 16; mean age = 25.9 (13.4) years, median age=24, 2–65 years) with patent *P. vivax* infection were enrolled from Chabahar district during cross-sectional surveys performed during 2008 to 2012. All participants in the study filled out a questionnaire for demographic and clinical information for assessment of malaria exposure (Table 1). For negative control in all tests, the blood samples (n = 39) were obtained from residents outside malaria settings (Tehran) with no known pervious exposure to malaria. Before blood collection, an informed consent was obtained from adults or parents/legal guardians of children who participated in this study. In total, 2 mL venous blood was collected from all participants in EDTA-containing tubes for both *P. vivax* DNA detection and serum collection. The diagnosis of vivax malaria was made by microscopic examination of blood smears stained with Giemsa staining in the study areas. In the main laboratory in Tehran, all blood samples were analyzed again for *P. vivax* DNA by nested-PCR amplification as described previously.[53] This investigation was approved by the Ethical Review Committee of Research of Pasteur Institute of Iran.

Haplotype selection

In our previous molecular work, genetic analysis of *pvama-1* gene was carried out in the same malaria endemic region of Iran.[51] In all 37 sequences analyzed, 29 haplotypes with different frequencies were recognized. The single nucleotide polymorphism (SNP) was identified in *ama-1* ectodomain based on *pvama-1* gene of Sal-1 strain (accession no. AF063138). Only three of these SNPs were located in B-cell epitope regions, and all of the detected SNPs were outside intrinsically unstructured/disordered regions (IURs).[51] Therefore, for further analysis, two of the haplotypes were selected in this study: PvAMA-1A (GenBank accession no. JX624741, total frequency of 5.4%) and PvAMA-1B (GenBank accession no.JX624758, total frequency of 8.1%) (Table 2). PvAMA-1A haplotype consisted of 13 SNPs, two of which were located in B-cell epitopes. However, PvAMA-1B haplotype had nine SNPs, three of which were located in predicted B- cell epitopes.[51] Interestingly, both of these selected haplotypes have been previously reported from different malaria settings, including India (EF025196)[54] and Sri Lanka (EF218694, EF218696, and EF218700).[47]

PCR amplification as well as cloning and sub-cloning of PvAMA-1

Parasite genomic DNA was prepared from 200 µL whole blood cells by phenol/phenol-chloroform extraction and ethanol precipitation as described previously.[53] The DNA was dissolved in 30 mL Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) and kept at -20°C until use. The *pvama-1A* and *pvama-1B* genes were amplified with the following primers designed in our laboratory according to the *pvama-1* gene of Sal-1 strain (accession no. AF063138):

AMAF: 5′-ATTATGGATCCGGGCCTACCGTTGAGAG- 3’ (underlined: *Bam*HI site; nucleotides 124-140)

AMAR: 5′-TTCACCCGGG*TTA*TAGTAGCATCTGCTTG- 3′ (underlined: *Sma*I site; Italic: stop codon; nucleotides 1446-1461)

The cycling conditions for amplification was 95°C for 5 min, followed by 30 cycles of 60°C for 1 min, 72°C for 1 min, and 94°C for 1 min with a final extension at 72°C for 30 min. Afterward, the PCR products were analyzed by electrophoresis on 1% agarose gel (Invitrogen, Carlsbad, CA) and observed on an ultraviolet transilluminator after ethidium bromide staining. Then, the PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Germany) following the manufacturer’s instructions. After sequencing confirmation, the gel-purified PCR products of the two variant forms were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5α. The transformed clones were selected on the Luria-Bertani (LB) agar medium, containing 100 µg/mL ampicilin, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 0.04% X-gal. Positive clones were confirmed by plasmid isolation, which was followed by *Eco*RI digestion, and the cloned fragments were

Table 1. Demographic and clinical information of the participants in this study.

Number of patients	Sex (%)		Nationality (%)			Mean age (SD)
	Male	Female	Iranian	Afghani	Pakistani	
110	94 (85.5)	16 (14.5)	60 (54.5)	23 (20.9)	27 (24.5)	25.9 (13.4)

SD = standard deviation

Table 2. Amino acid substitutions of the selected PvAMA-1 (AMA-1A and AMA-1B) haplotypes in the present study.

	Domains		
***P. vivax* strains**	**I**	**II**	**III**
Sal-1	D R K N N L A E E K P	E K L E	K R
AMA-1A	A T R K D . E A K Q .	. . R D	R H
AMA-1B	. K R . . I . A . . S	K I P .	. H

* Sequences were compared with the published Sal-1 reference strain (AF063138) sequence. Dots represent identical residues with Sal-1. Only highlighted amino acids in gray are located in B cell epitopes. No SNPs were found in intrinsically unstructured/disordered regions (IURs).

then sequenced. The fragments corresponding to the PvAMA-1 sequence were removed by *Bam*HI and *Sma*I restriction enzymes and ligated to the *Bam*HI-*Sma*I sites of vector pQE-30 (Qiagen, Germany) for subcloning PvAMA-1 into pQE30 vector. This process provided poly-histidine (6-His) tag in N-terminus of PvAMA-1 to facilitate further purification. The open reading frame was confirmed by sequencing, and this construct was then used to be transformed into *E. coli* M15 (pREP4) expression host (Qiagen, Germany).

Expression and purification of recombinant PvAMA-1

In this study, two recombinant proteins of PvAMA-1 were obtained using the same protocol. Briefly, overnight cultures from single colonies of recombinant PvAMA-1 were expanded in Terrific Broth (TB) medium containing ampicillin (100 µg/mL) and kanamycin (25 µg/mL) with shaking (150 rpm/min) until an OD at 600 nm of 0.6 to 0.8 was reached. The expression of PvAMA-1 was induced with 0.2 mM IPTG (Sigma, USA). The culture was further grown for 4 hr, and the *E. coli* cells were harvested by centrifugation and kept at -80ºC until use. Moreover, after induction, the protein expression was analyzed by 12% SDS-PAGE gels under reducing conditions. The PvAMA-1was expressed in inclusion bodies; therefore, the cell pellet was dissolved in denaturation buffer (8 M urea, 20 mM Tris-HCl, 1 M NaCl, 30 mM immidazol, and 1 mM PMSF, pH 7.9) and incubated at 4ºC for 1.5 hr. The cells were then lysed on ice by sonication (Ultraschallprozessor, Germany) with 10 cycles, each consisting of 20-second (s) pulses with 40-s intervals. The bacterial lysate was centrifuged at 14,000 ´ g at 4ºC for 30 min. The supernatant was incubated at 4°C for 2 hr using the equilibrated Ni^{2+}-nitrilotriacetic acid-agarose resin (Ni-NTA agarose, Qiagen, Germany, equilibrated in 8M urea, 20mM Tris-HCl, 40 mM immidazol, and 1 M NaCl, pH 7.9).The resin was packed into a column and washed with a 10-column volume of wash buffer (containing 6 M urea, 20 mM Tris-HCl, 1 M NaCl, and 60 mM imidazol, pH 7.9). The bound protein was eluted with elution buffer containing 4 M urea, 20 mM Tris-HCl, 300 mM NaCl, and 200 mM imidazol, pH 7.9. The elute containing PvAMA-1 was desalted with Econo-Pac 10DG columns (BioRad, USA) according to the manufacture's manual and then concentrated with a concentrator (Eppendorf, Germany). All elutes were analyzed by 12% SDS-PAGE gels under reducing and non-reducing (in the absence of 2 ME and boiling) conditions. Then, the fractions containing a clear single protein band were pooled, and the concentration of the protein was determined using Bradford's assay with a spectrophotometer (Eppendorf, Germany). Immuno-blotting was carried out by standard protocols, with both anti-His antibody (Qiagen, Germany) and the *P. vivax*-infected human sera in both reduced and non-reduced conditions.

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to recombinant PvAMA-1 (PvAMA-1A and PvAMA-1B variants) in human were evaluated by ELISA as described previously with some modifications.[41] Briefly, Maxisorp flat-bottomed, 96-well microplates (Grainer, Labortechnic, Germany) were coated duplicate with 250 ng of each affinity-purified PvAMA-1variant (based on checkerboard titrations) in 0.06 M carbonate-bicarbonate buffer (pH 9.6) and incubated at 4ºC overnight. After washing five times with phosphate buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBS-T, pH 7.4), the microplates were blocked with 200 µL PBS containing 2.5% bovine serum albumin (BSA; Sigma, USA) at room temperature for 1 hr. Then, the duplicate antigen-coated wells were incubated with 100 µL test sera (diluted 1:200 in PBS-T containing 0.5% BSA) for 1 hr. After washing with PBS-T, the plates were incubated with enzyme-labeled secondary antibody (horseradish peroxidase-labeled anti-human immunoglobulin) (IgG, 1:30,000; Sigma, USA) at room temperature for 1 hour. Then, the plates were washed again with PBS-T, and the enzyme reaction was developed with o-phenylediamine dihydrochloride-H_2O_2 (OPD, Sigma, USA) and stopped with 2N H_2SO_4. The OD was recorded at 490 nm by use of a microplate reader (BioTech, USA). The detection of human IgG subclasses among anti-PvAMA-1antibodies was achieved using ELISA as described above, but with secondary antibodies of biotin-conjugated isotype-specific mouse anti-human IgGs (Sigma, USA) at dilutions of 1:4,000 (IgG1) and 1:2,000 (IgG2, IgG3, and IgG4) at room temperature for 1 hr. After washing, streptavidin-peroxidase conjugate (1:2,500, Sigma, USA) was added and incubated at room temperature for 1 hr. The enzyme reaction was developed with OPD-H_2O_2 (Sigma, USA) and stopped with 2N H_2SO_4. The ELISA cut-offs were obtained from the average of the negative sera (n = 39) plus three standard deviations (SD).The serum of one of our patients, who had one of the highest values of IgG, was selected and added to duplicate

wells of all the tested plates as positive control.

Statistical analysis

A database was created in SPSS 20 (SPSS Inc., USA), NCSS 10 Statistical Software (2015)[55] and SAS 9.3 (Inc, USA) for windows. The comparison of antibody levels was tested using non-parametric Wilcoxon signed-rank tests (for paired samples). In addition, differences between the proportions of subjects positive for IgG and different subclasses were assessed using the exact type of McNemar's test (McNemar's test with continuity correction) and an effect size of this test and its 95% confidence limit (CL) were computed and reported with Asymptotic Wald C.C method. The association between the prevalence of positive responders with different age groups and rPvAMA-1A and rPvAMA-1B antigens was analyzed with Pearson's chi-squared test and the Fisher's exact test was computed for exact type of chi-square test (χ^2). The odds ratios [95% confidence intervals (CI)] were considered as the effect size. Moreover, the heterogeneity in antibody responses to both tested variants in subjects infected with *P. vivax* was also performed with Pearson's chi-squared test and the Fisher's exact test was computed for exact type of χ^2 association test. The symmetric Lambda criteria (95% CL) were considered as the effect size. Furthermore, the Pearson's correlation test was also used to assess the association between antibody levels with age. *P* values < 0.05 were considered statistically significant.

Results

Detection of P. vivax parasites by nested PCR

Based on both microscopy and nested-PCR results, all 110 patients were infected with *P. vivax*, as a mono infection, and none of the healthy control individuals had either *P. vivax* or *P. falciparum* infections (data not shown).

Antibody responses to PvAMA-1 variants

The PvAMA-1 variants (PvAMA-1A and PvAMA-1B) were expressed in *E. coli* M15-pQE30, and the purified proteins were analyzed by SDS-PAGE and had a molecular mass of ~52 kDa. The purity of the recombinant proteins was evaluated by Western blot assay. The result showed that the expressed proteins migrated at different sizes in the presence and absence of 2ME, indicating the existence of a disulfide bond. The result also showed that both recombinant proteins had a tertiary shape in their antigens and had proper conformation and folding (data not shown). Total IgG antibody responses to these two variant forms of PvAMA-1 were determined in 110 individuals (aged 2 to 65 years; median = 24 years; Table 1), of whom only 65.5% had positive IgG antibody responses to PvAMA-1 antigens (Figure 1). Furthermore, difference in the prevalence of anti-PvAMA-1 IgG responses in the studied population was not statistically significant to the two variants (PvAMA-1A: 65.5%, 72/110, cut-off = 0.29, mean OD_{490} = 0.9 (0.49); PvAMA-1B: 65.5%, 72/110, cut-off = 0.25, mean OD_{490} = 1.014 (0.545), *P* = 0.32, effect size = 0.000 (CL= -0.027 - 0.027), McNemar's test; Figure 1 and Table 3). However, a significant correlation was found in the level of IgG to PvAMA-1A and PvAMA-1B variants (r = 0.925, P < 0.001, Spearman's correlation test, Figure 2, Table 3). Moreover, analysis of the antibody responses to PvAMA-1A and PvAMA-1B showed high (6.36 % and 8.2%), medium (21.8% and 30%), and low (37.2% and 27.3%) responses to the allelic forms (OD > 2, high positive responses; 1< OD < 2, medium positive responses; cut-off < OD <1, low positive responses; OD < cut-off, negative responses, Figure 3). None of the sera from healthy individuals (control group) contained IgG antibodies to PvAMA-1, which confirms the specificity of the present results.

IgG subclass responses to PvAMA-1 antigens

The serum samples that were positive for total anti-PvAMA-1 IgG were evaluated for IgG subclass responses to PvAMA-1A and PvAMA-1B antigens. In individuals who were infected with *P. vivax*, the IgG1 (63.6%, OD_{490} = 0.89 for PvAMA-1A; 64.5%, OD_{490} = 1.1 for PvAMA-1B) was the dominant subclass, whereas the second frequent subclasses was IgG3 (33.6%, OD_{490} = 0.75 for PvAMA-1A; 37.3%, OD_{490} = 0.93 for PvAMA-1B) (Figure 1 and Table 3). Furthermore, a significant correlation was found between the antibody level of both IgG1 and IgG3 and the two variant antigens (Spearman's correlation test, *P* < 0.001, Figure 2

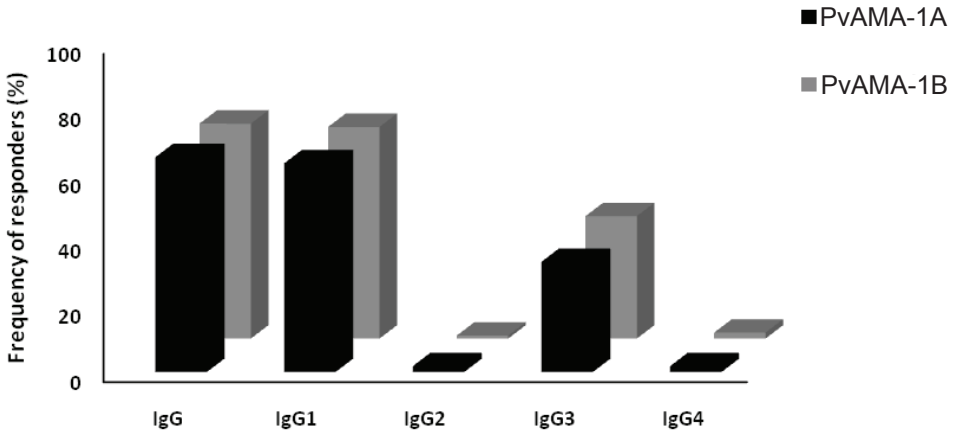

Figure 1. Prevalence of IgG and its subclass responses to two variant forms of PvAMA-1 antigen (PvAMA-1A and PvAMA-1B) in individuals with *P. vivax* patent infection (n = 110) from malaria endemic area of Iran. No significant difference was observed in the proportion of IgG, IgG1, IgG2, IgG3, and IgG4 responses to PvAMA-1A and PvAMA-1B variants (*P* = 0.32 for IgG, *P* = 1 for IgG1, *P* = 1 for IgG2, *P* = 0.34 for IgG3 and *P* = 0.32 for IgG4; McNemar's test). The cut-off values of the controls were as follow: PvAMA-1A: IgG (0.29), IgG1 (0.245), IgG2 (0.247), IgG3 (0.292), and IgG4 (0.207). PvAMA-1B: IgG (0.256), IgG1 (0.234), IgG2 (0.205), IgG3 (0.275), and IgG4 (0.229).

Table 3. Comparative analysis of the isotype-specific antibodies to PvAMA-1 variants among individuals with patent *P. vivax* infection in Southeastern Iran.

Antigen	Mean OD_{490} (SD)				
	IgG	IgG1	IgG2	IgG3	IgG4
PvAMA-1A	0.9 (0.49)	0.89 (0.76)	0.284 (0.002)	0.75 (0.66)	0.75 (0.44)
Cut-off	0.29	0.245	0.247	0.292	0.207
PvAMA-1B	1.014 (0.545)	1.1 (0.84)	0.376*	0.93 (0.81)	1 (0.98)
Cut-off	0.256	0.234	0.205	0.275	0.229
P-value (Spearman's correlation test)	< 0.001	< 0.001	< 0.001	< 0.001	= 0.424

The meanOD_{490} nm value was considered as a measure of the anti-PvAMA-1 specific antibody responses of each serum sample by ELISA assay. The cut-off values were calculated by mean ODs of normal human sera (n = 39) out of malaria endemic region plus three Standard Deviation (SD). There was a significant correlation between the mean ODs of IgG, IgG1, IgG2, and IgG3and to PvAMA-1A and PvAMA-1B variant antigens (IgG, $r = 0.925$, $P < 0.001$, IgG1, $r = 0.651$, $P < 0.001$; IgG2, r = 0.453, $P < 0.001$; and IgG3, $r = 0.856$, $P < 0.001$; Spearman's correlation test). *SD was not calculated because the positive sample for this subclass was only one.

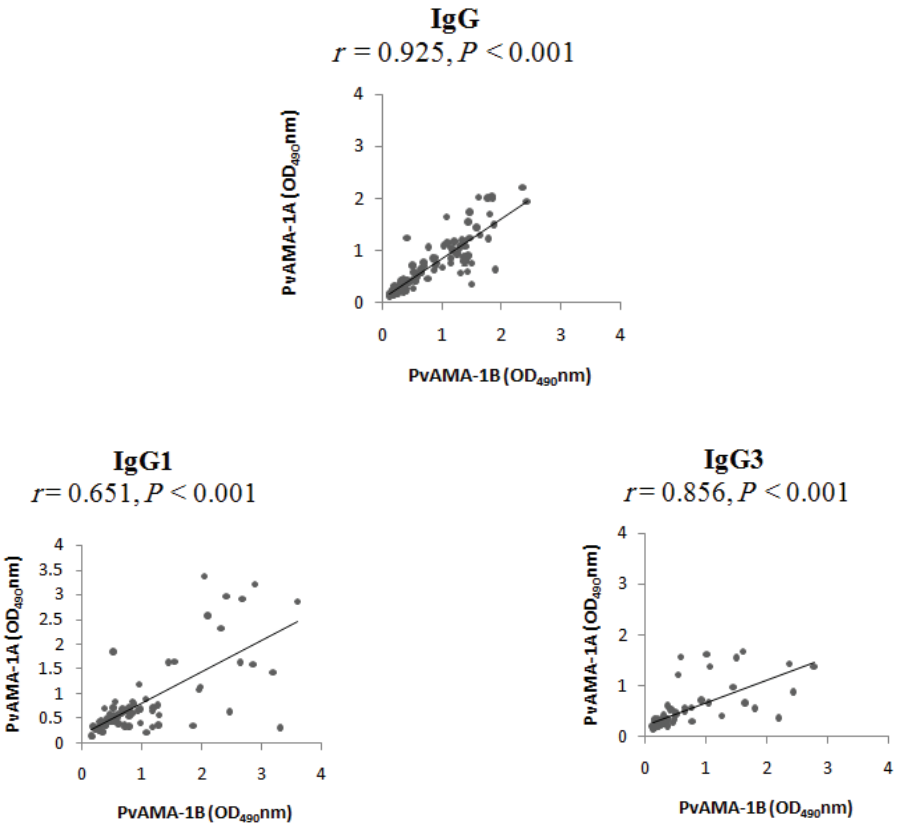

Figure 2. The correlation of IgG, IgG1, and IgG3 antibody responses and the two variant forms of PvAMA-1 antigen (PvAMA-1A and PvAMA-1B). A significant correlation was observed between the antibody levels of IgG, IgG1, and IgG3 and the two variant antigens ($P < 0.001$, spearman's correlation test).

and Table 3). Regarding IgG2 and IgG4, the frequency distribution of individuals was 1.8% and 1.8% for PvAMA-1A (mean OD_{490} = 0.284 and 0.75, respectively) and also 0.9% and 1.8% for PvAMA-1B (mean OD_{490} = 0.376 and 1, respectively) (Figure 1 and Table 3). Furthermore, heterogeneity in IgG1 and IgG3 isotype responses of the individuals was not statistically different in the two variant antigens ($P = 0.82$, effect size = 0.028 (CL = 0 - 0.144), χ^2 test; Table 4). The results indicated that IgG1 and IgG3 were predominant over IgG2 and IgG4 antibodies, and all of the tested antigens were equally recognized by IgG1 and IgG3 antibodies in individuals from hypoendemic malaria regions in Iran ($P = 1$, effect size = 0.009, (CL = -0.018–0.036) for IgG1 and $P = 0.34$ effect size = -0.036 (CL = -0.101–0.029) for IgG3, McNemar's test). In addition, significant differences were observed in the levels of IgG, IgG1, and IgG2 (but not for IgG3 and IgG4) for PvAMA-1A and PvAMA-1B ($P < 0.001$ for IgG, $P = 0.01$ for IgG1, $P < 0.001$ for IgG2, $P = 0.83$ for IgG3 and $P = 0.24$ for IgG4; Wilcoxon signed-rank test).

Age-dependent IgG, IgG1, and IgG3 responses

The levels of IgG, IgG1, and IgG3 antibodies to PvAMA-1A and PvAMA-1B variants did not correlate with age (PvAMA-1A: $r = -0.001$, $P = 0.98$ for IgG; $r = 0.03$, $P = 0.81$ for IgG1; and $r = 0.17$, $P = 0.14$ for IgG3; PvAMA-1B: $r = -0.049$, $P = 0.61$

patient code	Age	Antibody responses (OD_{490nm})					
		PvAMA-1A			PvAMA-1B		
		IgG	IgG1	IgG3	IgG	IgG1	IgG3
101	45						
64	27						
47	30						
48	15						
57	20						
109	42						
3	30						
24	20						
29	24						
65	25						
11	27						
74	17						
93	23						
43	18						
69	22						
52	22						
17	17						
75	30						
58	17						
12	17						
100	18						
28	22						
20	35						
66	19						
53	32						
78	28						
49	25						
5	61						
89	14						
85	25						
9	25						
68	30						
80	20						
4	25						
18	13						
98	15						
96	18						
39	53						
108	26						
88	20						
60	61						
10	38						
51	35						
22	14						
13	45						
79	30						
87	30						
31	17						
107	21						
15	38						
30	19						
16	60						
36	3						
67	9						
72	14						
86	13						
27	2						
104	22						
21	55						
71	32						
23	50						
45	15						
83	25						
33	41						
84	40						
106	19						
1	8						
40	34						
99	19						
90	26						
63	22						
42	18						

Figure 3. The patterns of IgG, IgG1, and IgG3 responses to two variant form of PvAMA-1 in positive sera (n = 72). The negative sera (n = 38, mean age (SD) = 25.8 (14.1)) are not shown in the Figure. Ages are given in years. Cut-off values are 0.29 and 0.256 for IgG, 0.245 and 0.234 for IgG1, and 0.292 and 0.275 for IgG3 responses to PvAMA-1A and PvAMA-1B variants, respectively. The OD means values are divided into the following groups: OD > 2: High-positive antibody responses (black), 1 > OD > 2: medium-positive responses (dark gray), OD < 1: low-positive responses (light gray), and OD < cut-off: negative (white).

for IgG; r = -0.072, P = 0.53 for IgG1; and r = 0.114, P = 0.32 for IgG3; Pearson's correlation test). However, the prevalence of positive sera for IgG and IgG1 to PvAMA-1A variant and IgG, IgG1, and IgG3 to PvAMA-1B variant was increased in the age group of 11-20 years (PvAMA-1A: P = 0.02, effect size = 0.149, (CI = 0.033 - 0.666) for IgG and P = 0.29, effect size = 0.186, (CI = 0.043 - 0.8) for IgG1; PvAMA-1B: P = 0.02, effect size = 0.149, (CI = 0.033 - 0.666) for IgG; P = 0.02, effect size = 0.149, (CI = 0.033 - 0.666) for IgG1 and P = 0.03, effect size = 0.97, (CI = 0.011 - 0.855) for IgG3; χ^2 test; Figure 4A and B).

Table 4. The heterogeneity in IgG1 and IgG3 responses to PvAMA-1A and PvAMA-1B variant antigens in individuals with patent *P. vivax* infection who are living in Chabahar district of Iran.

Antigen	$IgG1^+/IgG3^+$ No. (%)	$IgG1^+/IgG3^-$ No. (%)	$IgG1^-/IgG3^-$ No. (%)	$IgG1^-/IgG3^+$ No. (%)	χ^2 Test *P*-value
PvAMA-1A	35 (31.8)	35 (31.8)	38 (34.5)	2 (1.8)	= 0.82
PvAMA-1B	40 (36.4)	31 (28.2)	38 (34.5)	1 (0.9)	

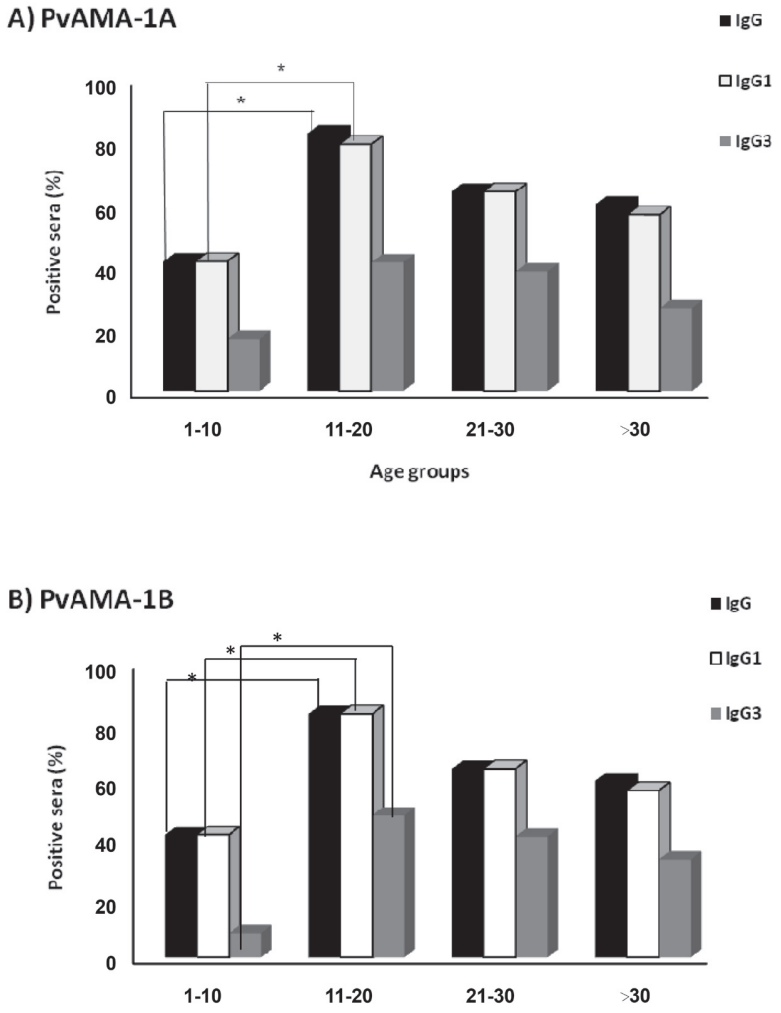

Figure 4. Association between the age and IgG, IgG1, and IgG3 antibody responses to PvAMA-1A (a) and PvAMA-1B (b) haplotypes of PvAMA-1 antigen. The prevalence of positive responders for IgG, IgG1, and IgG3 antibodies for each age group is shown in the Figure. The prevalence of responders to PvAMA-1A for IgG and IgG1 as well as PvAMA-1B for IgG, IgG1 and IgG3 was statistically higher in the age group of 11-20 years compared to age group of 1-10 years ($P < 0.05$, X^2 test). Age groups are: 1-10 years (n = 12), 11-20 years (n = 29), 21-30 years (n = 39), and ≥ 31 years (n = 30).

Discussion

According to the WHO recommendation, elimination and eradication of malaria is a worthwhile goal in many countries today. Since elimination and eradication of malaria requires complete removal of all parasites, and successes achieved by scaling-up use of existing tools are inadequate, additional or alternative strategies such as vaccine are highly needed.[56] Also, in development of malaria vaccine, immuno-epidemiological data from various populations with a different genetic background from diverse endemicity is necessary to provide information on mechanisms of immunity and its targets. Therefore, in the current investigation, naturally acquired antibody to two different variant forms of AMA-1, one of the important asexual stage vaccine candidates of *P. vivax* was analyzed and compared in individuals living in the malaria hypo-endemic and unstable transmission areas, in Iran.

It should be noted that immuno-epidemiological studies could help to identify the proper target antigens that might be used in vaccine development against malaria. In this regards, in the present study, 65.5% of the studied individuals had positive anti-PvAMA-1 IgG antibody responses to both variant forms of antigens, suggesting that PvAMA-1 is a well-recognized asexual-stage parasite antigen. However, the absence of such response in 34.5% of the individuals could be possibly explained by first or short

exposure to this antigen, which may be insufficient to induce considerable immune responses. This slow development of naturally acquired malaria immunity has been shown by others in low to moderate malaria transmission settings.[14,39,57]

In high-transmission regions, all individuals had many infections during their life; therefore, protective immunity developed with age/exposure in these individuals. In contrast, in low and unstable transmission regions, there was a lack of such correlation with age.[24,58–60] In our study area with unstable and hypoendemic malaria transmission, the level of antibodies to both variant antigens was not significantly correlated to the host age. This finding was also reported for an area with intense malaria transmission,[61] and also from similar malaria settings in north of Brazil.[24] However, the prevalence of positive sera for IgG and IgG1 to PvAMA-1 variants increased with age. This fact might indicate that the immune system in both younger and older children is boosted due to patent *P. vivax* infection, but such a response in adults may be induced by long-lived memory cells. This result again confirms the need for boosting the antibody response induced by vaccination in malaria.

Since the IgG subclasses produced in response to a given antigen may determine the function of the antibodies, in this work, the anti-PvAMA-1 IgG subclasses to both expressed variant forms of PvAMA-1 were analyzed. The total IgG and its isotype profiles to PvAMA-1 in this study are in agreement with those from previous immuno-epidemiologic studies performed in India,[39] Sri Lanka,[40] and Brazil,[24,35,38] in which the IgG1 and IgG3 isotype responses were high in vivax malaria. In our study, although anti-PvAMA-1 IgG1 was predominant, there was also a mixed IgG1/IgG3 response. This heterogeneity in IgG1 and IgG3 recognition could be related to either different epitopes in PvAMA-1 antigen recognized by these two IgG subclasses or short half-life of IgG3 antibody in the serum sample. On the other hand, since IgG3 has a relatively short half-life, detection of antibody response to PvAMA-1 for this isotype may be induced by frequent re-exposure to the parasite, which is required to increase the level of IgG3 antibody isotype.[62] Moreover, it is well established that IgG1 and IgG3 subclasses mediate opsonization and complement fixation of pathogens and they are involved in antibody-mediated protective immunity against Plasmodium blood stages.[28,63,64] Therefore, the finding that IgG subclasses to PvAMA-1 are mainly of the IgG1/IgG3 type may contribute to protective effect through cell-mediated mechanisms. Also, this result was comparable to the frequency observed in our recent studies to PvMSP-1_{19}.[65] and PvDBP.[42,66] during patent infection in the same study areas.

The present results extended the impact of the allelic polymorphism described for PvAMA-1 on antibody recognition of recombinant proteins representing different allelic forms of the protein. Our previous molecular epidemiological study[51] in the same area of the present study showed that PvAMA-1A (5.4%)- and PvAMA-1B (8.1%)-bearing parasite isolates are prevalent. Although the detected SNPs in the B-cell epitopes of *pvama-1* gene are different in these two variant forms, and it is supposed that these SNPs dramatically impair the binding of peptides to MHC molecules, the present study shows that the prevalence of responders to both tested variant antigens does not differ. This finding suggests that the majority of our individuals have cross-reactive antibodies against both PvAMA-1 variants. This result is in line with our earlier studies on other Plasmodium antigens, PfMSP-1_{19},[65] and PvDBP,[42] which were performed in the same study areas and indicated the presence of cross-reactive antibodies.

In summary, our results add more information to the available immuno-epidemiological data on the characteristics of naturally acquired antibodies to PvAMA-1 in populations with distinct degrees of exposure to *P. vivax*. In addition, the presence of mature, cytophilic isotype antibodies with equal immune responses to two genetically distinct variant forms of PvAMA-1 antigen in the studied individuals could be explained by the fact that this protein is immunogenic. Therefore, the persistence and the similarity of this result from different malaria endemic regions could support and emphasize the use of PvAMA-1 in designing a universal vivax malaria vaccine.

Disclosure Statement

No competing financial interests exist.

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