GENETIC DIVERSITY OF APICAL MEMBRANE ANTIGEN (AMA-1) IN ISOLATES OF *PLASMODIUM VIVAX* FROM BALOCHISTAN.

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ABSTRACT: Malaria is endemic in Pakistan but little research data is available on vaccine candidate antigens like Apical membrane antigen 1 (AMA-1), Circumsporozoite protein (CSP), Merozoite surface protein 1 (MSP-1) and Duffy binding protein. We found scanty information on genetic diversity of these antigen genes in *Plasmodium* species of Balochistan. The AMA-1 is one of the leading vaccine candidate antigen and required for the invasion of red blood cells by the malaria parasites. To the best of our knowledge, this is the first study focusing the genetic diversity of *Plasmodium vivax AMA-1 (Pvama-1*) gene among Pakistani isolates. The *AMA-1* gene was amplified at domain-1 in 62 isolates out of 100 symptomatic clinical cases collected from Quetta, Balochistan. The amplified PCR products were further verified by sequence analysis of selected isolates. The results indicated that *Plasmodium vivax* showed considerable degree of polymorphism at *AMA-1* gene domain-1. The comparison of *PvAMA1* nucleotide sequences and resulting amino acid sequences with sequences from other countries also showed a variation. This study will serve as base line data in this region for further molecular study of this gene from other parts of country as well.

Key words: Malaria, Vaccine candidate gene, Polymorphism, *AMA1*, *Plasmodium vivax.*

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INTRODUCTION

Malaria is considered as one of the major reason for mortality and morbidity in developing countries. The approximate incidence of malaria cases is 300-500 million worldwide and it is responsible for about 1-3 million deaths per year. Malaria is prevalent in tropical and subtropical regions around the equator (Hector, 2014) which include Asia, Sub-Sahara Africa and Latin America. Malaria has been one of the major infectious health problem in many regions of the world like South America, Oceania, and Asia resulting 80-300 million cases per year (Chenet *et al.,* 2012; Atemnkeng *et al*., 2013). There are 2.1 billion people at the risk of malarial infection in Asia pacific (WHO, 2014). In 2009, 4.2 million cases of malaria have been reported from Pakistan out of which 42% belonged to Balochistan, *Plasmodium falciparum* infection was about 30% (WHO, 2010). Pakistan is considered endemic for both *P. vivax* and *P. falciparum* (Yasinzai and Kakarsulemankhel 2008) causing 75% and 25% of malaria cases respectively. Malaria has been one of the most common reasons for acute fever in Pakistan and is recognized as a major health problem (Khan *et al.,* 2006). About 500,000 cases of malarial infection occur in Pakistan each year and number of deaths are more common in children, infants and pregnant females (Khattak *et al*., 2013; Ansari *et al.,* 2009). The prevalence of malaria varies in different regions and different provinces, 30% of reported cases are found each in Balochistan and Sindh although the population is 5% and 25% of the total population respectively while Punjab has 52% of the population with less than 10% of the total reported cases (Murtaza *et al.,* 2009). The clinical signs of mild or uncomplicated malaria are fever along with symptoms like chills and sweats, headache, vomiting, watery diarrhea, anemia, jaundice, and swelling of the spleen (splenomegaly) (WHO, 2000). *P. vivax* malaria is found across a large area of the globe and potentially affects larger number of people than *P. falciparum* malaria (Gething *et al.,* 2012).

The parasite passes through complex life stages in human host offering altered antigens during different phases of its life cycle. It gets in to body as a sporozoites which passes to lever and then released in to blood stream as merozoites. The apical membrane antigen (AMA-1) is expressed at the apical surface of merozoites and sporozoites during infection, previously named immunogenic type 1 integral protein, plays an important role in hepatocyte and erythrocyte invasion (Hodder *et al.,* 2001; Silvie *et al.,* 2004). AMA-1 shows limited genetic diversity in different geographical regions which is shown by few predominant haplotypes (Hisaeda *et al.,* 2005). Two common genotypes of *AMA-1* has been studied in East Asian countries like China and South Korea (Chung *et al.,* 2003), but more genetic information is required from South East Asia and neighboring

countries. To date, studies of genetic diversity of *Plasmodium vivax* in Pakistan have focused on a number of genes, including that encoding the PVCSP, PVMSP-1, PVMSP 3A, PVMSP-3B, but no work has been done on PVAMA-1. The present study had focused the molecular genetics of *AMA-1* in *P. vivax* species in Quetta, Pakistan. The study aimed to find genetic diversity of Apical Membrane Antigen (*AMA-1*) gene in isolates of *Plasmodium vivax* from Balochistan.

MATERIALS AND METHODS

Sample Collection: Samples collection of malaria patients was carried out for two seasons of the year 2013 and 2014. All the symptomatic clinical cases of malaria attending TB Clinic and Bolan Medical Complex Hospital, Quetta were included in study. Three ml of blood was taken in EDTA tubes after informed consent of the patients. Presence of *Plasmodium vivax* parasite by light microscopy examination was being confirmed. The characteristics of patients like age, sex, ethnic group, and clinical symptoms (fever, vomiting, shivering, headache and nausea) were also recorded. Blood samples were stored at -20^{o}C.

DNA extraction and PCR confirmation: DNA was extracted using QIAmp DNA blood Kit (Qiagen Germany) according to manufacturer's instructions. The DNA aliquots extracted were stored at 4^{o}C till further PCR amplification. All the samples were tested by diagnostic PCR using primer pairs forward 5′-CCTGTTGTTGCCTTAAACTTC-3′ and reverse 5′-TTAAAATTGTTG CAGTTAAAAC G- 3′ as described by Johnston *et al.*, 2006.

PCR Amplification of PvAMA-1: The polymorphic region of AMA-1 gene was amplified by polymerase chain reaction using the following oligonucleotide primers (Han *et al*., 2002) *Pvama*-1F (5′–CCAG CTGGAAGATGTCCTGT–3′) and *Pvama*-1R (5′–ATCCGAACTTGGCGTTTC–3′). The reaction mixture prepared for each sample consisting of 5 µl of template DNA, 10µl of master mix (Go Taq Green Master Mix 2x by Promega), 0.5µl of each of reverse and forward primer (from 10 µM working solution of each primer) and DPC water to make the final volume as 20µl. Reaction mixtures were subjected to 35 cycles of amplification in a thermocycler (Thermohybaid PCR express thermocycler). The cycling conditions applied for 35 cycles of 94^{o}C for 1 minutes, 56^{o}C for 1 minute and 72 ^{o}C for 2 minutes preceded by initial denaturation at 95^{o}C and followed by final extension at 72 ^{o}C for 6 minutes. Amplified products were analyzed by electrophoresis on 1.5 % agarose gel along with 100bp DNA marker (Norgen 100bp ladder, Norgenbiotek corporation) (Figure; 1). Amplified PCR products were sequenced directly by ABI373 automated DNA sequencer (Applied Biosystem CA). The representative sequences were submitted to GenBank by the accession numbers KT884598-KT884602.

RESULTS

The present study was carried out in Quetta Balochistan. Out of 100 samples collected 62 were confirmed for *P. vivax* infection and were amplified on *AMA-1* gene showing band size of about 450- 500bp on gel electrophoresis. The bands showed slight variation in their size when compared with 100bp marker DNA ladder through gel electrophoresis (Figure.1). The majority of samples were amplified as a segment of about 500 bp. The 10 selected PCR products were sent for sequence analysis to Macrogen Laboratory South Korea. The alignment of sequences was done using Clustal W programme and Bioedit software. A 395 bp region belonging to domain1 had been aligned with corresponding region of reference sequence PH-84 (Gen Bank accession number L27503) and compared with sequences from other regions of the world like India, Africa, Iran, Indonesia, Venezuela, Sri Lanka, South Korea and Iran. The alignment showed nucleic acid base substitutions (Figure 2).

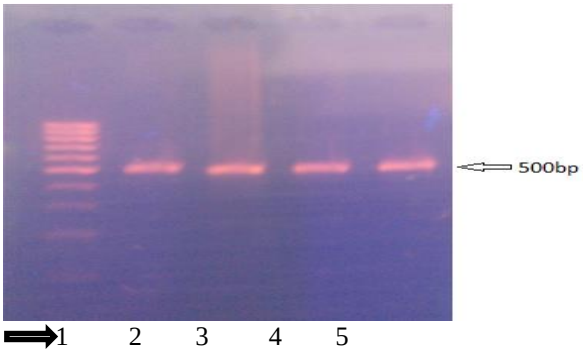

Figure-1: Representative PCR products with *PvAMA-1* specific primers amplifying region of *AMA-1* gene using DNA from infected individuals with *P. vivax*. Lane 1: molecular size marker (100 pb DNA ladder); lanes 2, 3, 4 and 5 are PCR products

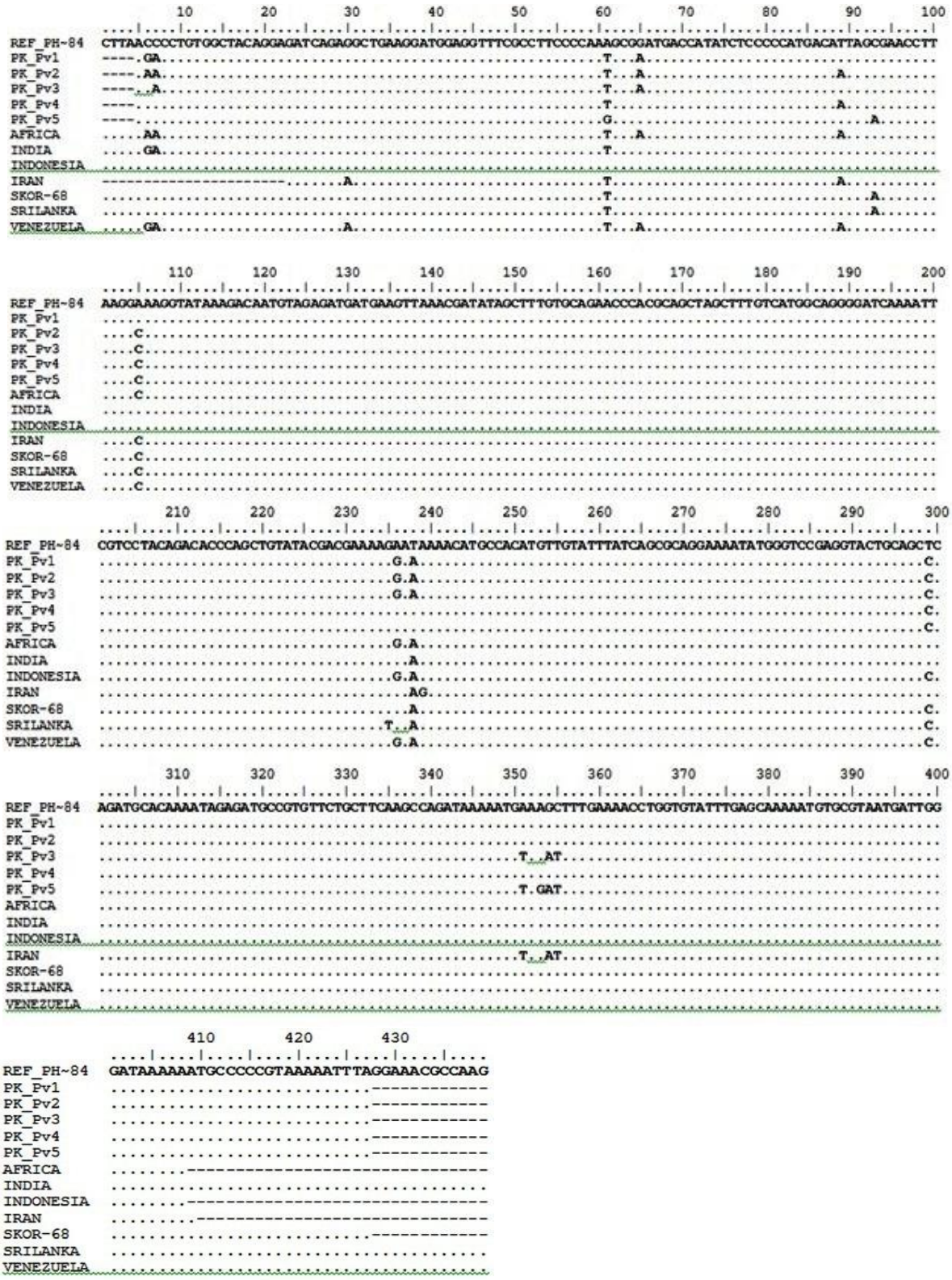

Figure-2: Multi-sequence alignment of Nucleotide polymorphisms in PvAMA-1gene (domain-1) in *P. vivax* isolates from Pakistan (PK Pv1, PK Pv2, PK Pv3, PK Pv4, PK Pv5) PH-84 (reference sequence), South Korea (SKOR-68a), Iran, India, Sri Lanka, Venezuela, Africa and Indonesia. Dots indicate the identical amino acid.

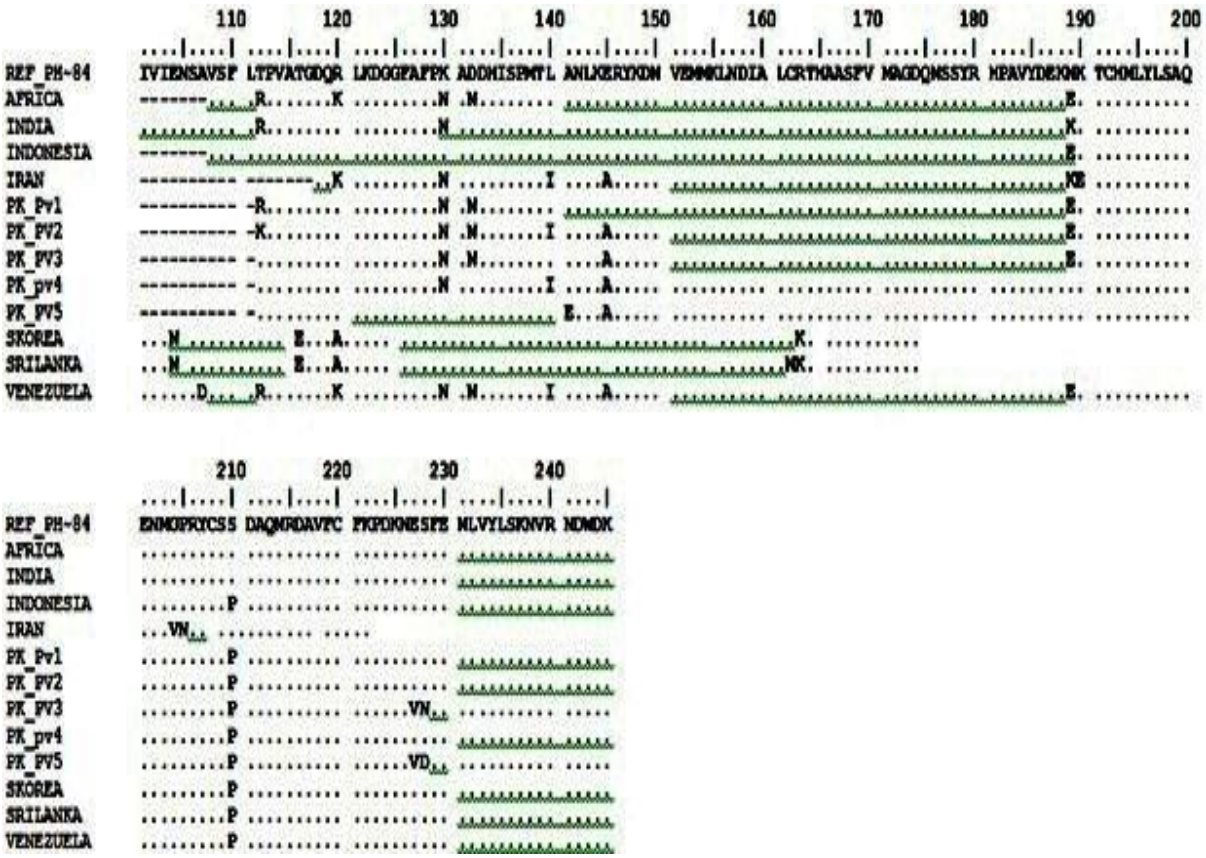

Figure-3. Multiple protein sequence alignment of PvAMA-1gene (domain-1) in *P. vivax* isolates from Pakistan (PK Pv1, PK Pv2, PK Pv3, PK Pv4, PK Pv5) PH-84 (reference sequence), South Korea, Iran, India, Sri Lanka, Venezuela, Africa and Indonesia. Dots indicate the identical amino acids.

DISCUSSION

Antigenic diversity is considered as one of the major problems to develop an effective anti-malaria vaccine against apical membrane antigen -1 (Kang *et al*., 2015). The detailed study of antigenic variants of parasite is the key step in designing a vaccine that will be effective in an endemic area like Pakistan. So present study had focused the genetic diversity of one of important vaccine candidate antigen AMA-1 in this region.

There is a wide distribution of *P. vivax* and *P. falciparum* in Pakistan and their primary vector species are *A. culicifacies* and *A. stephensi* (WHO, 2010). The development of sophisticated evasion of the human immune system is reflected by antigenic polymorphism in most *P. vivax* parasites, the understanding based on genetic variation of *Pvama-1* gene will provide a basis for development of effective vaccine against the parasite and further malaria control strategies (Rastaghi *et al*., 2014). Many antigens like DBP, MSP-1, and AMA-1 are required by the parasite for active invasion of red blood cells. AMA-1 is considered plausible vaccine candidate antigen, as studies show it to be conserved, with limited polymorphism reported when compared with other merozoite antigens (Remarque *et al.,* 2008). AMA-1 has been considered to be less variable as compare to other vaccine candidate antigens, however it shows a sequence polymorphism with a majority of variation in domain-I and domain-II (Arnott *et al*., 2013). Present study also reported sequence polymorphism at the domain-I of PvAMA-1 when compared with reference sequence (Figure 2). A recent study of amino acid polymorphism in Korean isolates of PvAMA-1 also revealed a different pattern of amino acid polymorphism in comparison to amino acid sequences from other regions (Kang *et al*., 2015), supporting the results of amino acid sequence polymorphism of present study (Figure3). Primers used in the present study also amplified the region of domain I of this antigen reported in Korean isolates as amplifying a band size of 511 bp from 22 isolates with a result of two genotypes of AMA-1 as SKOR-69 and SKOR-68 when analysed for polymorphism with PH-84 isolate. Although no size polymorphism was being observed among any of the isolates (Han *et al.,* 2002).

Cheng and Saul (1994) published one of the first study regarding complete sequencing of *P. vivax AMA-1* gene and found 12 single nucleotide substitutions causing

changes in nine amino acids. One hundred and twenty nine *P. vivax* isolates were being studied in Iran for sequence polymorphism at Domain-I and D-II, the study showed that domain I is more variable than Domain II. The present study also found that genetic diversity existed at domain-1 of *PvAMA-1* gene in this region along with conserved pattern, showing that domain-I of AMA-1 antigen is also variable in this endemic part (Rastaghi *et al*., 2014). The sequences found in the present study showed polymorphism at different positions when aligned with reference sequence PH-84 and sequences from India, Africa, Iran, Indonesia, Venezuela, Sri Lanka, South Korea and Iran (Figure 2). The amino acid alignment also showed variations at different positions in comparison to reference sequence PH-84 and other sequences (Figure 3).

Conclusion: Present study is the first report of genetic characterization of *AMA-1* gene from this region. The *AMA-1* gene showed genetic polymorphism at domain 1 in the study region resulting amino acid substitutions in the AMA-1 antigen. The polymorphism observed in *P. vivax* isolates will help in understanding the nature of these isolates and development of malaria vaccine based on this antigen

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