RESTRICTED T-CELL EPITOPE DIVERSITY IN THE CIRCUMSPOROZOITE PROTEIN FROM Plasmodium falciparum POPULATIONS PREVALENT IN IRAN

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Abstract. Parasite genotyping studies have indicated that the Plasmodium falciparum populations circulating in Iran are genetically diverse and that multiple genotype infections are observed regularly. We wished to extend the analysis to the Pfcsp gene, coding for the dominant sporozoite surface antigen on which the leading malaria vaccine candidate RTS,S is based. Infected blood samples were collected mainly from Iranian, as well as Afghan and Pakistani, patients on admission with falciparum malaria. DNA was purified from 90 isolates, and from these, 21 fragments corresponding to Pfcsp and 69 fragments corresponding to the 3′-end conserved domain were amplified and sequenced. Overall diversity was low. Six patterns were noted for the repeat region, but mixed genotypes were not observed in any of the isolates. T cell epitopes also displayed limited diversity, with only five haplotypes (combined Th2R/Th3R epitopes) noted, and of these, three were dominant, accounting for 94% of the 90 sequences. These observations are akin to those observed in Thai P. falciparum isolates, where a particular Pfcsp Th2R/Th3R haplotype seems to be maintained in an otherwise genetically diverse parasite population. The data imply that the selective pressure that maintains a restricted T cell epitope is caused by factors outside the mammalian host immune responses. Furthermore, they sustain the notion that protective responses induced by RTS,S vaccination are not strain-specific.

INTRODUCTION

The malaria eradication campaign initiated in the 1950s led to the elimination of this major public health disease in most temperate and subtropical countries and to a significant reduction in the cases recorded in many tropical countries. This was mainly achieved by combining vector control measures and mass treatment of febrile individuals. Subsequently, the efficacy of these measures waned as a result of the emergence and spread of drug-resistant Plasmodium falciparum, the development of insecticide resistance by the Anopheles mosquito vectors, climate changes, political instability, and population migration. Consequently, malaria is now resurgent in most tropical areas, and the disease is regaining a foothold in areas from which it had been eradicated or controlled. This is the case in Iran, where national malaria control programs initiated in 1958 achieved eradication for the temperate northern Caspian region by 1977 and significantly reduced transmission in the subtropical southeastern provinces of Sistan, Baluchistan, Hormozgan, and Kerman. In 2005, about 20,000 malaria cases were reported for the whole country, although the majority (85%) originated in the southeastern provinces where both P. vivax (80%) and P. falciparum (20%) species are present.

In the past decades, extensive efforts were made to develop a vaccine against falciparum malaria, because P. falciparum is not only the most prevalent of the four malaria parasites that infect humans but also because this parasite is associated with the highest proportion of severe malaria and nearly all the mortality ascribed to malaria. Many of the experimental vaccines are based on antigens from the asexual and sexual blood stages, but formulations targeting the pre-erythrocytic stages (the sporozoite and the liver stage parasite) are leading the race to deployment in endemic residents. Most experimental pre-erythrocytic stage vaccines are based or include the circumsporozoite protein (CSP) as an immunogen. CSP is the dominant surface protein of the sporozoite, the form injected by the infected mosquito. The gene coding for CSP was the first Plasmodium gene to be isolated and characterized, and the first P. falciparum subunit vaccine tested in human volunteers was based on this protein. Today, the most advanced vaccine against malaria, RTS,S, is based on the P. falciparum CSP (PfCSP). This vaccine has already undergone two Phase IIb clinical trials in adults and children from The Gambia and Mozambique, respectively, where it provided encouraging, although modest, levels of protection.

 Destruction of the liver stage parasite, where CSP can be found and where it is expressed during the early stages, is considered to primarily depend on cellular immune mechanisms. Extensive sequence diversity was found in the two immunodominant T-cell epitopes (Th2R and Th3R) identified in the carboxyl terminus of PfCSP. This raised concerns that this extensive antigenic diversity, which also characterizes many other malaria vaccine candidates, might be an obstacle to the design of a CSP-based vaccine that would be universally effective against P. falciparum. These concerns apply to the RTS,S formulation that make up a large portion of the PICSP antigen, which includes the carboxyl terminus, obtained from a cloned parasite line (i.e., a single haplotype). Nonetheless, molecular studies associated with the two RTS,S trials failed to reveal any evidence of selection of parasites with PfCSP Th2R and Th3R epitopes that differ from those of the vaccine in the P. falciparum parasites found during follow-up in the vaccinated volunteers. This raised the possibility that protective immune responses might not be strongly dependent on the variable Th2R and Th3R epitopes. In a separate extensive study of PfCsp diversity, this notion was supported by the finding of a geographically and temporally fixed dominance of a particular haplotype in otherwise
It is not known whether this pattern of parasite diversity will be found in other endemic regions. Therefore, at present, it is not clear which of the two hypotheses above, or whether a combination of both, accounts for the diversity of the Th2R and Th3R epitopes of PICSP.

In this context, the survey of Pfcspr diversity in parasites circulating in Iran that is presented here will be of interest. First, if vaccine formulations based on PICSP prove efficacious at preventing malaria in trials conducted in endemic countries, their deployment might be envisaged to control malaria in Iran. It will thus be important to carry out prospective baseline surveys of Pfcspr diversity in Iranian isolates. Second, it had been previously noted that P. falciparum populations circulating in Iran (predominantly in the southeastern provinces) display a relatively high degree of genetic diversity, despite the low endemicity of the infection.17 We wished to ascertain whether the diversity of Pfcspr reflects that observed for the Pfmsp1 and Pfmsp2 molecular markers used in the study above, and thus ascertain whether the parasite populations in Iran have a similar population structure to those surveyed in the studies carried out in Thailand.15,16

MATERIALS AND METHODS

Study area and sample collections. Samples were collected during the malaria transmission season (April to March) during 2001–2005 from patients with fever (age, 1 to > 60 years) who attended malaria clinics at primary health centers in the Chabahar district (Figure 1). The majority of the patients were male (80.5%), the average age of all patients was 27 years, and they were nationals of three countries, Iran (59%), Afghanistan (19%), and Pakistan (16.5%); the remainder was not recorded. Diagnosis was established by microscopic examination of Giemsa-stained smears made from fingerprick blood. Approximately 1 mL of whole blood was collected from P. falciparum–infected patients (N = 169 samples). Before sample collection, written informed consent was sought and obtained from the patients. This study was approved by the Ethical Review Committee of Research of the Pasteur Institute of Iran. Blood was collected in tubes containing EDTA, stored at 4°C, and transported to the main laboratory in Tehran.

Parasite DNA extraction. Parasite DNA was prepared by phenol/phenol-chloroform extraction and ethanol precipitation as described previously.18 The DNA template obtained from 250 μL of whole blood was dissolved in 30 μL of TE buffer (10 mm Tris-HCl, pH 8.0, 0.1 mm EDTA) and stored at −20°C until use.

Genotyping of csp polymorphic epitope sequences. Primers for amplification were designed according to the Pfcspr sequence present in the P. falciparum 7G8 line (accession no. AB121015). One microliter of the DNA was used as the template for the primary PCR amplification. The reaction was carried out in a total volume of 25 μL and in the presence of 1.5 mmol/L MgCl2, 1 unit of Taq DNA polymerase (Invitrogen, Cergy Pontoise, France), 10 pmol of each oligonucleotide primer (forward primer: 5’-GGCCTTATTCCAGGAAATACCCGTCG, reverse primer: 5’-ACGACATTAAACACACTGGAAC), and 2 μmol/L dNTPs. In this manner, most of the Pfcspr coding sequence (excluding the first and last 75 bp) could be amplified. The cycling conditions were as follows: 95°C for 2 minutes, 64°C for 2 minutes, 72°C for 2 minutes, 35 cycles of 64°C for 1 minute, 72°C for 1 minute, and 94°C for 1 minute, followed by 72°C for 10 minutes. In the secondary PCR amplification, 1 μL of the product obtained in the primary reaction was used as the template to amplify the conserved 3′ region of the gene, using previously described primers.19 The cycling conditions were as follows: 94°C for 3 minutes, 60°C for 2 minutes, 72°C for 2 minutes, 30 cycles of 60°C for 2 minutes, 72°C for 1 minute, and 94°C for 1 minute, followed by 72°C for 10 minutes.

Amplified DNA fragments were separated on agarose gels by electrophoresis and visualized on an UV trans-illuminator after ethidium bromide staining.

DNA sequence analysis. Amplified fragments were gel-purified using the QIAGEN DNA purification kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Direct sequencing of the DNA fragments was performed in both directions for each polymerase chain reaction (PCR) product using an ABI-3100 sequencer (Applied Biosystems, Foster City, CA). For the whole gene, three sequencing primers were used so as to obtain overlapping sequences that span the whole gene: the forward and reverse primers used for amplification (see above) and an internal reverse primer: 5’-TTCACTACATTGCCGTTTG. For the region corresponding to the carboxyl region of PICSP, the primers used for amplification were used for sequencing.

Nucleotide and amino acid sequences were aligned and compared with the 7G8 sequence (accession no. AB101215)

![Map of Iran indicating the location of the four primary health centers in Chabahar District situated in the southeastern corner of Baluchistan Province, from where the P. falciparum isolates were collected. S&B, Sistan and Baluchistan province.](image-url)
using CLUSTALX. Nucleotide sequences reported in this article are available in the European Molecular Biology Laboratory (EMBL), GenBank, and DNA Data Bank of Japan (DDBJ) databases under accession numbers DQ521752–DQ521752 (PfCsp) and DQ521663–DQ521731 (PfCsp C-terminal domain).

RESULTS

Sequence diversity in the repeat and non-repeat region of csp gene. Amplification of the PfCsp gene was successfully carried out for the 169 isolates collected from P. falciparum–infected patients presenting to clinics in the Chabahar district (Figure 1). Size diversity was observed for the amplified PfCsp gene fragments (range, 1,100–1,300 bp), although the 1,150-bp fragments were predominant. There was no evidence of mixed genotype infections in any of the samples tested, although minor differences in repeat number (repeat unit of four amino acids corresponding to 12 bp) would not be resolved by the agarose gels used. The PfCsp fragments were divided into groups that differ by 50 bp, and a subset of each group was randomly selected in a proportion reflecting their frequency for sequence analysis. A total of 21 fragments representing nearly the full-length gene were sequenced. In addition, PCR amplification of the PfCsp region (320 bp) coding for the C-terminal domain, where the Th2R and Th3R epitopes are found, was amplified from a further 69 samples and sequenced.

As expected, size variation was caused by differences in the number of repeat units, which ranged from 43 to 48. All the mutations observed in the gene derived from the 21 Iranian P. falciparum isolates had been previously reported. Taking the PfCsp sequence of the 7G8 line as a reference, all the synonymous point mutations observed between the sequences were found in the central repeat region. Two repeat units are known for PfCsp, NANP, and NVDP. All the non-synonymous mutations found in the repeated region led to the conversion of the first type of repeat unit to the other (Figure 2). Point mutations were found in only four positions outside the repeat region, and in all cases, these proved to be non-synonymous. The two mutations located in the pre-repeat region occurred together in 10 of the 21 sequences (Figure 2). It was interesting to note that these mutations in the pre-repeat region were associated with the mutations in the repeat units. The other two mutations were found in the region coding for the Th2R epitope in the conserved 3′-end region (Figure 2). When the previously described nomenclature for the Th2R and Th3R variants was adopted, two haplotypes were observed: 7 sequences were of the Th2R*1/Th3R*1 haplotype and 14 sequences were of the Th2R*5/Th3R*1 haplotype.

Sequencing of the region coding for the conserved C-terminal region was carried out for a further 69 samples, collected from patients who acquired the infection either in Iran (N = 37), Pakistan (N = 20), or Afghanistan (N = 12). In addition to the Th2R*1 and Th2R*5 haplotypes, a novel Th2R haplotype was observed in a single isolate collected from an Afghan patient and was denoted Th2R*N thereafter; thus, three Th2R variants were observed in total. Two Th3R variants were observed (Th3R*1 and Th3R*10) in total. In the 69 samples, three additional PfCsp haplotypes (Th2R*1/Th3R*10; Th2R*5/Th3R*10; Th2R*N/Th3R*1) were observed in addition to the two described above for the 21 Iranian isolates (Figure 3). The frequency of the five haplotypes was calculated for the 90 samples (Figure 4). The two minor haplotypes (Th2R*1/Th3R*10 and Th2R*N/Th3R*1) were only found in parasites originating outside Iran, whereas the three dominant haplotypes (Th2R*1/Th3R*1, 22.2%; Th2R*5/Th3R*1, 55.5%; and Th2R*5/Th3R*10, 17.7%) were found in both the Iranian and the non-Iranian parasite isolates. No significant association with age, sex, or season could be observed, and the dominant haplotypes observed in the samples collected in 2004–2005 were also those observed in those collected in 2001 and 2003, although the number of latter samples was small (N = 15).

DISCUSSION

In this report, we present the first survey of the genetic diversity of the gene coding for a major malaria vaccine candidate, the circumsporozoite protein, in P. falciparum parasites circulating in the tropical southeastern region of Iran. In addition to gathering baseline data on Plasmodium parasites found in an area with re-emerging malaria, the study was in part prompted by observation of higher than expected levels of genetic diversity for the P. falciparum populations in a region where transmission intensity is low. The salient finding was that the PfCsp genes display a restricted diversity, which is particularly marked for the Th2R and Th3R epitopes. The limited PfCsp diversity contrasted with the higher Pfmsp-1 and Pfmsp-2 diversity observed in Iranian P. falciparum isolates collected in the same location. The differences in diversity for these two sets of markers were noted not only for the total number of distinct haplotypes but were also reflected in the frequencies of mixed genotype infections, of which none were found when PfCsp was used for genotyping as opposed to a multiplicity of infection around 2.5 for either Pfmsp-1 or Pfmsp-2.

![Figure 2](image-url) Alignment of the nine different PfCsp types sequenced from 21 Iranian isolates. The first (5′-end) and the last (3′-end) 75 bp were not obtained or were part of the oligonucleotide primers. Only regions where non-synonymous mutations were observed are depicted: the pre-repeat region immediately after Region I, the central repeat domain, whereas the observed differences in the whole C-terminal part concerned only the Th2R epitope.
Numerous surveys of Pfcs expansion have been carried out in diverse endemic regions. However, in many cases, the number of isolates collected in a given area tended to be low. Outside the variable central repeat region, most non-synonymous mutations have been recorded for the Th2R and Th3R epitopes in the C-terminal region of the antigen. Indications that the degree of polymorphisms in these regions are high were initially obtained from a sequence analysis of Pfcs expansion derived from isolates collected in the Gambia. In this study, seven Th2R and five Th3R haplotypes were observed in five clinical isolates collected over a short period of time and in patients residing within a 5-mi. radius. This pattern of high polymorphism from African parasites was confirmed by studies carried out on isolates collected from different regions of Africa and in one study of Indian P. falciparum isolates. Studies of isolates collected in Brazil, Papua New Guinea, Thailand, and Myanmar indicated the opposite that Th2R and Th3R polymorphisms in the parasites circulating there were relatively limited. With the exception for the studies associated with the RTS,S trials, the above surveys were carried out with a relatively low number of isolates (a dozen or so), often collected at different times from diverse localities. One of the first large scale surveys of Pfcs expansion polymorphisms was carried out using samples collected in Vanuatu. It was noted that only two Th2R/Th3R haplotypes were found. In a large scale survey conducted in two sites in Thailand; samples from one were collected over a 5-year period, one Th2R/Th3R haplotype dominated despite the fact that the P. falciparum parasites present in these isolates were highly diverse with respect to three other genetic markers (Pfmsp1, Pfmsp2, and glurp). This last observation is reminiscent of the results obtained in the surveys of Iranian parasites presented here. Recently studies of Pfcs expansion in isolates collected from Myanmar, Vietnam, and Indonesia confirmed the predominance of a single Th2R/Th3R haplotype in the endemic parasite populations. Recently, comparative analysis of CSP and thrombospondin-related adhesive protein (TRAP), two P. falciparum antigens implicated in pre-erythrocytic protective immunity, provided evidence of differential natural selection, with TRAP but not CSP being under strong selection for amino acid sequence diversity and allele frequencies under balancing selection.

The observation of relatively low T-cell epitope diversity, combined with geographical and temporal fixation and dominance of a restricted number of haplotypes, has already prompted the suggestion that the polymorphisms in this region of Pfcs expansion might not actually be selected for by immune pressure but rather by the anopheline species responsible for the bulk of transmission in a particular area. One of the predictions of this hypothesis is that the dominant Th2R/Th3R haplotypes in different geographical locations are likely to differ, because different species of anopheline mosquitoes are likely to be responsible for transmission in geographically distant areas. To test this prediction, many large-scale surveys associated with solid entomologic studies would be needed. Nonetheless, analysis of the data published to date would seem to support the hypothesis of a mosquito-derived selective pressure on the C-terminal portion of Pfcs expansion. Thus, in two independent surveys in Brazil, a majority of the P. falciparum parasites were of the Th2R*1/Th3R*1 haplotype (65% for 17 isolates and 83% for 23 isolates, respectively), whereas in Papua New Guinea, all 22 isolates were of the Th2R*5/Th3R*4. This last observation is reminiscent of the results obtained in the surveys of Iranian parasites presented here. Recently studies of Pfcs expansion in isolates collected from Iran, Afghanistan, or Pakistan. One of the first large scale surveys of Pfcs expansion diversity have been carried out on isolates collected from patients who acquired the infection in Iran, Afghanistan, or Pakistan. The position of the residues are those of the protein found in the P. falciparum 7G8 line, where the epitopes are Th2R*1 and Th3R*1.

**Figure 3.** Alignment of the different Th2R and Th3R variants observed in the 90 Pfcs expansion sequences derived from isolates collected from patients who acquired the infection in Iran, Afghanistan, or Pakistan. The position of the residues are those of the protein found in the P. falciparum 7G8 line, where the epitopes are Th2R*1 and Th3R*1.

**Figure 4.** Frequency distribution of the different Th cell epitope haplotypes encountered in the sequences obtained from 90 isolates collected from Iran, Afghanistan, and Pakistan. Two haplotypes were only observed in non-Iranian (shaded in gray).
indicate that anopheline mosquitoes are more permissive to these haplotypes than others. It should be noted that the two relatively uncommon haplotypes found in the survey conducted in Iran were found in parasites collected from persons who had acquired the infection outside Iran, probably in Pakistan or Afghanistan. It would be interesting to compare the autochthonous Iranian *P. falciparum* isolates with those from these two countries and to determine whether the frequency of these two rare haplotypes will increase in southeastern Iran in future years. These studies must nonetheless be associated with detailed entomologic studies aimed at determining which anopheline species account for most malaria transmission and genetic analysis of *Pf* in the parasites found in the salivary glands of wild-caught mosquitoes of this species.

In conclusion, a large-scale survey of *Pf* in Iranian *P. falciparum* populations indicates that this antigen displays limited antigenic diversity. If the Th2R and Th3R epitopes are indeed implicated in protective immune responses, the low levels of polymorphisms found indicate that diversity of this candidate antigen is unlikely to influence the outcome of field trials of vaccines based on PICP6 adversely.

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