Acta Protozool. (2005) 44: 289 - 292

ACTA
PROTOZOOLOGICA

Plasmodium falciparum Pfs25 Gene Promoter has no Polymorphism in Natural Isolates of Eastern India

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Summary. Most of the potential vaccine candidate genes show polymorphism or point mutation, which affects the structure of antigen and thereby, production of antibody to protect the host against further infection. In the present study, we have analyzed the promoter regions of 415 base pair (nucleotide position -722 to -308) of *Pfs25* gene. Previous studies have shown that, removing these important regions drastically affects the promoter activity and Pfs25 protein expression. We for the first time, report that though promoter regions of *Pfs25* gene is AT rich, it doesn't show any polymorphism or point mutation in sequences important for its activity in 155 natural isolates of *Plasmodium falciparum* from hyper endemic malaria transmission regions. Since, *Pfs25* expression is vital for the survival of the parasite in the mosquito vector, a stable promoter sequence of *Pfs25* in all the strains may have fair chances of survival in the mosquito vector. Our finding of a stable promoter sequence in combination with previous reports of limited polymorphism in the coding regions of *Pfs25* indicates that Pfs25 might be a strong candidate for vaccine.

Key words: PCR-SSCP, *Pfs25* promoter, *Plasmodium falciparum,* point mutation, polymorphism.

INTRODUCTION

Malaria is a public health problem in more than 90 countries, inhabited by a total of some 2.5 billion people almost 40% of the world's population. There are 300 to 500 million clinical cases every year and between one and three million deaths, mostly of children, are attributable to this disease. Every 40 seconds a child dies of malaria, resulting in a daily loss of more than 2,000 young lives worldwide (Sachs and Malaney 2002). These estimates render malaria the pre-eminent tropical parasitic disease and one of the top three killers among communicable diseases. Vector control and malaria chemotherapy that were previously effective in controlling and treating malaria are now largely ineffective due to insecticide-resistant mosquitoes and drug-resistant parasites. As alternatives, immunological intervention will be needed to stop the resurgence of malaria, a disease with a devastating impact on the human health. Pfs25 (Kaslow *et al.* 1988, 1989; Kaslow 1990), cysteine-rich 25 kDa surface protein of *Plasmodium falciparum*, is a sexual

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stage specific potential vaccine candidate (Vermeulen *et al.* 1986), which is expressed on the surface of zygote and ookinete forms of the parasite in mosquito vector (Vermeulen *et al.* 1985). Monoclonal antibodies directed against native Pfs25 could block completely the development of *P. falciparum* oocysts in the midgut of the mosquito vector (Charoenvit *et al.* 1997). Thus, this 25-kD protein is a potential vaccine candidate antigen of *P. falciparum*, which may assist in the control of lethal forms of human malaria (Zou *et al.* 2003). Recombinant vaccines based on Pfs25 are in various stages of clinical trials (Barr *et al.* 1991, Kaslow *et al.* 1991). Numerous studies had shown that very limited or no polymorphism occurs in the coding regions of *Pfs25* (Niederwieser *et al.* 2000). Most part of the *Pfs25* coding regions are conserved and shows low titer yet highly potent antibody production in animal models and human hosts (Stowers *et al.* 2000).

Previous studies have demonstrated that promoter region (-722 to -308) of *Pfs25* is very important for the expression of *Pfs25* gene (Dechering *et al.* 1999). In this report, for the first time, we showed that this regulatory region of *Pfs25* gene has no polymorphism in Indian isolates of Orissa.

MATERIALS AND METHODS

Study area and *Plasmodium falciparum* isolates

We have selected Orissa, a state of India as our study area with longitude 30.20.N and latitude 85.54.E and the whole region comes under endemic zone of malaria transmission. With 38% of the India's population Orissa contributes 20% of the *P. falciparum* cases and 40% of malarial deaths of India. Blood samples (n = 155) were collected from *P. falciparum* infected individuals attending malaria clinic at primary health centers of hyper endemic regions of Orissa. Blood was collected from consenting volunteers as 1 ml samples in 0.08M EDTA and stored at -70°C for further use. The Institutional Ethical Committee approved this study.

DNA isolation

DNA was prepared from these samples using a rapid DNA isolation method described earlier by Foley *et al*. (1992), with little modifications. Briefly, 500 µl of ice-cold 5 mM sodium phosphate (pH-8) was added to 20 µl of venipuncture blood and vortexed. After centrifugation for 10 min in a microcentrifuge tube at 4°C, the supernatant was discarded. The pellet was suspended in 100 µl PBS containing 0.01% saponin and washed twice with same buffer by repeating the above steps of vortexing and centrifugation. After washing, the pellet was suspended in 50 µl of sterile water, vortexed and then boiled for 20 min at 100°C. After centrifugation in a micro centrifuge tube at 6,500 g for 10 min at 4°C, the supernatant was collected and used in PCR.

Primer designing and PCR

The sequence of the promoter regions of *Pfs25* gene was taken from GenBank, (Accession number- AF030628, Dechering *et al.* 1999). The sequence of *Pfs25* promoter is AT rich, so the primers were designed in such a manner that it included the regions important for promoter activity and few GC bases for optimum annealing in PCR. Primer sequences, P310F 5'- GAT ACT TCT CTA TGT ACA TAT ATA - 3', P310R 5'- CCA AAC TCT TAA GTA TCA GTA c-3' and P128F - 5'- GTA CTG ATA CTT ATA GAG TTT GGC - 3', P128R - 5'- TGT ATT TAT TTA CAA TGA TTA TAT AAA CGG - 3' were used for the amplification of 310 base pair (bp) (nucleotide position -722 to -413) and 128 bp (nucleotide position -435 to -308) promoter region fragments, respectively. PCR was carried out in 25 µl reactions with 1.5 U of Taq polymerase (Amersham, USA). The cycle parameters for first set of primers (for 310 bp fragment) includes, initial denaturation for 5 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The PCR conditions for second set of primers (for amplification of 128 bp fragment) were remained same except the change of annealing temperature from 50°C to 52°C.

Polymerase chain reaction and single strand conformational polymorphism(PCR-SSCP) analysis

PCR- SSCP was done as described earlier by Raj *et al*. (2004a) with little modifications. Briefly, 5 µl (20 ng) of the genomic DNA was amplified for 35 cycles as 20 µl reactions in the presence of [α^{32}P] dATP. The reaction conditions were same as described above for the second set of primers. 2 µl of the labeled PCR-product was mixed with 9 µl of loading dye (98% formamide, 20mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol). The samples were denatured at 95ºC for 10 min and immediately chilled on ice for 5 min. 1-2 µl of the above samples were electrophoresed through a non-denaturing 8% polyacrylamide gel containing 3% glycerol. Electrophoresis was performed at 250 V for 18-20 h at 25°C. The gel was then autoradiographed.

RESULTS AND DISCUSSION

The PCR showed a single step amplification of the required fragment even at low parasitaemia of 0.1% (Fig. 1). When same primer was taken for *P. vivax* DNA in PCR, we got the amplification of similar fragment size as obtained in case of *P. falciparum*. The data indicates that there might be a very similar gene in *P. vivax*, which is yet to be reported.

Previous reports have indicated that a change in the important promoter regions of a gene severely affects the gene expression in *P. falciparum* (Horrocks and Lanzer 1999, Osta *et al*. 2002, Porter 2002). But the

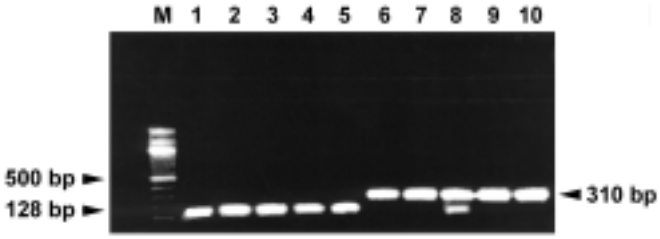

Fig. 1. A representative number of samples (out of 155) showing PCR amplification of promoter regions of 128 bp (Lane 2-5) and 310 bp (Lane 7-10) in *P. falciparum* natural isolates. M, 100 bp ladder. Lane 1 and 6, 3D7 clone of *Plasmodium falciparum.*

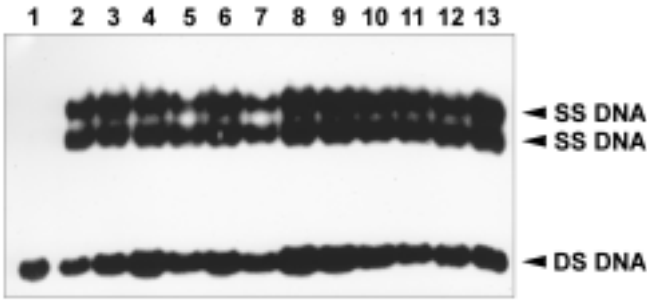

Fig. 2. A representative number of samples (12 out of 155) showing PCR-SSCP of 128 bp PCR amplified fragments. Lane 1: PCR amplified product of clone 3D7 DNA without denaturation showing only double stranded DNA; lane 2: Clone 3D7 DNA denatured with formamide showing both single and double stranded DNA; Lane 3-13: DNA of natural isolates of *Plasmodium falciparum* collected from an hyper endemic region.

PCR - SSCP analysis of the 128 bp important fragment of *Pfs25* promoter (Fig. 2) showed that none of the isolates out of 155 samples analyzed were polymorphic or having any point mutations. Similar results were obtained with 310 bp fragment (Figure not shown). Previous studies has shown that deletion of regions between nucleotide positions -648 to -484 reduces the *Pfs25* promoter activity by more than 16 fold and nucleotide position -484 to -368 completely abolishes *Pfs25* promoter activity (Dechering *et al.* 1999). Both the PCR amplified fragment of 310 bp and 128 bp generated by the two set of primers, contain binding sites for the transcription factor PAF I. To confirm the sequence of PCR amplified products of *Pfs25* promoter regions, we have sequenced the 128 bp and 310 bp fragments by PCR Sequencing Kit (Amersham) from few natural isolates and a 3D7 clone, which was taken as control.

Preliminary analysis of coding regions of *Pfs25* gene containing epidermal growth factor (EGF) like domain showed very limited polymorphism (data not shown). Most of the potential vaccine candidate genes of *P. falciparum* show polymorphism, which affect the vaccine potential by change of antigenic domain in the coding regions (Su and Wellems1994, Hanke *et al.* 1998, Tonhosolo *et al.* 2001, Raj *et al.* 2004b). The absence of polymorphisms or point mutations in the above regions showed that the *Pfs25* gene expression may not affected by the polymorphism of its promoter in natural isolates of *P. falciparum*. This study on promoter regions along with previous work on *Pfs25* coding sequence may be a supporting evidence for stable expression of Pfs25 protein, which is vital for the survival of the parasite in mosquito vector. Probably there is no alternative path exist for the survival of gametocytes after fertilization without expression of *Pfs25* gene. The reports of pervious studies on *Pfs25* shows antibodies to Pfs25 can completely block the transmission of malaria parasites when mixed with infectious blood and fed to mosquitoes through a membrane feeding apparatus (Lobo *et al.* 1999, Zou *et al.* 2003), but at this point of time we can't deny the involvement of other factors involved in the expression of *Pfs25* gene. Numerous studies on polymorphisms of *Pfs25* coding sequence in natural isolate showed that it displays limited or no polymorphisms in natural isolates of different geographical location of the world (Niederwieser *et al*. 2000). Again the effect of limited changes in the coding sequence of *Pfs25* on the vaccine potential of the gene is yet to be studied. Recombinant DNA vaccines by taking various coding regions of the gene may proved to be effective in animal models and human host (Barr *et al.* 1991, Kaslow and Shiloach 1994, Gozar *et al.* 1998). In conclusion, we believe that our study on sequence polymorphism of *Pfs25* promoter regions may provide additional information on Pfs25 to be used as a vaccine in case of *P. falciparum* malaria.

Acknowledgements. This work was supported by funds from Department of Biotechnology, Government of India. We thank Prof. P. C. Supakar for the critical review of the manuscript before submission.

REFERENCES

Barr P. J., Gibson H. L., Bathurst I. C., Quakyi I. A., Kaslow D. C. (1991) Recombinant *Pfs25* protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. *J. Exp. Med.* **174:** 1203-1208

Charoenvit Y., Fallarme V., Rogers W. O. (1997) Development of two monoclonal antibodies against *Plasmodium falciparum* sporozoite surface protein 2 and mapping of B-cell epitopes. *Infect. Immunol.* **65:** 3430-3437

Dechering K. J., Kaan A. M., Mbacham Wirth D. F., Eling W. M. C., Stunnenberg H. G. (1999) Isolation and functional characterization of two distinct sexual-stage -specific promoters of the human malaria parasite *Plasmodium falciparum. Mol. Cell Biol.* **19:** 967-978

Foley M., Cartwrought L. C. R., Babiker H. A. (1992) Rapid and simple method for isolating DNA from finger prick samples of blood. *Mol. Biochem. Parasitol.* **53:** 241-244

Gozar M. M., Price V. L., Kaslow D. C. (1998) *Saccharomyces cerevisiae*-secreted fusion proteins *Pfs25* and Pfs28 elicit potent *Plasmodium falciparum* transmission-blocking antibodies in mice. *Infect. Immunol.* **66:** 59-64

Hanke T., Schneider J., Gilbert S. C., Hill A. V., McMichael A. (1998) DNA multi-CTL epitope vaccines for HIV and *Plasmodium falciparum:* immunogenicity in mice. *Vaccine* **16:** 426-435

Horrocks P., Lanzer M. (1999) Mutational analysis identifies a five base pair cis-acting sequence essential for GBP130 promoter activity in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **99:** 77-87

Kaslow D. C. (1990) Immunogenicity of *Plasmodium falciparum* sexual stage antigens: implications for the design of a transmission blocking vaccine. *Immun. Lett.* **25:** 83-86

Kaslow D. C., Quakyi I. A., Syin C., Raum M. G., Keister D. B., Coligan J. E., McCutchan T. F., Miller L. H. (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. *Nature* **333:** 74-76

Kaslow D. C., Syin C., McCutchan T. F., Miller L. H. (1989) Comparison of the primary structure of the 25 kDa ookinete surface antigens of *Plasmodium falciparum* and *Plasmodium gallinaceum* reveal six conserved regions. *Mol. Biochem. Parasitol.* **33:** 283-287

Kaslow D.C., Isaacs I., Quakyi I.A., Gwadz R., Moss B., Keister D.B. (1991) Induction of *Plasmodium falciparum* transmission-blocking antibodies by recombinant vaccinia virus. *Science* **252:** 1310-1313

Kaslow D. C., Shiloach J. (1994) Production, purification and immunogenicity of malaria transmission-blocking vaccine candidate: TBV25H expressed in yeast and purified using nickel-NTA agarose. *Biotechnology (N Y)* **12:** 494-499

Lobo C. A., Dhar R., Kumar N. (1999) Immunization of mice with DNA-based *Pfs25* elicits potent malaria transmission-blocking antibodies. *Infect. Immunol.* **67:** 1688-1693

Niederwieser I., Felger I., Beck H. P. (2000) *Plasmodium falciparum*: expression of gametocyte-specific genes in monolayer cultures and malaria-positive blood samples. *Expl Parasitol.* **95:** 163-169

Osta M., Gannoun-Zaki L., Bonnefoy S., Roy C., Vial H. J. (2002) A 24 bp cis-acting element essential for the transcriptional activity of *Plasmodium falciparum* CDP-diacylglycerol synthase gene promoter. *Mol. Biochem. Parasitol.* **121:** 87-98

Porter M. E. (2002) Positive and negative effects of deletions and mutations within the 5' flanking sequences of *Plasmodium falciparum* DNA polymerase delta. *Mol. Biochem. Parasitol.* **122:** 9-19

Raj D. K., Das B. R., Dash A. P., Supakar P. C. (2004a) Identification of a rare point mutation at C- terminus of merozoite surface antigen - 1 gene of *Plasmodium falciparum* in eastern Indian isolates. *Expl Parasitol.* **106:** 45-4

Raj D. K., Das B. R., Dash A. P., Supakar P. C. (2004b) Genetic diversity in MSA-1 gene of *Plasmodium falciparum* in different malaria endemic localities. *Am. J. Trop. Med. Hyg.* **71:** 285-289

Sachs J., Malaney P. (2002) The economic and social burden of malaria. *Nature* **415:** 680-685

Stowers A. W., Keister D. B., Muratova O., Kaslow D. C. (2000). A region of *Plasmodium falciparum* antigen *Pfs25* that is the target of highly potent transmission-blocking antibodies. *Infect. Immunol.* **68:** 5530-5538

Su X. Z., Wellems T. E. (1994) Sequence, transcript characterization and polymorphisms of a *Plasmodium falciparum* gene belonging to the heat-shock protein (HSP) 90 family. *Gene* **151:** 225-230

Tonhosolo R., Wunderlich G., Ferreira M. U. (2001) Differential antibody recognition of four allelic variants of the merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum. J. Euk. Microbiol.* **48:** 556-64

Vermeulen A. N., Ponnudurai T., Becker P. J., Verhave J. P., Smits M. A., Meuwissen J. H. (1985) Sequential expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. *J. Exp. Med.* **162:** 1460-1476

Vermeulen A. N., van Deursen J., Brakenhoff R. H., Lensen T. H., Ponnudurai T., Meuwissen J. H. (1986) Characterization of *Plasmodium falciparum* sexual stage antigens and their biosynthesis in synchronized gametocyte cultures. *Mol. Biochem. Parasitol.* **20:** 155-163

Zou L., Miles A. P., Wang J., Stowers A. W. (2003) Expression of malaria transmission-blocking vaccine antigen *Pfs25* in *Pichia pastoris* for use in human clinical trials. *Vaccine* **21:** 1650-1657

Received on 18th March, 2005; revised version on 28th June, 2005; accepted on 1st July, 2005