USE OF PCR/DNA PROBES TO IDENTIFY CIRCUMSPOROZOITE GENOTYPE OF *PLASMODIUM VIVAX* IN CHINA

Han Guang-dong, Zhang Xiao-jing, Zhang Hong-hua, Chen Xi-xin and Huang Bing-cheng
Shandong Institute of Parasitic Diseases, Jining, Shandong, China

Abstract. The paper reports the rusult of identifying cirumsporozoite(CS) genotype of *Plasmodium vivax* by using PCR/DNA probe labeled with biotin. The sensitivity of this method to detect patient blood samples was 0.2 parasite/µl and also with high specific to *P.vivax*. CS genes from 52 blood samples collected from patients with *P.vivax* in Hainan and Yunnan Provinces were amplified by PCR and 49 were positive by gele electrophoresis analysis, positive rate was 94%. Then the amplified CS genes further were probed with special oligoprobes (PV_{210} and PV_{247}) that hybridized with the predominant CS repeat region and the variant CS repeat region. The results showed 46(88.5%) PV_{210} positive and 6 (11.5%) PV_{247} positive; 2 hybridized with both probes. The variant genotype was present only in samples from Yunnan Province.

The above results showed that the PCR/DNA probe labeled with biotin was highly sensitive and specific to *P.vivax* and found a CS variant genotype of *P.vivax* in Yunnan Province of China.

INTRODUCTION

At present, malaria is still one of main problems for human health in the world, though large scale epidemics have been controlled in China, the prevalence in some areas is rising, especially the tertian malaria epidemic areas are extensive. So recent efforts have focused on the development of a vaccine against the infective sporozoite stage for both *Plasmodium falciparum* and *Plasmodium vivax* (Gordon *et al,* 1990). Progress on a vaccine for *P.vivax* based on the predominant surface or circumsporozoite (CS) protein of *P.vivax* sporozoites has recently been complicated by the observation of genetic variation within the CS gene and phenotypic heterogeneity in the protein it encodes (Rosenberg *et al,* 1989). Fourteen percent of patients in Thailand were observed to be infected with a variant strain of *P.vivax* that produces a CS protein with a repeat unit ANGAGNQPG, distinct from the predominant form GDRAA/DGQPA (Kevin *et al,* 1991). The prevalence and world wide distribution of this variant will have implications for the efficacy of a vaccine based on the predominant CS form.

In order to know the situation of genetic variation of *P.vivax* in China, we used the polymerase chain reaction and DNA probe (PCR/DNA) system to detect and identify the CS genes of *P.vivax* from patient blood samples collected in Hainan and Yunnan Provinces.

MATERIALS AND METHODS

Blood samples

Blood (20-50 µl) was collected by venipuncture from patients with smear-positive *P.vivax* infection. 20 samples were from Hainan and 32 from Yunnan Provinces, the parasitemia ranged from 0.0014% to 3.2%. The control samples were for *P. falciparum, P. cynomolgi, P. berghei, P. yoellii* and non-infected human blood kept in our laboratory.

Sample handling

The DNAs were extracted by phenol/chloroform method (Wang, 1992) from confirmed *P.vivax* blood sample and above control samples were used to detect sensitivity and specificity. All patient blood samples were treated in 0.83% NH_4Cl 500 µl at 37°C for 20 minutes, then were centrifuged (8,000 rpm for 3 minutes), and discarded the supernatant and RBC pieces, the white pellet in the bottom of eppendorf tube was washed again with H_2O. Then was added 20 µl of a lytic solution (50mM KCl, 15mM Tris-HCl, pH 8.0, 2.5 mM $MgCl_2$, 1% SDS, 0.5% Tween-20, 100 µg/ml protein K), kept at 37°C for 30 minutes, then boiled for 15 minutes, finally centrifuged (15,000 rpm for 3 minutes), and the supernatant used for the amplification template.

PCR amplification

The 3 µl each of *P.vivax* DNA and the DNA diluted in gradient (1:10), control DNAs and DNA supernatant were amplified by PCR using oligonucleotide primers PV_5, 5′-GTC GGA ATT CAA TAA GCT GAA ACA ACC-3′, and PV_6, 5′-CAG CGG ATC CAC AGC TTA CAC TGC AT-3′, complementary to the conserved regions I and II of the *P.vivax* CS gene (Gordon *et al*, 1990). 50 µl of amplification reaction mixture consisted of 50mM KCl, 10mM Tris pH 8.0, 0.01% (wt/vol) bovine serum albumin (BSA), 2.5 mM $MgCl_2$, 250 µM dNTP, 200 pM of each primer. Three units of TaqE (Promega, USA) were added, denaturation was done at 95°C for 5 minutes, then 94°C for 1 minutes, and 60°C for 1 minute for 30 cycles, and final extension at 72°C for 10 minutes. After amplification, 10 µl of each PCR mixture was subjected to agarose gel electrophoresis to observe amplified sensitivity and specificity and 10 µl of each PCR products was spot blotted in two NC membranes for identification with DNA probes.

End biotin-labeled probe and spot-blotting hybridization

Using 30ng each of 2 synthetic oligoprobes, PV_{210} 5′-CCA GCA GGT GAT AGA GCA G-3′ and PV_{247} 5′-GGC AAT CAA CCA GGA GCA AAT GG-3′, complementary to the predominant and variant form of the *P.vivax* CS gene (Kevin *et al*, 1992), the 3′ end were labeled with 2 µl (0.5 mM) dio-11-dUTP and 8 U terminal transferase (Promega, USA), the membrane-bound DNA fragments were hybridized with the above probes respectively.

In general, pre-hybridization was carried out in 4 x SSC, 5 x Denhardt's, 5 mM EDTA, 20mM Tris, pH 8.0, 100 µg/ml denatured salmon sperm DNA at 65°C for 5 hours in a plastic bag and then the probe was added for hybridization at 68°C for 24 hours. High-stringency washes were done, finally, the NC was dyed by SA-AP, NBT-BCIP method (Wang, 1992), when a spot appears in color of blue-purple for positive.

RESULTS

The DNAs extracted from *P.vivax* samples and control samples were amplified and the results were read under ultraviolet lamp after gel electrophoresis and dyed with EB, only *P.vivax* DNA was present in amplification, and detecting sensitivity was in DNA volume of 2 parasites/µl and with a clear gradient difference wide band according to template concentration (Table 1).

The PCR products blotted on NC membrane were further analyzed by PV_{210} and PV_{247} probes mixture, the result of specificity was the same with that analyzed by gel electrophoresis, but the sensitivity was 10 times higher than that of gel electrophoresis, a visible hybridized-spot in color of blue-purple appeared with amplified products in template DNA volume of 0.2 parasites/µl (Table 2).

Each of 52 patient sample supernatants amplified was analyzed by gel electrophoresis, 49 of 52 gave a special DNA band (94% positive rate) and analyzed by PV_{210} and PV_{247} probes respectively, PV_{210} DNA was identified in 46 (88.5%) including negative by gel electrophoresis. Six (11.5%) were positive for PV_{247}, 4 for single PV_{247}, 2 for both probes simultaneously. The total identification rate was 96% (50 of 52) by both probes compared with microscopic examination (Tables 3 and 4).

In further analysis of results from samples from Yunnan Province, the CSP gene form was 78% (25 of 32) for PV_{210}, 18.8% (6 of 32) for PV_{247}, and 6.3% (2 of 32) for both forms (Table 5).

DISCUSSION

The method of PCR/DNA probe was very sensitive and specific to detect *P.vivax* in our experiment. The *P.vivax* DNA amplified was identified with a detective sensitivity of 2 parasites/µl, but the probe hybridization was 10 times more sensitive. The DNAs released from boiling blood samples and amplified were examined. The coincidental rate with microscopy was 94% by gel electropthoresis, and 96% by probe (PV_{210} and PV_{247}) hybridization.

The genotype analysis showed that 88.5% were positive for PV_{210} and 11.5% for PV_{247}. There were evident geographical differences in this study: the PV_{247} genotype was not observed in Hainan Province. The forms of *P.vivax* CSP gene in samples of Yunnan Province were basically the same as that of Rosenberg *et al* (1998), but the rate of mixed infec-

Table 1
Specificity of the PCR/DNA probe system.

Samples	Pv	Pf	Pb	Py	Pc	Tox	HB
Results	+	-	-	-	-	-	-

Table 2
Sensitivity of the PCR/DNA probe system.

No. parasite	2,000	200	20	2	0.2	0.02
Results	+	+	+	+	+	-

Table 3
Comparison between results of gel electrophoresis and probe hybridization after PCR amplification.

Samples	No.	PCR/Gel		PCR/Probes	
HY		No.	(%)	No.	(%)
Patient	52	49	94	50	96

HY: Samples from Hainan and Yunnan Provinces.

Table 4
Detection results by both probes of PV_{210} and PV_{247}

Samples	No.	PV_{210}		PV_{247}		PV_{247} and PV_{210}	
PV		No.	(%)	No.	(%)	No.	(%)
HY	52	46	88.5	6	11.5	2	3.8

HY: Samples from Hainan and Yunnan Provinces.

Table 5
Geographical difference of *Plasmodium vivax* CSP genotypes.

Samples	No.	Positive	PV_{210}		PV_{247}	
		No.	No.	(%)	No.	(%)
Yunnan	32	31	25	78	6	18.8
Hainan	20	19	19	95	0	0

tion from Kevin *et al* (1992) was higher than ours. There had been an earlier small study of *P.vivax* CSP genotyping in China (Victoria *et al,* 1994) of *P.vivax* from the western part of Guangxi Province close to Yunnan Province: of the 6 isolates examined, 3 different sequences were obtained, 3 isolates had identical sequences, and the sequencing data also showed polymorphism of the *P.vivas* CSP gene.

In conclusion, the PCR/DNA probe labeled with biotin is a good method to detect and identify *P.vivax*

infection and genotype. The results indicate that the PV_{247} variant of *P.vivax* exists in China and the patients can be simultaneously infected with both PV_{210} and PV_{247} forms of *P.vivax*. The observation suggests that a single-epitope vaccine based on the predominant PV_{210} form may be ineffective, and also gives information for preparation of vaccines in the future in China. Further studies will be needed because the samples were only collected from limited regions and also the numbers were not large in this experiment.

REFERENCES

Gordon DM, Cosgriff TM, Schneider CI, Wasserman GF. Safety and immunogenicity of a *Plasmodium vivax* sporozoite vaccine. *Am J Trop Med Hyg* 1990; 42: 527-31.

Kevin CK, Arthur EB, Webster HK, *et al.* Circumsporozoite genotyping of global isolates of *Plasmodium vivax* from dried blood specimens. *J Chin Microbiol* 1992; 30: 1863-6.

Kevin CK, Keystone J, Eileen DF, David EL. Global distribution of a variant of the circumsporozoite gene of *Plasmodium vivax. J Infect Dis* 1991; 164: 208-10.

Rosenberg R, Wirtz RA, Lanar DE. Circumsporozoite protein heterogeneity in the human malaria parasite *Plasmodium vivax. Science* 1989; 245: 973-6.

Victoria HM, Tianyi H, Cheng Q. Saul A. Sequence variation in the cirumsporozoite protein gene of *Plasmodium vivax* appears to be regionally biased. *Mol Biochem Parasitol* 1994; 68: 45-52.

Wang SW. Techniques of gene diagnosis. Beijing: Medical Universities of Beijing and Xidhe, 1992.