Indonesian Journal of Biotechnology, June (2000) 441 - 447

Preliminary report on allelic variation of Th2R and Th3R region of the circumsporozoite protein of *Plasmodium falciparum* from Kokap, Yogyakarta Province, Indonesia

Supargiyono[1], Conway D.J.[2], Alloueche A.[2], Widjayanti A.W.[1] and Herdiana E.[1]

1) Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia.
2) Department of Infectious Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom.

Abstract

Allelic variation on the Th2R and Th3R non-repetitive epitope regions of the Circumsporozoit Protein (CSP) is a well known phenomenon among Plasmodium falciparum *population within different regions or geographical situations. However, such information originated from Indonesian plasmodium population is still not available. Studies have been initiated on Indonesian* Plasmodium *populations, aiming to identify the sequence variation of the gene encoding Th2R and Th3R epitope region of the CSP molecules of* P. falciparum *from malaria endemic areas of Indonesia.*

At the initial stage of these studies P. falciparum *infected blood had been collected from patients living in endemic areas of Kokap district, Yogyakarta Province, Indonesia. Genomic DNA were isolated from microscopically positive samples and the gene encoding non-repetitive T cell epitope region of the CSP molecules were amplified by Polymerase Chain Reaction (PCR) using primers designed based on conserved regions of published sequences. The DNA PCR amplification products were purified and sequenced directly using ABI Prism 377 type Automatic DNA Sequencer, and the allelic type of the Th2R and Th3R epitopes were analyzed. The parallel PCR products were also dotted onto replicate nylon membranes and the Th2R and Th3R epitopes were typed using Sequence Specific Oligonucleotide Probes.*

*The results indicated that from 18 samples which were successfully sequenced the Th2R epitope types were all Th2R*05 (100%), whiles on the Th3R locus 72.2% were Th3R*01; and 27.8% were Th3R*04. If these data combined with sequences from other countries, it seems that* P. falciparum *from Indonesian isolates is more closely related to PNG and Thailand rather than to Gambia and Brazil populations.*

Keywords: *Plasmodium falciparum* – Th2R – Th3R – Circum Sporozoite Protein – SSOP

Introduction

Plasmodium falciparum is one of the most pathogenic human malaria agents which in non immune individuals may cause malaria with severe or even complicated clinical manifestations. In Indonesia this parasite is prevalent in most of the endemic areas all over the country. Since drug and insecticide resistant strains of plasmodium and anopheline mosquitoes are widely spread throughout Indonesian islands, an effective and efficient malaria control method needs to be established.

Antigenic polymorphism is a well known phenomenon among plasmodium populations (Conway *et al.*, 1992; Conway, 1997; Creasey *et al.*, 1990) and therefore, characterization of immunogenic epitopes on the surface molecules of the parasite are an important step in the development of protective malaria vaccine. The surface molecule of the sporozoite the Circum Sporozoite Protein (CSP) has been reported as an important vaccine candidate which contain many different immunogenic epitopes both for humoral as well as T cell responses. Allelic variation on these molecules have also been reported among plasmodium populations of different countries and continent, but so far, such information on *P. falciparum* originated from endemic areas of Indonesia which have thousands of islands with different geographical situations and levels of malaria endemicity, still not available.

In order to understand the distribution and the magnitude of allelic variation of the gene encoding the Th2R and Th3R region of the CSP of Indonesian falciparum isolates studies on this has been initiated and a short research visit to Department of Infectious Diseases, London School of Hygiene and Tropical Medicine U.K. is the initial part of it. During this 6 weeks visit genomic DNA have been extracted from *P. falciparum* infected blood collected from malaria patients living in endemic area of Kokap, Yogyakarta province, Indonesia. The gene encoding the non-repetitive, Th2R and Th3R region, on the CSP molecules were amplified by polymerase chain reaction (PCR). The PCR amplification products were sequenced using Automatic DNA Sequencer and the allelic types of the Th3R and Th2R epitopes of each isolates were evaluated. The parallel PCR amplified product of each samples were also typed for Th2R and Th3R using Sequence Specific Oligonucleotide Probes (SSOP) and the results were compared.

Materials and Methods

Blood sample collection and DNA preparation

Samples were collected from clinically malaria patients both by active and passive case detection, in the village of Kokap, Yogyakarta Province, Indonesia from March to July 1999. Thin and thick blood smears were prepared from each patient, Giemsa stained and microscopically examined. Peripheral blood were taken from *P. falciparum* positive patients by venepuncture and collected in Citric Acid Dextrose (ACD) containing eppendorf tubes. DNA from each samples were prepared by Proteinase-K digestion followed by two extractions in phenol:chloroform:isoamyl aclcohol (25:24:1), one extraction in chloroform and precipitated in absolute ethanol. The DNAs pellete were then dissolved in sterile nanopure water and used as template in PCR amplification .

Amplification of the non-repetitive region of CSP gene

The gene encoding non-repetitive, T-cell epitope region of the CSP molecules were amplified by Polymerase Chain Reaction using primer designed according to published sequence. PCR reaction mixture were prepared in 50 µl volume using 0.2 µM primers: 5' CCAAGTGATAAGCACATAGAAC 3' (forward) and 5' CTATTTA CGACATAAACACACTGG 3' (reverse), 1 µM of each dNTP, lx Bioline Taq Polymerase Buffer including 1.5 mM $MgCl_2$ and 1.2 units of Bioline Taq Polymerase. Amplifications were done in 96 well plates employing an initial denaturation cycle of 94°C for 3 minutes, 58°C for 2 minutes and 72°C followed by 32 cycle of 94°C for 1 minutes, 58°C for 2 minutes and 72°C for I minutes. The PCR amplification products were monitored in 1.2% agarose gel to check the quality and size

of the DNA before proceeding to direct sequencing or dot blotting.

Molecular typing of Th2R and Th3R by direct sequencing

The DNA PCR amplification product of each sample was purified using QIAQuick PCR product purification system, and the quality and quantity of the purified products were checked in 1.2% agarose gel. Sequencing reactions were then prepared in PCR tubes containing 4 µL Big Dye mix, 1 µL Bioline 10x buffer, 30-90 ng of DNA, 3 pmol of primer and top up with PCR quality distillated water to 20 µL. After gentle vortexing the tubes were put in PCR machine and amplified using PCR condition: initial denaturation 96°C for 1 minutes followed by 30 cycles of 96°C for 30 seconds, 45°C for 15 seconds and 60°C for 4 minutes. The amplification products were then ethanol precipitated, and the DNA pelletes were dissolved in 6 µl loading buffer. After a brief gentle vortexing 2 µl of each sample was loaded in the sequencing gel and run in ABI 377 type DNA sequencer.

Molecular typing using dot blotting and SSO probing

This dot blotting technique was designed by Alloueche *et al.* (1999, submitted for publication), briefly; PCR amplification products were denatured at 95°C for 5 minutes and 1.5 µl volumes were dotted onto replicate gridded MagnaGraph nylon membranes (Micron Separations Inc., USA) and left to dry at room temperature. DNA was cross-linked by exposure to ultraviolet light (UV) for 15 seconds and membranes were stored at 4°C until further processed.

Probes were designed according to the published sequences of the field and laboratory isolates for both the Th2R and Th3R epitope regions (Jongwutiwes *et al.*, 1994; Zevering *et al.*, 1994; Lalvani *et al.*, 1996). The probes designed were complementary to the single nucleotide polymorphisms described. These polymorphisms defined 15 allelic types for the Th2R epitopes (CSP-Th2R*01 to CSP-Th2R* 15, Table 1), and 13 allelic types for the Th3R epitopes (CSP-Th3R01 * to CSP-Th3R*13, Table 2). Oligonucleotide probes were 15 to 18 bases long (Perkin Elmer, Applied Biosystems, Warington, UK), labeled using a 3' end-digoxigenin labeling kit (Boehringer Mannheim, UK) and were a generous gift of Ali Alloueche (London School of Hygiene and Tropical Medicine, UK). Once labeled, probes remain stabil and were stored at -20°C. Membranes were blocked with blocking solution (4xSSPE, 0.1% laurylsarcosin and 1% skimmed milk powder) for 30 minutes at 37°C and prehybridized in TMAC buffer (Sigma, Poole, UK; 3M TMAC, 50 mM Tris pH 8.0, 0.1% SDS and 2 mM EDTA) at 54°C for 10 to 15 minutes. Replicate membranes were hybridized at 54°C for 90 minutes in separate tubes with 5 ml TMAD buffer containing 2 pmol/ml of each DIG-labeled oligonucleotide. Membranes were washed in 2xSSPE/0.1% SDS twice for 10 minutes at room temperature and twice in TMAC buffer for 10 minutes at 56°C for Th2R probes and at 57°C for Th3R probes. The membrane were then rinse in neutralization buffer (0.1 M pH 7.5, 0.15M NaCl) at room temperature for 5 minutes. Finally they were washed in buffer 1 for 5 minutes and left to dry on 3 MM filter paper before further processed.

Chemiluminescence detection and scoring

Membranes were blocked second time in blocking solution 2 (0.IM Tris pH 7.5, 0.15M NaCl and 1% skimmed milk powder) for 30 minutes at 37°C. One µl of antidigoxygenin alkaline phosphatase antibody (1:5000, Boehringer Mannheim, UK) was added and the membranes were incubated at 37°C for 40 minutes. They were then washed 3 times in neutralization buffer for 10 minutes at room temperature and equilibrated for 5

minutes in buffer 3 (0.1 M Tris pH 9.5, 0.1 M NaCl). Chemiluminescence reaction was performed using CSPD (Boehringer Mannheim, UK) diluted 1:10 in buffer 3. This reaction was carried out at room temperature under a lightproof box for no longer than 5 minutes. Membranes were incubated at 37°C for 15 minutes and exposed to Hyper-ECL X-ray film (Amersham Life Science, UK) for 1 to 2 hours. Autoradiograph were examined for the presence or absence of dots which correspond to the hybridization of specific probes.

Results and Discussion

During the 6 weeks research visit at London School of Hygiene and Tropical Medicine 68 DNA samples were extracted from clinically malaria patients' blood and the gene encoding Th2R and Th3R epitopes on CSP were amplified by PCR in a 96 well plates. The DNA amplified product were electrophoresed in 1.5% agarose gel and visualized under UV light.

The results indicated that not all samples showed expected band which were around 350 base pairs. Some wells showed vary faint bands but some other wells none at all. These could have been due to the different quality of the DNA samples, which may as results of different parasitemia levels or the presence of some enzyme inhibitors. Re-

Table 1. Allelic variation on the Th2R region of CSP of *P. falciparum.*

	Amino acid and nucleotide sequence																
Th2R*01 (7G8)	P 326	S	D	K	H	I	E	Q	Y	L	K	K	I	K	N	S	I 342
	cca	agt	gat	aag	cac	ata	gaa	caa	tat	tta	aag	aaa	ata	aaa	aat	tct	att
Th2R*02 (LE5)	-	-	-	-	-	-	-	-	-	-	-	T -c-	-	Q c--	-	-	L c--
Th2R*03 (3D7)	-	-	-	-	-	-	K a--	E g--	-	-	N --c	-	-	Q c--	-	-	L c--
Th2R*04 (BRA2)	-	-	-	Q c--	-	-	-	K a--	-	-	-	R -g-	-	Q c--	-	-	L c--
Th2R*05 (Wellcome)	-	-	-	-	-	-	-	-	-	-	-	-	-	Q c--	-	-	L c--
Th2R*06 (Gam366 & 399)	-	-	-	Q c--	-	-	-	K a--	-	-	-	T -c-	-	-	-	-	L c--
Th2R*07 ($Gam366_{8,9}$)	-	-	-	Q c--	-	-	-	K a--	-	-	Q c--	-	-	Q c--	-	-	L c--
Th2R*08 Gam406&419)	-	-	-	Q c--	-	-	-	K a--	-	-	Q c--	-	-	-	-	-	L c--
Th2R*09 Gam427)	-	-	-	-	-	-	-	-	-	-	N --c	T -c-	-	Q c--	-	-	L c--
Th2R*10 ($Gam366_{2-7}$)	-	-	-	Q c--	-	-	-	K a--	-	-	Q c--	-	-	R cg-	-	-	L c--
Th2R*11 (Thai)	-	-	-	Q c--	-	-	-	K a--	-	-	-	-	-	Q c--	Y t--	-	L c--
Th2R*12 (Thai II)	-	-	-	-	-	-	T ac-	E g--	-	-	-	-	-	Q c--	-	-	L c--
Th2R*13 (Thai IV)	-	-	-	-	-	-	-	-	-	-	-	-	-	Q c--	Y t--	-	L c--
Th2R*14 (Thai VII)	-	-	-	-	-	-	K a--	E g--	-	-	T -c-	-	-	Q c--	-	-	-
Th2R*15 (Thai V)	-	-	-	-	-	-	-	E g--	-	-	-	-	-	Q c--	-	-	L c--

Table 2. Allelic variation on the Th3R region of CSP of *P. falciparum*

	Amino acid and nucleotide sequence											
Th3R*01 (7G8)	N 367	K	P	K	D	E	L	D	Y	E	N	D 378
	aat	aaa	cct	aaa	gac	gaa	tta	gat	tat	gaa	aat	gat
Th3R*02 (LE5)	-	-	-	-	-	Q c--	-	-	-	A -a-	-	-
Th3R*03 (3D7)	-	-	-	-	-	-	-	-	-	A -a-	-	-
Th3R*04 (Well, FCR3)	D g--	-	-	-	-	Q c--	-	-	-	-	-	-
Th3R*05 (Gam336b, 406b,419)	-	-	-	-	-	Q c--	-	N a--	-	-	-	-
Th3R*06 (Gam406c)	-	-	-	R cg-	-	-	-	-	-	A -a-	-	-
Th3R*07 ($Gam406_d$)	-	-	-	-	-	-	-	-	-	A -a-	D g--	-
Th3R*08 AE7, PNG, Thai VIII	-	-	-	-	-	Q c--	-	-	-	-	-	-
Th3R*09 HB3, D10, Bra1	G gg-	-	S t--	-	-	-	-	-	-	-	-	-
Th3R*10 (Bra S34, ThaiV)	-	-	-	-	-	-	-	N a--	-	-	-	-
Th3R*11 (PNG3)	D g--	-	-	-	-	Q c--	-	-	C -g-	-	S -g-	-
Th3R*12 (Thai VI)	-	-	-	-	-	-	-	-	-	-	D g--	-
Th3R*13 (Thai VII)	G gg-	-	-	-	-	-	-	E --a	-	-	-	-

amplification were then also done to all negative samples using the same primer and first round PCR product as the template. Using this way some more positive PCR amplification products were achieved. Among all, good quality PCR amplified products were purified using QIA QUICK PCR Product Purification System (Promega) and sequenced using ABI 377 type DNA Sequencer.

The 18 successful sequences were edited and aligned carefully with P. *falciparum* 3D7 sequence to make sure that the sequence are right. The CSP sequences were then analyzed for Th2R and Th3R types using published sequence as shown in Table 1 and Table 2 (Lockyer *et al.*, 1989; Shi *et al.*, 1992; Doolan *et al.*, 1992; Jongwutiwes *et al.*, 1994). Analysis of the Th2R and Th3R type of Kokap samples are shown in Table 3. From 18 samples which are successfully sequenced, all (100%) showed a Th2R*5 type. No other type of Th2R were yet observed within these 18 samples. This Th2R*5 type are also predominant in PNG (92%) and Thailand (61%) falciparum populations but very rare in the Gambia and Brazil. While on the Th3R locus it was calculated that

Table 3. CSP-Th2R and Th3R allel frequencies of *Plasmodium falciparum* Kokap (Indonesia) Isolates and compared to published data

Isolate origin No. of sample	Kokap 18	Thailand** 23	PNG* 38	Gambia* 16	Brazil* 41
CSP-Th2R Types					
01	-	-	0.026	-	0.732
02	-	-	0.053	0.063	-
03	-	-	-	0.063	-
04	-	-	-	-	0.171
05	1.000	0.609	0.921	-	0.098
06	-	-	-	0.438	-
07	-	-	-	0.125	-
08	-	-	-	0.125	-
09	-	-	-	0.063	-
10	-	-	-	0.125	-
11	-	-	-	-	-
12	-	0.130	-	-	-
13	-	0.174	-	-	-
14	-	0.043	-	-	-
15	-	0.043	-	-	-
CSP-Th3R Types					
01	0.722	0.522	0.132	0.063	0.902
02	-	-	-	0.375	-
03	-	-	-	0.250	-
04	0.278	0.174	0.737	-	-
05	-	-	-	0.188	-
06	-	-	-	0.063	-
07	-	-	-	0.063	-
08	-	0.043	0.053	-	-
09	-	0.130	0.053	-	0.098
10	-	0.043	-	-	-
11	-	-	0.026	-	-
12	-	0.043	-	-	-
13	-	0.043	-	-	-

* Shi *et al.*, 1992.
** Jongwutiwes *et al.*, 1994.

72.2% of Kokap isolates were Th3R*1 and 27.8% were Th3R*4 type. These figures also similar with Thailand and at a lesser degree to PNG population. Since the sample only 18, other type of Th2R or Th3R are still expected to come with the increase of sample to be analyzed. So far no novel type were found from these 18 Indonesian isolates. From these preliminary data probably can be speculated that *P. falciparum* in Kokap or

probably in all Java are originated from Thailand and slightly contaminated with population from PNG.

The results of Th2R and Th3R typing using dot blotting method demonstrated a very (100%) identical results (data not shown), indicated that this techniques are as accurate as sequencing method, and therefore, can be used as an alternative whenever the sequencing apparatus is not available. Further studies elaborating more samples and from other Indonesian endemic areas still in progress and sequence variation analysis of other gene locus need to be done to elucidate in more depth on the allelic variation and frequencies of the T cell epitopes on the surface molecules of *P. falciparum*.

Summary

A preliminary studies have been initiated to analyzed the sequence variation of the gene encoding Th2R and Th3R epitopes on the CSP of *Plasmodium falciparum* isolated from Kokap, Yogyakarta using direct sequencing technique and compared the result with sequence specific oligonucleotide probing. Eighteen sequences have been analyzed and typed for the Th2R and Th3R epitopes. Within the Th2R region, all (100%) were Th2R*5 type, while on the Th3R locus 72.2% were Th3R*I type and 27.8% were Th3R*4 type. Other type of Th2R and Th3R epitopes were expected to arise with increase of samples. These figures indicated that *Plasmodium falciparum* of Kokap isolates is more closely related to PNG and Thailand rather than to Brazil and Gambia.

References

Alloueche, A., Silveira, H., Conway, D.J., Bojang, K., Doherty, T., Cohen, J., Pinder M. and Greenwood, B.M. (1999) High-throughout sequence typing of T-cell epitope polymorphisms in *Plasmodium falciparum* circumsporozoit protein. (Submitted for publication).

Conway, D.J. (1997) Natural selection on polymorphic malaria antigen and the search for a vaccine. *Parasitol. Today* 13: 26-30.

Conway, D.J., Greenwood, B.M. and McBride, J.S. (1992) Longitudinal study of *Plasmodium falciparum* polymorphic antigen in malaria endemic population. *Infect. Immun.* 60: 1122-27.

Creasey, A.M., Fenton, B., Walker, A., Thaithong, S., Oliviera, S., Mutambu, S. and Walliker, D. (1990) Genetic diversity of Plasmodium falciparum shows geographical variation. *Am. J. Trop. Med. Hyg.* 42: 403-413.

Doolan, D.L., Saul, A.L. and Good, M.L. (1992) Geographically restricted heterogeneity of the *Plasmodium falciparum* CSP: Relevance for vaccine development. *Infect. Immun.* 60: 675-82.

Jongwutiwes, S., Tanabe, K., Hughes, M.K., Kanbara, H. and Hughes, A.L. (1994) Allelic variation in the circumsporozoite protein of *Plasmodium falciparum* from Thai field isolates. *Am. J. Trop. Med. Hyg.* 51 (5): 659-68.

Lalvani, A., Hurt, N., Aidoo, M., Kibatala, P., Tanner, M. and Hill, A.V.S. (1996) Cytotoxic T lymphocytes to *Plasmodium falciparum* epitopes in an area of intense and perennial transmission in Tanzania. *Eur. J. Immun.* 26 (4): 773-9.

Lockyer, M., Marsh, K. and Newbold, C.I. (1989) Wild isolates of *Plasmodium falciparum* show extensive polymorphisms in T cell epitopes of the circumsporozoite protein. *Mol. Biochem. Parasitol.* 37: 275-80.

Shi, Y.P., Alpers, M.P., Povoa, M.M. and Lal, A.A. (1992) Diversity in the immunodominant determinants of the circumsporozoite protein of *Plasmodium falciparum* parasite from malaria endemic region of Papua New Guinea and Brazil. *Am. J. Trop. Med. Hyg.* 47 (6): 844-51.

Zevering, Y., Khamboonruang, C. and Good, M.F. (1994) Effect of polymorphism of sporozoite antigens on T-cell activation. *Res. Immunol.* 145 (6): 469-76.