GENOTYPING OF THE DUFFY BLOOD GROUPS BY PCR-RFLP METHOD IN BACAN ISLAND, MALUKU

Betty Roosihermiatie*

ABSTRAK

*Studi ini dilakukan untuk menentukan genotip golongan darah Duffy di Pulau Bacan, Maluku dengan mempergunakan metode PCR-RFLP (*Polymerase Chain Reaction-Restriction Fragment Length Polymorphism*). Metode ini dapat menentukan genotip sampel darah yang dibawa dari Indonesia secara akurat. Di samping itu manfaat lain penentuan genotip golongan darah Duffy dengan metode PCR-RFLP bila dibandingkan dengan metode standard antigen-antisera test adalah: dapat mempergunakan sediaan darah beku, dapat menentukan genotip banyak sampel pada sekali pemeriksaan, dan dapat melakukan pemeriksaan ulang bila diperlukan. Dari 146 sampel yang diambil, masing-masing terdiri dari 67 kasus bukan malaria, 13 kasus malaria vivax tidak disertai gejala demam, 50 kasus malaria vivax disertai gejala demam, dan 16 kasus malaria falciparum disertai gejala demam. Hasil penentuan genotip dengan metode PCR-RFLP, didapatkan frekuensi golongan darah Duffy di antara subyek tersebut sebagai berikut : Fy(a+b-) sebanyak 89,7%; Fy(a+b+), 9,6%; Fy(a-b+), 0,0%; dan Fy(a-b-), 0,7%. Subyek yang berasal dari Pulau Bacan asli lebih banyak yang mempunyai golongan Fyb+ dibandingkan dengan golongan Fyb-. Subyek yang berasal dari Pulau Bacan asli memiliki korelasi yang tinggi dengan golongan darah Duffy, Fyb+, menurut analisis koefisien korelasi Pearson ($p<0,05$). Kasus Duffy-negative, Fy(a-b-), yang dideteksi terdapat mutasi GATA-1 promoter (-365T→C), ditemukan pada laki-laki berumur 40 tahun yang berasal dari Pulau Bacan dengan campuran keturunan Arab. Kemungkinan besar dia mewarisi gen Duffy-negative dari Afrika melalui Arabia. Satu-satunya kasus Duffy-negative dari studi ini mengalami malaria vivax disertai gejala demam.*

Keywords : *Genotip, Golongan darah Duffy, pulau Bacan.*
Running title : Genotyping of the Duffy blood groups in Bacan island, Maluku

INTRODUCTION

The Duffy blood group system is a group of antigenic determinants on the red cell membrane which consists of two principal antigens, Fy^a and Fy^b, produced by the Fy^a and Fy^b alleles. Antisera, anti-Fy^a and anti-Fy^b define four major phenotypes, Fy(a+b-), Fy(a+b+), Fy(a-b+), and Fy(a-b-)[1]. Neither antiserum agglutinates Fy(a-b-) erythrocytes, however, Duffy-negative is extremely rare in racial groups other than Blacks[2].

According to Furuhata, the gene frequencies of Fy^a and Fy^b among Japanese were calculated to be 0.8966 and 0.1034, respectively. Each phenotype frequency is as follows: Fy(a+b), 80.39%; Fy(a+b+), 18.54%; Fy(a-b+), 1.07%; and Fy(a-b-), 0.00%[3]. Duffy frequencies in Whites

* Pusat Penelitian dan Pengembangan Pelayanan dan Teknologi Kesehatan Surabaya.

reported from Race et al., are the following: Fy(a+b-), 18.23%; Fy(a+b+), 47.35%; Fy(a-b+), 33.02%; and Fy(a-b-), 0.00%[2]. On the other hand, Duffy frequencies in Blacks are: Fy(a+b-), 9.82%; Fy(a+b+), 2.68%; Fy(a-b+), 19.87%; and Fy(a-b), 67.63%[2]. Such data show that Duffy phenotype frequencies show racial differences.

The Duffy blood groups antigen has been characterized by its role as a receptor for malarial parasites and as a promiscuous receptor for the chemokine superfamily[4]. The Duffy blood groups associated with glycoprotein D (gpFy) have recently been cloned[5]. Furthermore, Iwamoto et al reported that the Duffy blood groups alloantigen system is associated with a polymorphism at the 44-amino acid residue[6,7], and genotyping of the Duffy blood group antigen has become possible using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method.

Previous studies reported that Fy(a-b-) erythrocytes cannot be infected with the malaria parasites, *Plasmodium knowlesi* and *P. vivax*[8,9]. Duffy-negative erythrocytes are refractory to in vitro invasion by *P. knowlesi,* a simian malaria that can infect humans and invade Duffy-positive erythrocytes[8]. Furthermore, a retrospective study involving blood typing of 11 Black and 6 White volunteers, who had been exposed to the bites of *P. vivax*-infected mosquitoes, revealed that the five who had been resistant to infection were all Duffy-negative; while the others who had contracted malaria were Duffy-positive[9]. Support for this explanation of the refractoriness of vivax malaria has been provided by studies in Ethiopia, East Africa and in Honduras, Central America[10,11]. *Plasmodium vivax* is not endemic in West Africa, where the majority of people are Duffy-negative, and is rare in other regions of Africa. According to Marsh, this is usually interpreted as malaria selecting for humans who are negative. However, *P. vivax* causes little mortality[12].

In Southeast Asia, data on Duffy blood groups distribution using PCR-RFLP in malaria endemic area is not available. Hence, we carried out a study to genotype Duffy blood groups from frozen blood samples collected in Bacan island, a mesoendemic area of vivax malaria[13], using the new method of PCR-RFLP based on Iwamoto et al[6,7].

MATERIALS AND METHODS

1. Pilot area

(1) Location of Bacan island

The research was carried out in Bacan island, Maluku Province from October to November, 1998. Bacan island is one of three main islands in the Bacan subdistrict located 127°-125° E and 0°15'-0° S (Figure 1). Almost all villages are located on the coast at 0-500 m above sea level. There is much stagnant water, many ditches and a big bush swamp covers one third of the island, all of which are suitable malaria vector breeding locations.

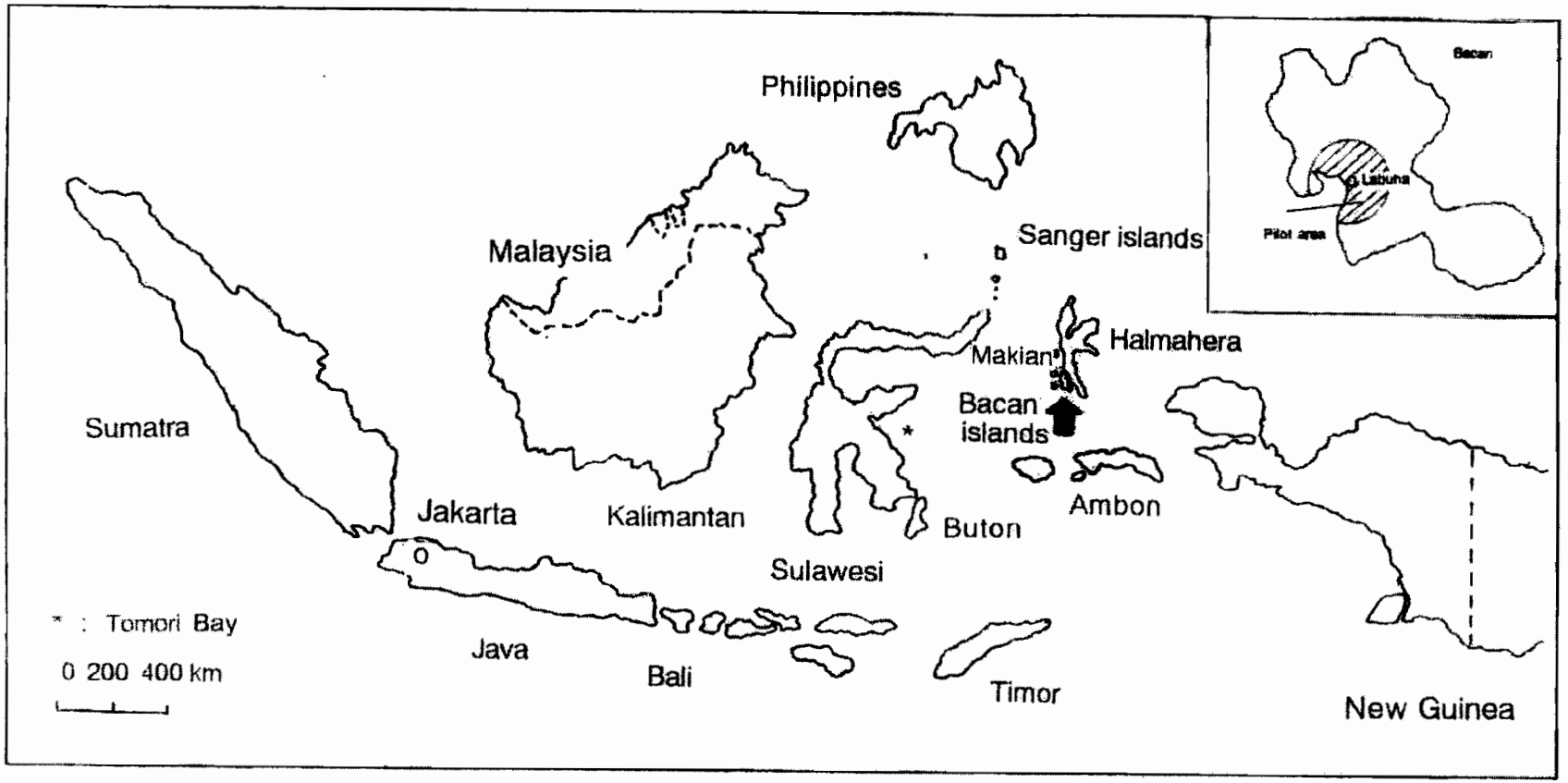

Figure 1.

Bacan is one of the three main islands in the Bacan subdistrict. The island is located adjacent to Halmahera island, North Maluku, in between Sulawesi and New Guinea islands, South of the Philippines.
Samples were taken from 11 villages surrounding Labuha, the capital of the Bacan subdistrict. There were people of 4 origins other than Bacan origin in the pilot area : Makian origin from Makian island North of Bacan island, Sanger origin from Sanger islands North of Sulawesi, Tomori origin from areas nearby Tomori Bay in Central Sulawesi Province, and Buton origin from Buton island in the Southeast of Sulawesi island.

(2) Situation of malaria infection

Bacan is categorized as a priority area for the malaria control program in areas outside of Java-Bali[14]. The malaria control activities consist of insecticide spraying, malariometric survey, case detection and malaria treatment in a Health Centre. Insecticide spraying has routinely been done in the 9 villages under study, however was not scheduled in 1997. Two villages declined the spraying since DDT was used. At present, the malaria control program uses lambda-cyhalothrin for the spraying. In spite of the malaria control program, the Health Center's data showed that clinical malaria was the leading cause of morbidity in 1993 and 1997, and was second in the years between. The Annual Parasite Incidence (API) based on passive case detection was about 20 per 1000 population[13]. The predominant type of confirmed cases is *P. vivax. Plasmodium falciparum* composed 10%-20% of the infections, while mixed infections were rare[13-15]. The major malaria vector in Maluku is *Anopheles farauti*[14].

2. Subjects

Subjects were selected from all 11 villages, the work area of the Health Center surrounding Labuha with the coordination of head of the Health Center. Inhabitants of the 11 villages were informed the purpose of the study and were asked to voluntarily participate. They were people who either had experienced febrile illness during the previous week when a blood smear was made, or those who had not experienced febrile illness during the proceeding month. The examinations were done at the village head office. Temperature was recorded at the time of blood smear was made. Febrile cases received antipyretic treatment while the nonfebrile cases were given symptomatic treatment or vitamins for healthy persons.

Each village was visited by the team three times over three days. The first day was for blood smear collection, examination, and ABO blood group tests. Eligible samples were asked to come again on the second or third day for a physical examination and interview. Confirmed febrile and nonfebrile malaria cases were given radical treatment.

A physical examination and interview were done by the investigator. For children aged 15 years or younger, the questionnaire were directed to their mothers. Place of origin was determined from the ethnicity of the samples by interview. Finally, villages where people of different origins lived were grouped together.

3. Smears and ABO blood group examinations

Thick and thin smears were stained with Giemsa. Blood smears were examined by the Health Center's two microscopists using x500 oil immersion. Thick smears were used to determine malaria cases, while thin smears were done to diagnose malaria types. One hundred fields of each thick film were examined before being declared negative. Parasitemia were graded to determine the severity of infection. Parasitemia 1+ and 2+ were categorized if one to ten and eleven to a hundred parasites were found in 100 fields of the thick films, respectively. While Parasitemia 3+ and 4+ were categorized if one to ten and eleven to a hundred parasites were found in every field of thick films, respectively[12]. The smears were reexamined and validated by one of the

microscopists who had been trained at the Maluku Provincial Health Office.

ABO blood groups were examined using a standard agglutination technique. In 19 (13%) samples, the ABO blood groups were determined using elution test.

4. Genotyping of Duffy blood groups

Blood samples were collected from 146 persons who returned at four-week intervals after the blood smears. Informed consent for participation in the study was provided by the subjects themselves or their legal guardians. There were 67 non-malaria, 13 nonfebrile vivax, 50 febrile vivax, and 16 febrile falciparum cases.

Genomic DNA was extracted from 200µl of the frozen blood using a DNA extractor QIAamp DNA mini kit (Qiagen GmbH, Germany) following the manufacturer's instructions. For typing of the Duffy antigen, genomic DNA samples were amplified by PCR in an automatic thermal cycler (Techne) using oligonucleotides gpFy1 (sense, 5'-GGCTTCCCCAGGACTGTTCCTG-3') and gpFy735 (antisense, 5'-GTGACAGGCAGTGTCAGTAG-3')[6]. Each Amplification reaction contained about 30-40 ng of genomic DNA, 2pmol of each promer, 2mmol/L $MgCl_2$, 0.16 mmol/L of each deoxynucleotide, and 2.5 units of Goldtag DNA polymerase (Perkin Elmer) with a PCR buffer supplied from Perkin Elmer in a final volume of 50 µl. The PCR reaction had an initial melting temperature of 50°C (5 mins) followed by 35 cycles denaturating (95°, 1 min), annealing (58°C, 1 min), extension (72°C, 3 mins) and a final extension (70°C, 10 mins). The amplified product was digested by a *BanI* restriction enzyme overnight. Each digestion product was loaded on a 5% polyacrylamide gel, electrophoresed at 150 volts for 2 hours, stained with ethidium bromide, and visualized with UV light. Duffy-negative, Fy(a-b-), individuals yielded the same fragments as Fy(a-b+) individuals.

To determine whether there was a mutation in Duffy-negative, Fy(a-b-), individuals, the same PCR prochedure with oligonucleotides gpFy-535 (sense,5'-TTCCTGAGTGTAGTCCCAAC-3') and gpFy-279 (antisense,5'-CTGTGCAGACAGTTCCCCAT-3') and a restriction enzyme *EcoT141* were used[7]. Each digestion product was loaded onto a 12% polyacrylamide gel, electrophoresed at 100 volts for 4 hours, stained with ethidium bromide, and visualized with UV light.

5. Statistical analysis

The characteristic differences between different Duffy blood group were analyzed with X^2-test and Pearson's correlation coefficient using the Statistical Package for Social Sciences (SPSS) Software.

RESULTS

As shown in Figure 2, *BanI* cleaved two common cutting sites, while the specific site from Fy(a+b-) individuals resulted in four fragments of 306-, 213-, 109-, and 107-bp, respectively. The *BanI* digestion product from Fy(a-b+) individuals resulted in three fragments of 519-, 109-, 107-bp. Fy(a+b+) individuals yielded the combined restriction fragments. Duffy negative, Fy(a-b-), individuals yielded the same as Fy(a-b+) individuals.

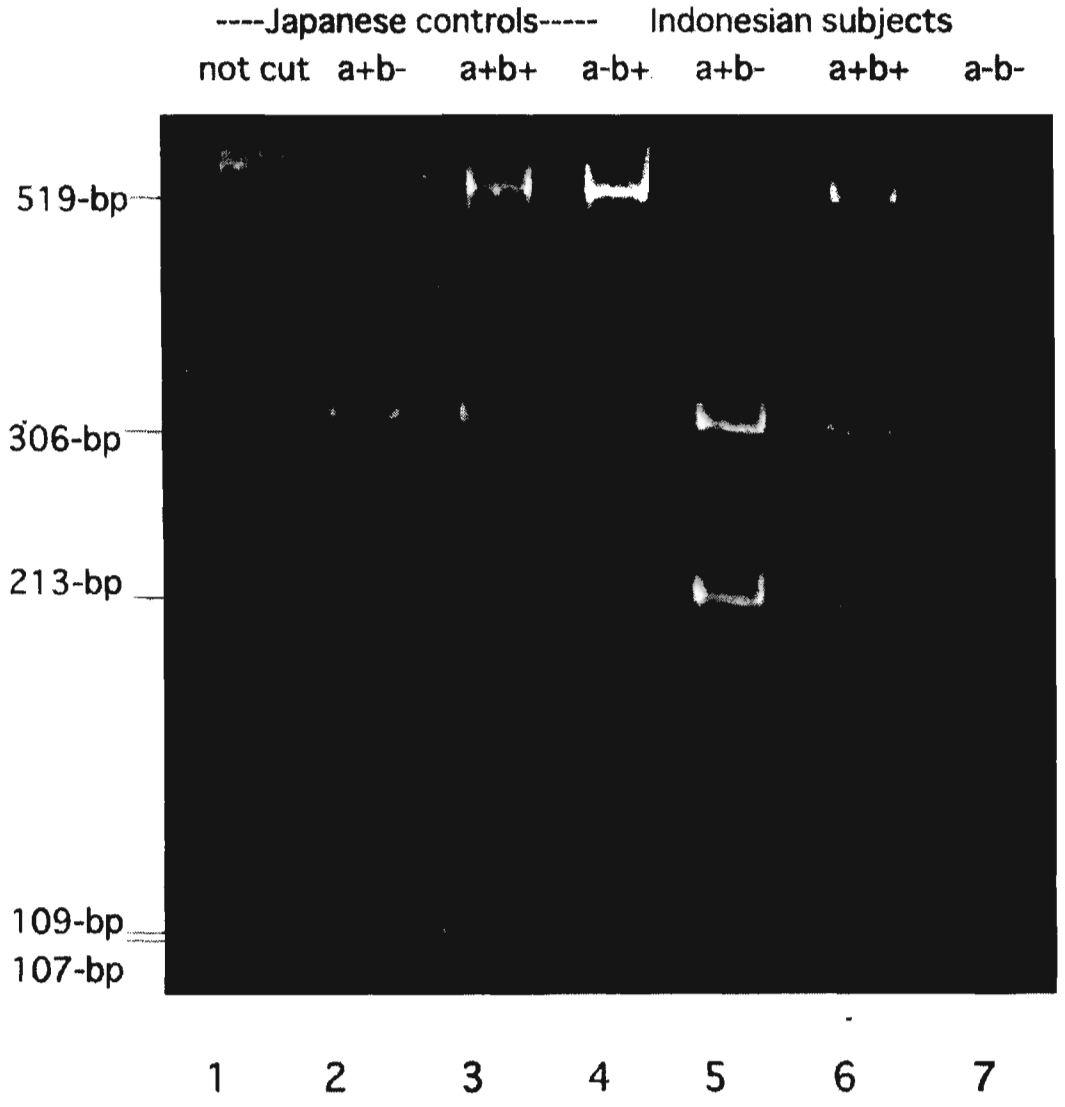

Figure 2.

The first PCR using oligonucleotides gpFy1 and gpFy735 with a restriction enzyme *BanI*was done to type the Duffy antigen. The electrophoresed gel shows lane1: not cut, lane2-4: the Duffy Fy(a+b-), Fy(a+b+), Fy(a-b+) among Japanese controls, respectively and lane 5-7: the Duffy Fy(a+b-), Fy(a+b+), Fy(a-b+) among Indonesia samples. The Duffy-negative, Fy(a-b-), individual yielded the same fragments as Duffy Fy(a-b+) individual.

Duffy negative, Fy(a-b-), individuals showed the GATA promoter mutation at a base nucleotide (-365T→C) of the Fy allele cleaved 77-bp fragment. The *EcoT141* digestion product from Fy(a-b-), individuals resulting in fourth restriction fragments site and yielded 108-, 65-, 51-, 21-bp fragments (Figure 3).

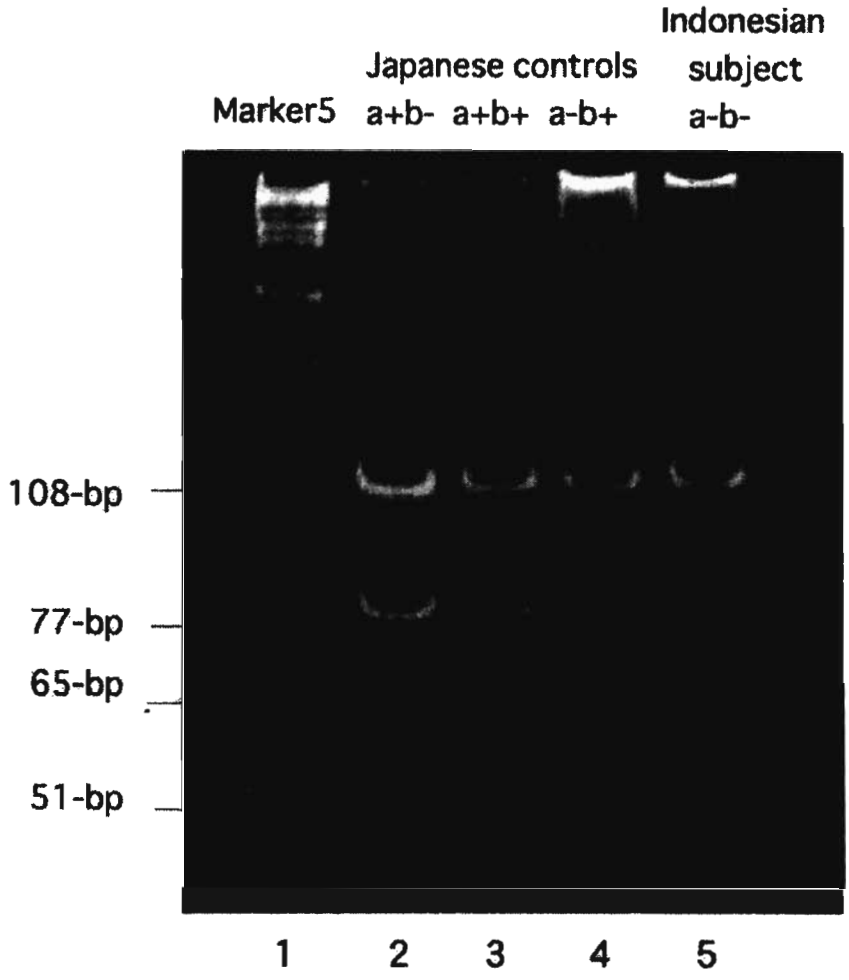

Figure 3.

The second PCR using oligonucleotides gpFy-535 and gpFy-279 with a restriction enzyme *EcoT141* to determine if there was a GATA-1 promoter mutation in Duffy-negative, Fy(a-b-), individual. The electrophoresed gel shows lane1: marker DNA, lane2-4: the Duffy Fy(a+b-), Fy(a+b+), Fy(a-b+) among Japanese controls, and lane 5: the Duffy-negative, Fy(a-b-), in an Indonesian sample shows a cleave at 77-bp confirmed to be a mutation.

Table 1 shows the majority 89.7% (131/146) of subjects were Duffy Fy(a+b+). Duffy Fy(a-b+) accounted for 9.6% (14/146) persons. Only one (0.7%) person was found to be Duffy-negative, Fy(a-b-), but no one were found to be Duffy Fy(a-b+).

People of Bacan origin made up the highest proportion of Duffy Fy(a+b+) than people of other origins from outside of Bacan island. The proportion of Duffy Fy(a+b-) among those of Bacan origin was significantly higher than those of sanger origin (X^2-test=5.45, $p<0.05$). The only Duffy-negative, Fy(a-b-), case was detected in 40-year-old male of Bacan and Arab origin.

Table 1. Distribution of Duffy blood group by origins.

Origins	No.	Fy(a+b+)	Fy(a-b+)	Fy(a+b-)	Fy(a-b-)
Bacan	28	21 (75.0%)	6 (21.4%)	0 (0%)	1 (0.7%)
Tomori	15	13 (86.6%)	2 (13.4%)	0 (0%)	0 (0%)
Makian	13	12 (92.3%)	1 (7.7%)	0 (0%)	0 (0%)
Buton	45	41 (91.3%)	4 (8.9%)	0 (0%)	0 (0%)
Sanger	45	44 (97.7%)	1 (2.3%)	0 (0%)	0 (0%)
Total	146	131 (89.7%)	14 (9.6%)	0 (0%)	1 (0%)

Table 2 shows the characteristics of samples. A slightly more than a half, 52.8% (77/146) were females. The majority, 88 (60.3%) of the subjects were blood group O. Only 2 (12.5%) febrile falciparum cases had severe malaria with a degree of parasitemia 3+ and accompanied by high fever and shivering symptoms. Most febrile vivax or febrile falciparum cases had mild malaria with a degree of parasitemia 1+. And all non febrile vivax cases had a degree of parasitemia 1+.

Table 2. The characteristics of the samples (n=14).

Variables	No.	%
Age:	146	(100%)
Mean ± SD (years)	21.55±21.54	
Sex:		
males*	69	(47.2%)
females	77	(52.8%)
ABO blood groups:		
O*	88	(60.3%)
A	25	(17.1%)
B	28	(19.2%)
AB	5	(3.4%)
Malaria types:		
non malaria	67	(45.9%)
non febrile vivax	13	(8.9%)
febrile vivax*	50	(34.2%)
febrile falciparum	16	(11.0%)
Degree of parasitemia:		
-	67	(45.9%)
Plasmodium vivax		
+*	52	(35.6%)
++	11	(7.5%)
Plasmodium falciparum		
+*	10	(6.8%)
++	4	(2.7%)
+++	2	(1.4%)

* : indicates the Duffy negative, Fy(a-b-), individual.

A correlation matrix among Duffy blood groups (Fyb+ or Fyb-) and the characteristics of the samples is presented in Table 3. The proportion of people of Bacan origin was higher among the Fyb+ group than the Fyb- group. Bacan origin had a significantly higher correlation with the Fyb+ group (r=.196, p<0.05). Duffy blood group had low correlation with febrile vivax, as well as with febrile falciparum (r>.1), but the correlations were not statistically significant (p>0.05).

Table 3. The characteristics differences between the Fyb+ and Fyb- groups.

Variables	Fyb+ n=14	Fyb- n=132	r (P)
Mean ± SD (years)	20.57±16.36	21.66±22.07	.015 (.858)
Sex:			
males/females	6 / 8	61 / 71	.064 (.439)
ABO blood groups:			
blood group O / non O	10 / 4	78 / 54	.099 (.235)
Malaria types:			
Fever / non fever	9 / 5	57 / 75	.125 (.133)
Febrile vivax and non-malaria (n=117):			
febrile vivax / non-malaria	6 / 4	44 / 63	.107 (.252)
Febrile falciparum and non-malaria (n=83):			
febrile falciparum / non-malaria	3 / 4	13 / 63	.181 (.101)
Origin:			
Bacan / non-Bacan	6 / 8	22 / 110	.196 (.018)

R : correlation, P : p-value of Pearson's correlation coefficient, * : p-value < 0.05.

DISCUSSION

The Duffy antigens are located on a red cell membrane glycoprotein, encoded by a single exon of the Duffy gene on chromosome 1[5]. We used two PCR-RFLP methods for genotyping of Duffy blood group system using two pairs of primers to determine a base difference at nucleotides 306G[6] and -365C mutation according to Iwamoto et al[7]. We firstly checked these methods with Japanese samples serotyped with Fy^a and Fy^b antigens and confirmed the accuracy of the methods.

Genotyping of the Duffy blood groups can be done if the standard agglutinin test is not available in an area. The standard agglutinin test is the same procedure as for examining the ABO blood groups. We could also accurately genotype samples transported from across the Indonesia archipelago. The PCR-RFLP method has many advantages, it can use frozen blood samples, it can examine many samples at once, and the examination can be repeated as necessary. But the PCR-RFLP method requires trained laboratory staffs, excellent laboratory facility, and reagents for PCR examination, which are relatively expensive. On the other hand, the standard agglutinin test or antigen-antisera test which is a simple test needs simple laboratory type and antigen-antisera reagens which are cheap.

The major Duffy Fy(a+b-) was similar to that found in previous studies in

Japan, China, Malaysia, Thailand and Bali, Indonesia detected by an antigen-antisera test[3,16-19]. In Papua New Guinea, all study subjects were found to have the Fy^a antigen using a PCR method[20]. In the present study, overall 89.7% Fy(a+b+) cases were found, this was higher than among Japanese (80.39%)[3] and Mongolians in China (85.3%)[16], but lower than Proto-Senoi Malays in Malaysia (93.0%)[17], Thai northern ethnic groups in Thailand (93.3%)[18], Balinese in Indonesia (98.4%)[19] and Papua New Guineans (100%)[20].

The proportion of of people of Bacan origin was higher among the Fyb-group than the Fyb+ group. Variation in Duffy blood groups among the subjects is likely due to the mixture of people of Polynesian and Melanesian origins. People in eastern Indonesia are a mixture of Polynesian and Melanesian. The significantly higher Fy(a-b+) in those of Bacan origin than those of Sanger origin would possibly suggest there was more Polynesian than Melanesian blood among those of Bacan origin. The higher number of Fy(a+b+) cases found in those of Bacan origin was similar to Japanese[3]. On the other hand, those of Sanger origin would possibly suggest more Melanesian than Polynesian blood similar to the higher number of Fy(a+b-) cases found among Papua New Guineans[20]. Dialect sources indicated those of Bacan origin were from central Austronesia, while those of other origins were from western Austronesia [21].

The only Duffy negative case, Fy(a-b-), was found among the subjects in Bacan island. The frequency of Duffy-negative people is high among Blacks[2]. The frequencies of Fy(a-b-) were 66-79.5% among 4 tribes in Central Africa Republic[22]. Iwamoto et al have identified a novel first exon of the Duffy gene and two inverse GATA motive, and one base substitution (-365T→C) was found in the proximal GATA motive from three Blacks with Fy(a-b-)[7]. The serologically defined Duffy-negative, Fy(a-b-), individuals in Thai northern ethnic groups totalled 8 persons who expressed $Fy^{a(weak)}$ antigens[18]. The Duffy-negative case was found in a 40-year old male of mixed Bacan Arab origins. He most probably inherited the Duffy-negative gene from an African via Arabia[23].

Black, Duffy-negative, Fy(a-b-), people lack Fy^a and Fy^b antigen because of the absence of the Duffy glycoprotein from erythrocytes but it can be detected on certain endothelial cells[4]. This Duffy glycoprotein known as the Duffy antigen receptor for chemokines (DARC), acts as a receptor for several pro-inflamatory cytokines. Besides being a receptor for chemokines, the Duffy glycoprotein is a receptor from *P. vivax* merozoites. Red cells Duffy-negative, Fy(a-b-), individual lacking Duffy glycoprotein, therefore, are resistant to invasion by *P. vivax* merozoites.

The only Fy(a-b-) case among the subjects in Bacan island, experienced febrile vivax. Although those infected with *P. vivax* by mosquito bites or Blacks who contracted vivax malaria in Vietnam were all Duffy-positive[24], Duffy-negatives could have *P. vivax* infection in areas where the majority of people were Duffy-negative, as in Ethiopia[11]. Furthermore, a survey on blood groups and malaria among the Blacks, Kuna indians, Katio indians and mixed population in Colombia showed that 8.9% of 146 *P. vivax* cases and 33.7% of 151 *P. falciparum* cases in the mixed population were Duffy-negative. While Duffy-negative were found to be

completely uninfected by *P. vivax* in the Blacks, Kuna indians and Katio indians[25].

Duffy-negative persons can be infected by *P. vivax*. Whether there is another receptor pathway[26] or if Duffy-negative persons could still express the Duffy antigen, post capillary venule endothelial cells in Duffy-negative Blacks were found to have the Duffy antigen receptor for chemokines (DARC). What chemically and genetically expresses the Duffy antigen receptor for chemokines (DARC) and what actually occurs in erythrocytes needs to be studied further.

CONCLUSION

1. We could accurately genotype the samples transported from Bacan island, East Indonesia using two pairs of primers to determine a base difference at nucleotide 306G and a -365C mutation.
2. The PCR-RFLP method has advantages. It could use frozen blood samples, and examine many samples at once, moreover this examination can be repeated as necessary.
3. As a result of genotyping by PCR-RFLP, the frequencies of the Duffy genotype among the subject was as follows: Fy(a+b+) was 89,7%; Fy(a-b+), 9,6%; Fy(a+b-), 0,0%; and Fy(a-b-), 0,7%.
4. The proportion of people of Bacan origin was higher in the Fyb+ group than the Fyb- group. Bacan origin had a high correlation with the Duffy blood groups ($p<0.05$).
5. The only Fy(a-b-) subject from our study experienced febrile vivax malaria.

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