P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : *https://tci-thaijo.org/index.php/SciTechAsia* Science & Technology Asia
Vol.26 No.1 January - March 2021 Page: [180-188]
Original research article

Detection of Phenotype and Genotype of Duffy Blood Group in Thai Blood Donors

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Received 2 September 2019; Received in revised form 3 March 2020
Accepted 1 June 2020; Available online 16 March 2021

ABSTRACT

The Duffy blood group system is important in many branches of medicine, especially blood transfusion and malariology. Serological methods are standard, and can be used to detect the phenotypes of blood groups. In parallel, use of polymerase chain reaction-sequence specific primer (PCR-SSP), a molecular method, is done to detect blood group genotype in cases where phenotyping detection cannot be done. Recently, the frequency of use of Duffy blood group and detection by PCR-SSP in Thailand were rarely employed. To compare the reliability of PCR-SSP with serological methods, the prevalence of different Duffy blood groups was analyzed. Two hundred EDTA blood samples were collected from Thai blood donors (Tak and Yala, Thailand). The frequency of Duffy phenotypes were 78.5% Fy(a+b−), 19.5% Fy(a+b+), and 2.0% Fy(a−b+). The frequency of Duffy genotypes were 76.0% *FY*A*/*FY*A*, 22.0% *FY*A*/*FY*B*, and 2.0% *FY*B*/*FY*B*. These results showed perfect agreement of the 2 methods ($\kappa = 0.931$). These results indicated that PCR-SSPs can be used as alternative method to analyze Duffy blood group, *i.e.*, for screening and selecting appropriate blood for patients.

Keywords: Duffy blood group; Duffy antigen receptor for chemokines; Polymerase chain reaction-sequence specific primer; Serological method

1. Introduction

The blood group systems are classified by the presence of antigen presented on the surface of red blood cells. The Duffy blood group system was discovered in the 1950s from work on Hemophilia patients [1]. The Duffy (Fy) blood group gene is located on chromosome 1 (1q22-1q23) and encodes the Duffy antigen receptor for chemokines (DARC) [2]. Two major antigens, Fy^a and Fy^b, encoded by two codominant alleles, *FY*A* and *FY*B*, are expressed on red blood cells and endothelial cells [3]. Fy^a is differentiated from Fy^b by a G>A substitution at nucleotide position 125 (G125A) in exon 2 which results in the change of glycine to aspartic acid at position

***Corresponding author:** jirajira28@yahoo.com **doi:** 10.14456/scitechasia.2021.16

42 (Gly42Asp) [4-5]. The Duffy null phenotype or Fy(a−b−) is the result of the mutation in the GATA box of *FY*^{ES}* (erythrocyte silence) promoter which results in the prevention of Duffy antigen expression on the surface of erythrocytes [4, 5]. Therefore, there are 4 main phenotypes of Duffy blood group including Fy(a+b−), Fy(a+b+), Fy(a−b+), and Fy(a−b−). Moreover, the C>T substitution at nucleotide position 265 of the *FY*X* gene results in the change of arginine to cysteine at position 89 (Arg89Cys). This amino acid alteration leads to the weakly positive Fy^{b} antigen which causes false negative results in serological testing [6].

The detection of Duffy blood group type can be performed by phenotyping and genotyping [7]. For phenotyping, serological methods such as an indirect antiglobulin test are commonly used [7]. The limitation of this method is an inability to detect the exact phenotype of the low-expression Fy^{b} antigen caused by the mutation in *FY*X* gene and inability to distinguish the silent expression of *FY*^{ES}* alleles [7, 8]. For genotyping, PCR-SSP, PCR-RFLP, and real-time PCR are commonly used to detect the Duffy blood group genotype with high precision. Among these, PCR-SSP proved to be useful and safe for multi-transfusion patients. This is a technique with lower cost compared to the other molecular methods [7, 9, 10]. The microarray is one of the advanced modern technologies used for large scale blood group genotyping, but the cost of this method is high [7].

There are 3 common Duffy alleles: *FY*A*, *FY*B*, and *FY*B^{ES}* [11]. The *FY*A* allele is highly prevalent across Southeast Asia and is observed as the Fy(a+b−) and Fy(a+b+) phenotypes, whereas the *FY*B* allele was mostly distributed in Europe and some parts of the Americas and observed as the Fy(a−b+) and Fy(a+b+) phenotypes. The silent *FY*B^{ES}* allele was predominant in sub-Saharan Africa in the form of the Fy(a−b−) phenotype [11]. In central Thailand, *FY*A/*A* was the predominant genotype, followed by *FY*A/*B* and then *FY*B/*B* [2]. Presently, there are few studies that have reported the frequencies of Duffy blood groups in the kingdom of Thailand [2, 12]. Therefore, this study was performed to investigate the phenotype and genotype frequencies of Duffy blood groups in Thai blood donors who reside in Tak and Yala, Thailand.

2. Materials and Methods

2.1 Sample collection

Two hundred EDTA blood samples from Thai blood donors were supplied by the National Blood Center, Thai Red Cross Society, Thailand, 100 samples of which were from Tak (western areas) and the other 100 samples were from Yala (southern areas). This study was approved by the Human Research Ethical Review Board No.3 of Thammasat University and Research Ethics Committee, National Blood Centre, Thai Red Cross Society.

2.2 Detection of Duffy phenotype by serological method

Initially, 2-5% red blood cell suspensions in 0.85% normal saline or normal saline solution (NSS) were prepared for all 200 EDTA blood samples. Thirty microliters of this red blood cell suspension and 60 µL of anti-Fy^{a} or anti-Fy^{b} (CEIMMUNDIAGNOSTIKAGmbH, Eschelbronn, Germany) were mixed inside clean 12 x 75 mm-plastic test tubes and incubated in a 37°C water bath for 30 minutes. The red blood cells were washed 3 times in NSS in order to remove unbounded globulin. Subsequently, 60 µL of anti-human globulin (AHG) (National Blood Centre, Bangkok, Thailand) was added into the tubes and centrifuged at 3,400 rpm for 15 seconds. The results were determined by presence of red blood cell agglutination. Negative agglutinations were checked further by visualizing under light microscope to observe the weakly positive results. Finally, 30 µL of Coomb control cell

was added into the negative test tubes and visualized under light microscope to confirm the result as true negative.

2.3 DNA extraction

Genomic DNA was prepared from blood leukocytes using the GeneiusTM Micro gDNA extraction kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) following manufacturer's instructions. The genomic DNA was then stored at -20°C until use.

2.4 Detection of Duffy genotype by PCR-SSP

*2.4.1 Amplification of FY*A and FY*B*

The PCR-SSP identification of the Duffy blood group was performed according to a procedure previously described with some modifications [13]. Two PCR-SSP reactions were carried out to detect the 711-bp amplified products of *FY*A* and *FY*B*. These two PCR-SSP reactions used the same components, except primer sets, and the same PCR conditions. *FY*A* was amplified using the forward primer FY-AB-F-5'-CTCATTAGTCCTTGGCTCTTAT-3' and the reverse primer FY-A-R-5'AGCTGCTTCCAGGTTGGCAC-3', and *FY*B* was amplified using the forward primer FY-AB-F-5'-CTCATTAGTCCTTGGCTCTTAT-3' and the reverse primer FY-B-R-5'-AGCTGCTTCCAGGTTGGCAT-3' [14]. Co-amplification of the human growth hormone (HGH) gene was used as an internal control to detect 434-bp amplified products from a pair of primers by using the forward primer HGH-F-5'-TGCCTTCCCAACCATTCCCTTA-3' and the reverse primer HGH-R-5'-CCACTCACGGATTTCTGTTGTGTTTC-3' [2]. The gene was amplified using T100^{TM} thermal cyclers (Bio-Rad, California, USA). The total volume of each reaction was 10 µl, comprised of 1X Taq buffer including 10mM Tris-HCL (pH 8.8 at 25°C), 50 mM KCL (Thermo scientific, Massachusetts, USA), 3 mM MgCl2 (Thermo scientific, Massachusetts, USA), 0.4 mM dNTPs (Bioline, London, United Kingdom), 0.3 µM FY-AB-F, FY-A-R, and FY-B-R primers (Sigma, Iowa, USA), 0.2 µM HGH-F and HGH-R primers (Sigma, Iowa, USA), and 0.05 U Taq polymerase (Thermo scientific, Massachusetts, USA). The PCR conditions were as follows: denaturation at 94°C for 2 minutes for the first cycle, followed by 10 cycles of 94°C for 10 seconds, 69°C for 1 minute, 20 cycles of 94°C for 30 seconds, 62°C for 1 minute, 72ºC for 30 seconds, and 72ºC for 5 minutes for the last cycle. The amplified PCR products were stained with Ultra Power DNA safe dye (Gellex International Co., Ltd., Machida, Tokyo), separated by 1.5% agarose gel electrophoresis with 100 V for 40 minutes and visualized under UV illumination (Omega FluorTM, San Francisco, USA).

*2.4.2 Amplification of FY*X*

The mutation of *FY*X* at nucleotide position 265 was identified by the PCR-SSP procedure previously described with some modifications [6]. The 180 bp of *FY*X* was amplified using the forward primer FY-X-F-5'-CCTTCCCAGATGGAGACTATGA-3' and the reverse primer FY-X-R-5'-CAGGGCAGAGCTGCCAGCA-3'. Co-amplification of the *HGH* gene was performed and used as an internal control [2]. PCR amplification was performed using T100^{TM} thermal cyclers (Bio-Rad, California, USA). The total volume of each reaction was 10 µl comprised of 1X Taq buffer including 10 mM Tris-HCL (pH 8.8 at 25°C), 50 mM KCL (Thermo scientific, Massachusetts, USA), 1.5 mM MgCl2 (Thermo scientific, Massachusetts, USA), 0.2 mM dNTPs (Bioline, London, United Kingdom), 0.4 µM FY-X-F and FY-X-R primers (Sigma, Iowa, USA), 0.2 µM HGH-F and HGH-R primers (Sigma, Iowa, USA), and 0.1 U Taq polymerase (Thermo

scientific, Massachusetts, USA). The PCR conditions were as follows: denaturation at 95°C for 1 minute at the initial cycle, 95°C for 30 seconds, 67°C for 30 seconds, 72ºC for 45 seconds for the subsequent 30 cycles, 72ºC for 30 seconds at the additional 1 cycle, and 72ºC for 5 minutes at the last cycle. The amplified PCR products were stained with Ultra PowerTM DNA safe dye (Gellex International Co., Ltd., Machida, Tokyo), separated on 1.2% agarose gel electrophoresis with 100 V for 40 minutes and visualized under UV light (Omega FluorTM, San Francisco, USA).

2.5 Data analysis

Statistical analysis was performed using the SPSS statistical package (version 15.0 SPSS Inc., Chicago, Illinois, USA). Pearson's chi-squared (χ^2) test was used to determine the distribution of phenotype and genotype frequencies. The chi-squared (χ^2) test was used to determine whether the observed genotypes were in accordance with the expected genotypes in a population in Hardy-Weinberg equilibrium. One-way ANOVA followed by Bonferroni post hoc test were used to compare the allelic frequencies of Duffy blood groups in different geographical areas. Statistical significance level was set at $\alpha = 0.05$. Cohen's kappa statistic was used to assess the agreement between the serological method and the PCR-SSP method. The Kappa coefficient (κ) results were interpreted as follows: values ≤ 0 indicate no agreement, 0.01–0.20 none to slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, and 0.81–1.00 almost perfect agreement.

3. Results and Discussions

3.1 Phenotypes of Duffy blood group

Three phenotypes of Duffy blood groups in 200 blood samples were identified, Fy(a+b−), Fy(a+b+), and Fy(a−b+). The observed phenotypes and genotypes are in agreement with that of the Hardy-Weinberg equation. The most prevalent Duffy blood group at the provinces of Tak and Yala was Fy(a+b−), followed by Fy(a+b+) and then Fy(a−b+). The phenotype Fy(a−b−) was not found in this study (Table 1). This result is concordant with that observed in a previous study in Thailand, which reported 81.2% prevalence of Fy(a+b−) and 0.2% prevalence of Fy(a−b−) [14].

The Duffy blood group phenotypes of Tak (western) and Yala (southern) were considered and a significant difference was observed (*p*-value = 0.002). Fy(a+b−) was shown to be significantly more prevalent in Tak (*p*-value = 0.002), while Fy(a+b+) was shown to be significantly more prevalent in Yala (*p*-value = 0.012). Fy(a−b+) was shown to be common in Yala, but the prevalence was not significantly different from other geographic areas in Thailand (*p*-value = 0.121). Most importantly, the Fy(a−b+) phenotype was rarely found in this study, 4.0% in Yala and 0.0% in Tak.

3.2 Genotypes of Duffy blood group

The observed genotypes were consistent with that of the Hardy-Weinberg equation ($\chi^2 = 0.1503$, *p*-value = 0.963). Two allelic genotypes, *FY*A* and *FY*B*, for Duffy blood group were examined in all 200 blood samples. Three genotypes for Duffy blood group were identified, *FY*A*/*FY*A*, *FY*A*/*FY*B*, and *FY*B*/*FY*B* as presented in Fig. 1. The most common genotypes for Duffy blood group were *FY*A*/*FY*A*, followed by *FY*A*/*FY*B* and then *FY*B*/*FY*B* (Table 1). The genotypes for Duffy blood group between Tak and Yala province were compared and a significant difference was found (*p*-value = 0.023). The *FY*A*/*FY*A* genotype was shown to be significantly more common in Tak (*p*-value = 0.031), while the *FY*A*/*FY*B* and FY*B/FY*B genotypes were shown to be more common in Yala, yet the differences were not significant (*p*-values = 0.124, 0.121, respectively). Of this, the *p*-value of

the *FY*A*/*FY*B* genotype was inconsistent with that of the Fy(a+b+) phenotype, possibly due to a decreasing number of Fy(a+b−) and an increasing number of *FY*A*/*FY*B*.

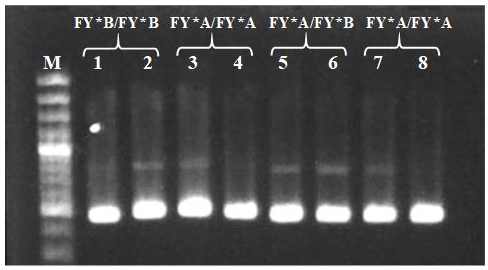

Fig. 1. The electrophoresis of the Duffy blood group genotyping by PCR-SSP. *FY*A* allele detection = lanes 1, 3, 5, 7. *FY*B* allele detection = lanes 2, 4, 6, 8. Lanes 3, 5, 7 presented 711 bp for *FY*A* allele. Lanes 2, 6 presented 711 bp for *FY*B* allele. M = 100 bp ladder marker (Thermo Scientific, Massachusetts, USA). Human Growth Hormone (internal control) presented 434 bp for Human Growth Hormone detection in human.

Table 1. The distribution of phenotypes, alleles and genotypes of Duffy blood group in 200 samples from Tak and Yala, Thailand.

	Tak province	Yala province	Total samples
n	100	100	200
Phenotypes[a]			
Fy(a+b−)	88 (88.0)[b]	69 (69.0)	157 (78.5)
Fy(a+b+)	12 (12.0)[c]	27 (27.0)	39 (19.5)
Fy(a−b+)	0 (0.0)	4 (4.0)	4 (2.0)
Fy(a−b−)	0 (0.0)	0 (0.0)	0 (0.0)
Allele frequencies[d]			
*FY*A*	183 (91.5)	165 (82.5)	174 (87.0)
*FY*B*	17 (8.5)	35(17.5)	26 (13.0)
Genotype[e]			
*FY*A/FY*A*	83 (83.0)f	69 (69.0)	152 (76.0)
*FY*A/FY*B*	17 (17.0)	27 (27.0)	44 (22.0)
*FY*B/FY*B*	0 (0.0)	4 (4.0)	4 (2.0)

Data are presented as numbers (%).

[a]Statistically significant difference of phenotype between samples from Tak and Yala (*p*-value = 0.002).

[b]Statistically significant difference with samples from Yala (*p*-value = 0.002).

[c]Statistically significant difference with samples from Yala (*p*-value = 0.012).

[d]Statistically significant difference of allele frequency between samples from Tak and Yala (*p*-value < 0.001)

[e]Statistically significant difference of genotype between samples from Tak and Yala (*p*-value = 0.023).

[f]Statistically significant difference with samples from Yala (*p*-value = 0.031).

The distribution of the *FY*A* and *FY*B* alleles in Tak were significantly different (*p*-value < 0.001) when compared to those in Yala. Finally, the *FY*B*/*FY*B* genotype is rare in Thailand, but an increased proportion of *FY*B* alleles was found in Yala. Further studies using a larger sample size, however, could confirm the frequencies of the *FY*A* and *FY*B* alleles in this part of Thailand.

In this study, a high frequency of the *FY*A* allele (87.0%) and a low frequency of the *FY*B* allele (13.0%) were observed in the 200 samples from Tak and Yala, Thailand. This result was in fact similar to that observed in the samples from other Thais in 2002 [14].

3.3 Comparison of phenotypes and genotypes of Duffy blood group detection

The frequency of phenotypes for Duffy blood group detected by serological method were 95% concordant with that of the genotypes analyzed by the PCR-SSP method. The Cohen's kappa statistic was used to find agreement between the methods. The results showed that these 2 methods are in perfect agreement, with a kappa value of 0.931. Of these, 78.5% of Fy(a+b−) was found by serological method and 76.0% of *FY*A*/*FY*A* was found by the PCR-SSP method. In addition, 19.5% of Fy(a+b+) was found by serological method, and 22.0% of *FY*A*/*FY*B* was found by PCR-SSP method (Table 1). The low amount of Fy(a+b−) and high amount of *FY*A*/*FY*B* found may occur from a mutation of *FY*X* gene, thus detection of an *FY*X* mutation was performed in order to find the mutation of *FY*X* gene using PCR-SSP. The results were identical with previous data [6], the mutation of the *FY*X* gene at nucleotide position 265 was not

found. Of these, the inconsistence of results occurs only in the samples from Tak , which was collected for 1-2 weeks before sending to laboratory, while the samples from Yala were being collected with a maximum of 3 days waiting before sending them to the laboratory. For this reason, sample collection and long storage duration may have resulted in RBC hemolysis and a decrease of antigens on RBCs. It can be suspected that the serological method may produce false negatives from the collection process. Nevertheless, the discrepancy between the phenotypes and genotypes was probably caused by a mutation in the promoter gene, *i.e.* $FY*A^{ES}$ and $FY*B^{ES}$, which are rare in Thailand [14, 15]. However, Fy(a−b−) was not present in the phenotyping of Duffy blood group detected by serological method, the mutation of *FY*B* and promoter region could be confirmed by sequencing.

3.4 Comparison of Duffy blood group in Thailand and other countries

The distribution of phenotypes, alleles, and genotypes for Duffy blood group were compared with One-way ANOVA followed by Bonferroni post hoc test and presented in Table 2. A similar pattern of allelic frequencies was found in a study of a Thai population in 2002 [14] (*p*-value=1.000) and a Chinese population [16] (*p*-value=0.952).

However, there were significant differences of allelic frequencies among Thais, 2015 [2] (*p*-value = 0.010), Sudanese [17] (*p*-value < 0.001), Indian [18] (*p*-value < 0.001), and Brazilian Amazon [19] (*p*-value < 0.001) populations. Studying the prevalence of different blood groups among the various populations is beneficial for managing alloimmunization and helps to find compatible blood for patients who are likely to develop antibodies against minor blood group antigens, especially in multi-transfusion patients, such as thalassemia patients [7]. Therefore, highly efficient methods are necessary. The serological method used in this study is suitable in cases of emergency, as it takes less time than the molecular method, which is appropriate for detection at the gene level to find low-expression antigens or silent phenotypes. PCR-SSP can also be used as a complementary method to the serological method.

4. Conclusion

The most common Duffy blood groups in Tak and Yala, Thailand are first Fy(a+b−), followed by Fy(a+b+), and then Fy(a−b+). The serological and PCR-SSP methods were in perfect agreement in this study. PCR-SSP might be used as an alternative method for identification of Duffy blood group, particularly in those with low-expression antigens.

Acknowledgements

This research was supported by the Thammasat University Research Fund, Contract No. TUGG 142/2562 and the National Research Council of Thailand in the new researcher development program according to research and innovation strategic plan for graduate students, Contract No.19/2562.

Table 2. The distribution of phenotype, allele and genotype of Duffy blood groups in different geographical areas.

	Sudanese [17]	**Indian** [18]	**Brazilian Amazon** [19]	**Chinese** [16]	**Thais 2002** [15]	**Thais 2015** [2]	**Thais** Present Study
n	60	3,072	164	146	500	200/500	200
Phenotype, *n* (%)							
Fy(a+b−)	7 (11.7)	1,295 (42.1)	30 (18.3)	128 (87.67)	406 (81.2)	177/200 (88.5)	157 (78.5)
Fy(a+b+)	51 (85.0)	377 (12.3)	77 (47.0)	18 (12.33)	90 (18.0)	22/200 (11.0)	39 (19.5)
Fy(a−b+)	2 (3.3)	1,392 (45.3)	51 (31.1)	0 (0.00)	3 (0.6)	1/200 (0.5)	4 (2.0)
Fy(a−b−)	0 (0.0)	8 (0.3)	6 (3.6)	0 (0.00)	1 (0.2)	0/200 (0.0)	0 (0.0)
Allele frequencies[a,b]							
*FY*A*	65 (54.2)	2,966 (48.25)	119 (36.3)	274 (94.0)	902 (90.2)	962 (96.2)	348 (87.0)
*FY*B*	55 (45.8)	3,162 (51.45)	148 (45.1)	18 (6.0)	96 (9.6)	38 (3.8)	52 (13.0)
*FY*ES*	0 (0.0)	18 (0.30)	61 (18.6)	nt	2 (0.2)	0 (0.0)	nt
Genotype, *n* (%)							
*FY*A/FY*A*	7 (11.7)	1,295 (42.1)	12 (7.3)	28 (87.67)	406 (81.2)	464/500 (92.8)	152 (76.0)
*FY*A/FY*B*	51 (85.0)	377 (12.3)	77 (47.0)	8 (12.33)	90 (18.0)	34/500 (6.8)	44 (22.0)
*FY*B/FY*B*	2 (3.3)	1,392 (45.3)	20 (12.2)	0 (0.0)	3 (0.6)	2/500 (0.4)	4 (2.0)
*FY*A/FY*^{ES}*	nt	nt	18 (11)	nt	nt	nt	nt
*FY*B/FY*^{ES}*	nt	nt	31 (18.9)	nt	nt	nt	nt
FY^{ES}/FY*^{ES}*	0 (0.0)	8 (0.3)	6 (3.6)	nt	1 (0.2)	0/500 (0.0)	nt

Note: nt = not tested
Data are presented as numbers (%)
[a] Statistically significant difference of allele frequencies between present study and the study in Sudan, India, Brazil (p-value < 0.001)
[b] Statistically significant difference of allele frequencies between present study and Thailand 2015 (p-value = 0.01).

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