Improved Allele-Specific PCR Technique for Kidd Blood Group Genotyping

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Background: We developed an allele-specific polymerase chain reaction (AS-PCR) technique for Kidd blood group genotyping. **Methods:** Altogether, 340 blood samples from Thai blood donors at the National Blood Centre, Thai Red Cross Society, were tested with anti-Jk^a and anti-Jk^b using the gel technique and the direct urea lysis test was used for screening Jk(a–b–) phenotype. For AS-PCR technique, different types of primers were used for JK*01 and JK*02 allele detections in known DNA controls. **Results:** Regarding JK*02 allele detection, the pseudopositive amplification products were found when using correctly matched forward primer and a single mismatch forward primer. Interestingly, one

type of two mismatch pairing at the 3' end of the forward primer can be used together with the newly designed reverse primer for Kidd blood group genotyping. It was found that the typing results in all samples obtained by serological techniques and newly developed AS-PCR technique were in agreement and this PCR technique also gave 100% concordance of results in 30 samples randomly tested twice and demonstrated reproducible results. **Conclusion:** This study shows that the in-house AS-PCR is simple, cost-effective, and convenient for Kidd blood group genotyping in routine laboratories, especially, in resolving serologic investigations. J. Clin. Lab. Anal. 27:53–58, 2013. © 2012 Wiley Periodicals, Inc.

Key words: allele-specific PCR; AS-PCR; Kidd blood group; genotyping; Thais

INTRODUCTION

The Kidd (JK) blood group system (ISBT009) was discovered in 1951 (1). The JK gene; SLC14A1 is located on chromosome 18 (18q12-q21) and organized in 11 exons distributed across more than 30 kbp of DNA. However, the mature JK glycoprotein of 389 amino acids is encoded by exon 4 through exon 11 with the translation start point in exon 4 (2). The Jk^a and Jk^b antigens are the products of two alleles (JK*01/JK*02) that are inherited in a co-dominant fashion. The JK*01/JK*02 polymorphism (838G > A in exon 9) results in an Asn280Asp substitution (3), which encodes for three common phenotypes; Jk(a+b–), Jk(a–b+) and Jk(a+b+), respectively. Additionally, the rare Jk(a–b–) phenotype is generally inherited as a homozygosity for silent JK allele. Various mutations of the silent JK allele are found among popula-

tions (4–6). In general, the Kidd antibodies are a significant cause of delayed hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (HDFN); the severity of the disease varies but tends to be mild. However, when patients produced Kidd antibodies, group- and type-specific red cells that are compatible are needed (7).

Routinely, Kidd antigen detection is performed using unknown red blood cells (RBCs) with anti-Jk^a and anti-Jk^b by indirect antiglobulin test or enzyme test (7), which

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is not suitable for mass screening in blood donors, especially in emergency transfusions. Moreover, for mass screening of the Jk(a–b–) phenotype, the urea lysis test is suggested; however, RBCs from other phenotypes, Jk(a+b+), Jk(a+b–), and Jk(a–b+), cannot be discriminated (8,9).

The hemagglutination test for Kidd blood group phenotyping has limitations such as it is not suitable to type RBCs from multitransfused patients and it is difficult to type RBCs with the positive direct antiglobulin test (DAT) (10,11). The implementation of DNA technology for Kidd blood group genotyping allows us to detect specific Kidd blood group single nucleotide polymorphisms (12,13). In addition, the PCR techniques including PCR-restriction fragment length polymorphism, allele-specific PCR (AS-PCR), sequence-specific PCR as single or multiplex assays, real-time quantitative PCR, and high-resolution melting analysis have been used for several blood groups genotyping (14–20). Among these, AS-PCR technique is widely used because it is simple and cost effective; nevertheless, when PCR is carried out under inappropriate reaction conditions, the pseudopositive problem may occur (21). This study aimed to develop AS-PCR for Kidd blood group genotyping, which is suitable for routine testing in the Thai population.

MATERIALS AND METHODS

Subjects

Altogether, 340 ethylenediaminetetraacetic acid (EDTA) blood samples were obtained from blood donors at the National Blood Centre, Thai Red Cross Society. Informed consent was obtained from each subject. This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Thammasat University, Pathumtani, Thailand.

DNA Standards

Twenty known DNA samples consisting of five Jk(a+b–), five Jk(a–b+), six Jk(a+b+), and 4 Jk(a–b–) phenotypes, confirmed by DNA sequencing from a previous study (6), were used as controls.

Primers

Primers for Kidd blood group genotyping used in this study are shown in Tables 1–3. The specific primers for *JK* gene were designed by PRIMER 3 software (<http://frodo.wi.mit.edu/primer3>) and some primers were identical to those previously described (3,18,22). All primers were tested for their specificities with known DNA of Kidd blood group genotypes.

TABLE 1. Sequences of the Primers for Kidd Blood Group Genotyping by AS-PCR Technique

Primer	Primer sequence (5'→3')	Product size (bp)	Final concentration (μM)
Set A			
JK-a-F (3)	CATGCTGCCAT AGGATCATTGC	301	0.5
JK-a-R (3)	CCAGAGTCCAA AGTAGATGTC		0.5
JK-b-F (3)	AGTCTTTTCAGC CCCATTGAGA	121	0.5
JK-b-R (3)	GAGCCAGGAG GTGGGTTTGC		0.5
Set B			
JK-b-838A-f2 (18)	AGTCTTTTCAGCC CCATTGcGA	171	0.5
JK-b-171-R ^a	TAGTCATGAGC AGCCCTCCCC		0.5
Internal control			
HGH-434-F (22)	TGCCTTCCCAA CCATTCCCTTA	434	0.3
HGH-434-R (22)	CCACTCACGGATT TCTGTTGTGTTTC		0.3

^aNewly designed primers.

TABLE 2. Sequences of the Two Mismatch Forward Primers and Reverse Primer for *JK*02* Allele Detection

Primer	Primer sequence (5'→3')
Set C	
JK-b-2M-F-01	AGTCTTTTCAGCCCCATTTGtcA
JK-b-2M-F-02	AGTCTTTTCAGCCCCATTTGtaA
JK-b-2M-F-03	AGTCTTTTCAGCCCCATTTGttA
JK-b-2M-F-04	AGTCTTTTCAGCCCCATTTGccA
JK-b-2M-F-05	AGTCTTTTCAGCCCCATTTGctA
JK-b-2M-F-06	AGTCTTTTCAGCCCCATTTGcaA
JK-b-2M-F-07	AGTCTTTTCAGCCCCATTTGcA
JK-b-2M-F-08	AGTCTTTTCAGCCCCATTTGgtA
JK-b-2M-F-09	AGTCTTTTCAGCCCCATTTGgaA
JK-b-171-R	TAGTCATGAGCAGCCCTCCCC

TABLE 3. Sequences of the AS-PCR Primers for Kidd Blood Group Genotyping

Primer	Primer sequence (5'→3')	Product size (bp)	Final concentration (μM)
JK-a-F (3)	CATGCTGCCA TAGGATCATTGC	301	0.5
JK-a-R (3)	CCAGAGTCCAA AGTAGATGTC		0.5
JK-b-2M-F-09 ^a	AGTCTTTTCAGC CCCATTGGA	171	0.6
JK-b-171-R ^a	TAGTCATGAGCA GCCCTCCCC		0.6
HGH-434-F (22)	TGCCTTCCCAAC CATTCCCTTA	434	0.3
HGH-434-R (22)	CCACTCACGGATT CTGTTGTGTTTC		0.3

^aNewly designed primers.

Methods

Kidd blood group phenotyping by the gel technique

A 5% RBC suspension in Diluent-I (BIO-RAD, Morat, Switzerland) was prepared. The cell suspension was incubated for 15 min at room temperature. The appropriate microtube of the ID-Card "Diaclon Anti-Jk^a" and/or "Diaclon Anti-Jk^b" (BIO-RAD) was labeled. Ten microliters of RBC suspension was added to the appropriate microtube and the ID-Card was centrifuged for 10 min in the ID-centrifuge (DiaMed AG, Morat, Switzerland). The results were read and recorded according to the manufacturer instructions.

Jk(a-b-) screening by urea lysis test

Screening for Jk(a-b-) phenotype by direct urea lysis test was performed in all blood samples, as previously described (9).

DNA extraction

For Genomic DNA extraction, EDTA blood samples were extracted with the Genomic DNA extraction kit (REAL Genomics, RBCBioscience, Taipei, Taiwan).

Kidd Blood Group Genotyping by AS-PCR Technique

AS-PCR using primer set A

DNA samples of known *JK*01* and *JK*02* alleles were used as controls. In the PCR, 2 µl of genomic DNA (50 ng/µl) was amplified in a total volume of 20 µl using 0.5 µM of JK-a-F and JK-a-R primers for a set of *JK*01* allele detection and 0.5 µM of JK-b-F and JK-b-R for *JK*02* allele detection. Moreover, 0.3 µM of HGH-434-F and HGH-434-R were included in both sets of *JK*01* and *JK*02* allele detections. Sequences of the primers used for this AS-PCR are shown in Table 1. PCR of each allele detection was performed with 10 µl of DreamTaqTM DNA polymerase (Thermo Fisher Scientific Inc., Glen Burnie, MD) consisting of 2× Dream Taq green buffer, dNTPs, 0.4 mM each, and 0.4 mM MgCl₂ in a G-STORM GS1 thermal cycler (Gene Technologies Ltd., Essex, UK). The PCR program consisted of one cycle of 95°C for 5 min, followed by 30 cycles at 95°C for 30 sec, 61°C for 40 sec, 72°C for 30 sec with a final extension at 72°C for 5 min. The PCR products were separated in 2% agarose gel in 1× tris borate ethylenediamine tetraacetic acid containing 0.5 µg/ml ethidium bromide 30 min at 100 V and visualized under a ultraviolet (UV) transilluminator.

AS-PCR using primer set B

DNA samples of known *JK*01* and *JK*02* alleles were used as controls. AS-PCR was performed for only *JK*02* allele detection using 0.5 µM of JK-b-838A-f2 and JK-b-171-R primers and 0.3 µM of HGH-434-F and HGH-434-R primers. Sequences of these primers are shown in Table 1. The PCR conditions were identical to AS-PCR using primer set A.

AS-PCR using primer set C

DNA samples of known *JK*01* and *JK*02* alleles were used as controls. AS-PCR was performed for only *JK*02* allele detection using 0.6 µM of nine types of forward primers with two mismatch pairing at the 3' end and JK-b-171-R primers. Sequences of primers are shown in Table 2. In addition, 0.3 µM of HGH-434-F and HGH-434-R primers were used as the internal control. The PCR conditions were identical to AS-PCR using primer set A.

AS-PCR for Kidd blood group genotyping

Altogether, 340 DNA samples of blood donors were genotyped for *JK*01* and *JK*02* alleles using the newly developed AS-PCR technique. Primers for Kidd blood group genotyping are shown in Table 3. In the PCR, 2 µl of genomic DNA (50 ng/µl) was amplified in a total volume of 20 µl using 0.5 µM of JK-a-F and JK-a-R primers for a set of *JK*01* allele detection and 0.6 µM of JK-b-2M-F-09 and JK-b-171-R for *JK*02* allele detection. Moreover, 0.3 µM of HGH-434-F and HGH-434-R were included in both sets of *JK*01* and *JK*02* allele detections. The PCR conditions were identical to the AS-PCR using primer set A.

To increase the validity and reliability of the evaluation, the technicians were blinded from the serological results. The sensitivity, specificity, positive and negative predictive values, and accuracy of AS-PCR technique were calculated and compared with serological techniques. The sensitivity of the AS-PCR was tested using known *JK*01* and *JK*02* allele samples with concentrations ranging from 25 to 200 ng/µl. Additionally, 30 samples were randomly repeated for Kidd blood group genotyping using the AS-PCR technique, to test for reproducibility.

RESULTS

The distribution of Kidd blood group phenotypes among 340 Thai blood donors was studied. It was found that Jk(a+b+) was the most common phenotype (45.3%), followed by Jk(a+b-), 27.6%; Jk(a-b+), 27.1%; and Jk(a-b-) was not found. Moreover, all samples were negative for Jk(a-b-) phenotype screening by urea lysis test.

Fig. 1. The amplified products of the *JK*02* allele detection using different types of forward primers (set A, set B, and set C) separated on a 2% agarose gel (lanes 1–9). Samples from individuals with the following JK phenotypes were used: Lanes 1, 4, and 7 = Jk(a–b+) and 2, 5, and 8 = Jk(a+b–). Lanes 3, 6, and 9 = negative controls. Arrows indicate the size of *JK* gene fragments (right): *JK*02* products = 121 bp and 171 bp and internal control (HGH) = 434 bp. M: 100 bp ladder marker (Fermentas, Carlsbad, CA).

AS-PCR Using Primer Set A

Known Jk(a+b–), Jk(a–b+), and Jk(a+b+) DNA controls were tested for Kidd blood group genotyping using primer set A. It was found that for *JK*01* allele detection, the results were in agreement with serological testing; however, for *JK*02* allele detection, a pseudopositive band (121 bp) was found in the Jk(a+b–) phenotype as shown in Figure 1.

AS-PCR Using Primer Set B

Known Jk(a+b–), Jk(a–b+), and Jk(a+b+) DNA controls were tested for *JK*02* allele detection using primer set B. It was found that a pseudopositive band (171 bp) was found in the Jk(a+b–) phenotype as shown in Figure 1.

AS-PCR Using Primer Set C

Known Jk(a+b–), Jk(a–b+), and Jk(a+b+) DNA controls were tested for *JK*02* allele detection using two mismatch allele-specific primers at the penultimate of 3' end. Altogether, nine types of specific forward primers for *JK*02* alleles were designed. It was found that a positive band (171 bp) was demonstrated in known Jk(a–b+) and Jk(a+b+) phenotypes only when using the JK-b-2M-F-09 forward primer (5'-AGTCTTTCAGCCCCATTTGgaA3') together with the JK-b-171-R (5'-TAGTCATGAGCAGCCCTCCCC 3') and a negative result was found in Jk(a+b–) phenotype as shown in Figure 1.

Fig. 2. A representative gel showing Kidd blood group genotyping by AS-PCR technique. The 434 bp amplification product of the HGH control primer is present in all lanes (lanes 1–6), which shows that amplification has occurred optimally. The genotype was deduced from the presence or absence of amplification products specific for *JK*01* and *JK*02* alleles. From left to right: Lanes 1–2 = Jk(a+b–), 3–4 = Jk(a–b+), 5–6 = Jk(a+b+), and 7–8 = negative control for *JK*01* and *JK*02* alleles detections, respectively. Arrows indicate the size of *JK* gene fragments (right): *JK*01* = 301 bp, *JK*02* = 171 bp, and internal control (HGH) = 434 bp. M: 100 bp ladder marker (Fermentas).

AS-PCR for Kidd Blood Group Genotyping

Known Jk(a+b–), Jk(a–b+), Jk(a+b+), and Jk(a–b–) DNA controls and 340 DNA samples of Thai blood donors were tested for Kidd blood group genotyping using the AS-PCR. Two sets of primers were used. The first set comprised the *JK*01*-specific primers, similar to that previously described (3). The second set comprised the *JK*02*-specific primers that were our own newly designed, forward primer: JK-b-2M-F-09 (5'-AGTCTTTCAGCCCCATTTGgaA3') and reverse primer: JK-b-171-R (5'-TAGTCATGAGCAGCCCTCCCC 3'). Moreover, the HGH primers; HGH-434-F (5'-TGCCTTCCCAACCATTCCCTTA3') and HGH-434-R (5'-CCACTCACGGATTCTGTGTGTTTC 3') were used as an internal control (22) for both *JK*01* and *JK*02* allele detection. Following the PCR reactions, it was found that for *JK*01* allele detection with the first set of primers, a positive band of 301 bp was found in Jk(a+b–) and Jk(a+b+) phenotypes. For *JK*02* allele detection with the second set of primers, a positive band of 171 bp was found in Jk(a–b+) and Jk(a+b+) phenotypes. Moreover, in each allele detection, the HGH internal control gave an expected band of 434 bp. The Kidd blood group genotyping results by AS-PCR are shown in Figure 2.

Comparison of Kidd Blood Group Typing Results

In all, 340 samples including 94 Jk(a+b–), 92 Jk(a–b+), and 154 Jk(a+b+) samples were tested for Kidd blood group genotyping by AS-PCR. All 94 Jk(a+b–) and 92 Jk(a–b+) samples were positive with

only the first set of primers and the second set of primers, respectively. Additionally, 154 Jk(a+b+) samples were positive with both sets of primers. The DNA controls were also tested with these two sets of primers and the results were in agreement.

The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the AS-PCR technique were calculated and compared with those of the serological techniques. It was found that the AS-PCR technique gave a sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of 100%. In addition, repeated AS-PCR testing was performed on 30 blinded samples and demonstrated reproducible results. The sensitivity of the AS-PCR technique for *JK*01* and *JK*02* allele detections was tested at different DNA concentrations using known Jk(a+b-) and Jk(a-b+) samples. It was found that the bands were most clearly detected at the concentration ranging from 25 to 200 ng/ μ l.

Moreover, when four genomic DNA samples from Thai individuals with the Jk(a-b-) phenotype were tested by AS-PCR, three samples gave results compatible with *JK*02/JK*02* genotypes and one sample gave the result compatible with *JK*01/JK*01* genotypes despite their Kidd-negative phenotype, in concordance with a previous study (6).

DISCUSSION

Until now, the gold standard for blood group antigen and antibody detection is routinely performed by serological techniques. Selection of suitable techniques for red cell serology testing depends on the sensitivity and specificity of each technique, cost, test time, and throughputs. Several advantages of the gel technique are that it is less time consuming and requires less technical skills. Although the gel technique is expensive compared with the conventional tube technique; however, it is simple and the exposure to blood bank personnel is low. This study shows the Kidd blood group distribution in 340 Thai blood donors. The Jk(a+b+) phenotype was the most common, 45.3% followed by Jk(a+b-) and Jk(a-b+), 27.6% and 27.1%, respectively, comparable with other studies in the Thai population (23, 24). The Jk(a-b-) phenotype, which is considered a rare phenotype among Thai people, was not found in this study.

In Thailand, the prevalence of unexpected antibodies in 2,821 multitransfused Thai patients was reported. The most common unexpected antibodies are antibodies in Rh system (42.2%), followed by MNS system (31.9%), and Kidd system (10.5%) (25). Therefore, it is essential to facilitate the identification of antibodies that may be formed in the future in order to provide compatible blood for these multitransfused patients.

In this study, the AS-PCR technique was initially set up using a set of primers similar to a previous study (3) in order to genotype *JK*01* and *JK*02* alleles. When testing DNA controls; Jk(a+b-) and Jk(a-b+) using a set of specific primers for *JK*01* allele, the results of positive and negative bands were in agreement. Nevertheless, pseudopositive bands were found when testing Jk(a+b-) DNA controls with a set of specific primers for the *JK*02* allele. Thereafter, the PCR products were confirmed for *JK*01* and *JK*02* alleles by DNA sequencing and it was proven that the nt 838 in exon 9 of the *JK* gene was G for the Jk(a+b-) DNA control. The pseudopositive results may have been generated because Taq DNA polymerase extends mismatched allele-specific DNA primers; hence, to improve the specificity and reliability of AS-PCR, the incorporation of additional mismatches in the allele-specific primer near the 3' end is suggested (21, 26).

High-throughput multiplex PCR genotyping for 35 RBC antigens was used for large-scale blood donor screening (18). Interestingly, for *JK*01* and *JK*02* allele detections, the single mismatch forward primers at the 3' ends were used. Thus, the single mismatch forward primer (JK-b-838A-f2: 5' AGTCTTTCAGCCCC ATTTGcGA3') and our own designed reverse primer (JK-b-171-R: 5' TAGTCATGAGCAGCCCTCCCC 3') for *JK*02* alleles were used in this study in order to solve the pseudopositive results. However, the pseudopositive results were shown in Jk(a+b-) DNA controls even when testing with different annealing temperature. Consequently, for *JK*02* allele detection, nine types of forward primers with two mismatch pairs adjacent to the terminal base pair were designed. Surprisingly, only one type of two mismatch forward primer can be used to differentiate the *JK*02* allele without pseudopositive results. Hence, the AS-PCR technique detection of *JK*01* and *JK*02* alleles was implemented to genotype 340 DNA samples of Thai blood donors. The Kidd blood group typing results of gel technique and newly developed AS-PCR technique were in agreement and this PCR technique also gave 100% concordance of results in 30 random samples. Moreover, the cost of the AS-PCR is approximately only \$1(US) per test.

A limitation of the used *JK*02* mismatch primer (JK-b-2M-F-09) is its decreased extension efficacy compared with *JK*01* fully matching primers, which makes it inapplicable for a single-tube AS-PCR. Therefore, for multiplex PCR genotyping of other RBC antigens, it is noteworthy to separate specific primer of *JK*01* and *JK*02* alleles. Unfortunately, the AS-PCR technique cannot differentiate four known DNA samples of Jk(a-b-) phenotype from a previous study (6), which is similar to other PCR techniques used in previous studies (3, 18). However, the Jk(a-b-) phenotype is exceedingly rare in most population except in Polynesians and only 0.025% in the

Thai population (7, 9). Routinely, Jk(a–b–) phenotype screening by the direct urea lysis test has been used for mass screening in blood donors and can be performed in parallel with AS-PCR.

In conclusion, the main advantages of the AS-PCR technique for Kidd blood group genotyping are a small amount of blood is used and the results can be obtained even with a low concentration of DNA (25 ng/μl). Moreover, it is simple, convenient and the cost is low. Therefore, this technique can be used not only as an alternative to serological techniques for population screening in routine blood bank laboratories, but also as a confirmation test for cases of unexpected incompatible cross-match or unexplained cases of HDFN.

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