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Short communication

VALIDATION OF A POLYMERASE CHAIN REACTION TECHNIQUE FOR KIDD BLOOD GROUP GENOTYPING

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Abstract. The Kidd blood group antigens, Jk^a and Jk^b, are two of the main surface markers which are found on the membrane of red blood cells. The determination of whether a donor or a recipient has the Jk^a and/or the Jk^b antigens is crucially important to have a successful transfusion without the development of adverse incompatibility-related reactions. In Malta, routine serological-based tests are applied with the purpose of differentiating between homozygous and heterozygous states for the Jk antigens respectively. Although these tests are highly specific and sensitive, there are particular clinical scenarios where haemagglutination assays are not suitable for determining the individual's Kidd blood group status. Additionally, the alternative genotyping procedure has never been applied in Malta within the context of blood grouping. The current study was therefore carried out to determine whether a molecular-based technique such as Polymerase Chain Reaction – Restriction Fragment Length Polymorphism analysis (PCR-RFLP) is a suitable alternative procedure for distinguishing amongst the three different Kidd phenotypes. After extracting deoxyribonucleic acid (DNA) from 50 blood samples obtained from serologically-tested healthy blood donors who expressed at least one of the Kidd antigens, PCR-RFLP analyses were carried out. The results of the latter were then compared with those previously obtained with haemagglutination and a complete match was observed between the two. Therefore, this PCR-RFLP method was confirmed as a suitable alternative laboratory technique that can be used to determine efficiently the Kidd blood group of both donors and recipients, in an accurate manner without subjectivity as encountered in the case of haemagglutination. This research further facilitates the introduction of molecular-based techniques in molecular blood transfusion.

Keywords: Kidd, Jk^a, Jk^b, haemagglutination, PCR-RFLP, molecular transfusion

1 Introduction

Red blood cells have surface markers on their membrane, known as blood group antigens. The three main blood group antigens that are the most clinically significant out of the total 600 are the A and B antigens of the ABO blood group system and the Rh-D antigen of the Rh blood group system. Nonetheless, there are also other blood group antigens which are particularly important for a successful transfusion process.

Two of these are the Jk^a and Jk^b antigens of the Kidd blood group system (Daniels & Bromilow, 2010).

The Kidd blood group system (ISBT 009; symbol *Jk*) was discovered in 1951 by Race et al. The antigens of the Kidd blood group system are expressed on the Kidd transmembrane glycoprotein, and the gene which is responsible for the production of the Jk antigens is the *SLC14A1* gene, which in old literature was referred to as *Jk*, *HUTII* (National Center for Biotechnology Information, 2014). The Jk gene is located on chromosome 18q11-q12 and has 11 exons spanning a total of 30kb (Geitvik et al., 1987).

There are three antigens within the Kidd blood group system: Jk^a, Jk^b and Jk³. The Jk^a antigen is found in 77% of Caucasians, 92% of Blacks and 73% of Asians. The Jk^b antigen is detected in 74% of Caucasians, 49% of Blacks and 76% of Asians (Dean, 2005). The Jk³ antigen is present on all the red blood cells which express the Jk^a and/or the Jk^b antigens, thus 99.8% of all the individuals have the Jk³ antigen, regardless of their ethnicity. In fact, the Jk³ antigen is only absent in 1% of Polynesians and Finns who do not express either Jk^a or Jk^b on their erythrocytes (Reid & Shine, 2012). Therefore, when it comes to determining the phenotype of the person, testing for the presence of the Jk^a and the Jk^b antigens is performed.

The Jk^a and Jk^b antigens are the products of the Jk-1 and the Jk-2 alleles of the *SLC14A1* gene respectively. When one examines the coding sequences of the mentioned co-dominant alleles, two fundamental differences can be detected; the wildtype Jk-1 allele has an Adenine at nucleotide 588 on exon 7 and a Guanine at nucleotide 838 on exon 9 whereas the mutant Jk-2 allele has a Guanine at nucleotide 588 with an accompanying Adenine at nucleotide 838 (Wester, 2010).

This Kidd blood group polymorphism was described for the first time almost two decades ago (Olivès et al., 1997). The 588A → G single nucleotide polymorphism (SNP) is considered to be silent since both resulting codons code for Proline (Pro) as amino acid 196 of the final gene product (Hong, Gong & Zhou, 2012). Conversely, the 838G → A missense SNP causes the change of amino acid 280 from Asparagine (Asn) to Aspartic acid (Asp) (Intharanut et al., 2013). Ultimately, this Asn280Asp amino acid substitution can be used to differentiate between the Jk^a and the Jk^b antigens (Olivès et al., 1997).

Since the Jk-1 and the Jk-2 alleles can be inherited in a co-dominant fashion, there are three possible phenotypes: homozygous for the Jk^a antigen Jk (a+b-), homozygous for the Jk^b antigen Jk (a-b+) or heterozygous for both antigens Jk (a+b+). In addition, there is also the null phenotype, depicted as Jk (a-b-), which is only expressed by 1% of Polynesians and Finns that lack both Kidd antigens (Irshaid et al., 2002).

In Malta, the three main Kidd phenotypes are differentiated from one another by use of the haemagglutination techniques which are carried out at the state general hospital's Blood Bank. During this serological procedure, a red cell suspension is prepared, which is then

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divided into two aliquots and mixed with two anti-sera containing monoclonal IgM anti-Jk^a and anti-Jk^b antibodies. Then, depending on the resultant agglutination pattern, one would determine whether the individual has a homozygous or heterozygous expression of the Kidd blood group antigens. This assay relies on the concept of antigen-antibody reactions, which typically consist of the Red Cell Sensitisation phase and the Agglutination phase, to detect the antigens of interest (Overfield, Dawson & Hamer, 2011).

Haemagglutination is considered as the gold standard technique for Kidd phenotyping due to its high levels of specificity and sensitivity. Other advantages include the simplicity of the technique and its quick and rapid process which ranges from 25 minutes to a maximum of 40 minutes, leading to a fast turnaround time (Reid, 2009). However, like all laboratory techniques, serological haemagglutination has its own limitations. It is a highly subjective test and there is the possibility of having batch-to-batch variations of the anti-sera that are used.

Furthermore, serology-based investigations cannot be applied on blood samples obtained from recently transfused patients, massively transfused patients who had a severe haemorrhagic event and chronically transfused patients who suffer from thalassaemia, sickle cell disease or aplastic anaemias where regular blood transfusions are considered to be part of the treatment regime (Westhoff, 2006). These patients have the donor's red blood cells in their circulation, which would directly interfere with the test since haemagglutination techniques are incapable of differentiating between the patient's and the donor's red blood cells (Castilho & Pellegrino, 2004).

Moreover, individuals who suffer from some form of autoimmune haemolytic anaemias (AIHA) such as Cold Agglutinin Disease, Warm AIHA, Paroxysmal Cold Haemoglobinuria and Drug-Induced AIHA, cannot be typed by haemagglutination. These patients have circulating auto-antibodies which coat their red cells and cause spontaneous agglutination. Consequently, false positive reactions can be observed with haemagglutination-based tests since the observed agglutination would not have occurred due to a reaction between the red blood cells and the anti-sera reagents but due to the reaction that transpired between the erythrocytes and the individual's own auto-antibodies (Westhoff, 2006).

Thus, a number of conditions can result in a higher risk of Kidd blood group status mistyping. This may lead to transfusion with incompatible red cells, therefore increasing the risk of haemolytic transfusion reactions (HTR) (Overfield, Dawson & Hamer, 2011). In order to overcome these limitations, genotyping procedures are being introduced to complement the routine serological techniques.

In Malta, the antigens of the Kidd blood group system have never been genotyped by molecular techniques, due to the fact that they are phenotyped on a routine basis with serological tests. The purpose of this study was therefore to validate Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) analysis, a molecular-based procedure which is specific, sensitive and capable of differentiating between the Jk^a and Jk^b antigens and their respective genotypes in a non-subjective manner.

2 Methods

2.1 Selection of samples

Fifty volunteer, regular, healthy Maltese blood donors from Malta's national blood transfusion centre were recruited for this study. These blood donors were chosen on the premise that they had already been tested by haemagglutination during past donations and thus, their phenotype for the Kidd blood group antigens was known. Donors were selected in such a way as to have the three different Kidd phenotypes represented in the study. Other selection criteria, such as age and gender, were not considered. Once each chosen donor voluntarily underwent the donation process, 9 mL of blood were transferred from the blood donation bag's sample diversion pouch into a 10 mL purple-

capped vacutainer containing ethylenediaminetetraacetic acid (EDTA) anticoagulant. Approval for the collection of blood samples was obtained from the University of Malta's Research Ethics Committee.

2.2 Deoxyribonucleic acid (DNA) extraction

The salting out technique (Miller, Dykes & Polesky, 1988) was implemented to extract the deoxyribonucleic acid (DNA) from the 50 whole blood samples. The extracted DNA was dissolved in sterile Tris EDTA buffer (TE) and quantified using the Nanodrop 2000c UV-VIS Spectrophotometer. The samples were diluted to a concentration of 50 ng/ μ L in a total volume of 20 μ L using TE buffer. In addition, a random batch of DNA samples was analysed by 1% agarose gel electrophoresis to re-confirm the quality of the extracted DNA.

2.3 Polymerase Chain Reaction (PCR)

The JK1S forward primer [5'-TGAGATCTTGCTTCCTAGG-3'] and the JK2 reverse primer [5'-ATTGCAATGCAGGCCAGAGA-3'] were the two published primer sequences which were used to target the Kidd blood group system gene (Denomme, Rios & Reid, 2000).

The Master Mix that was prepared during the optimised PCR runs is shown in Table 1, whereas Table 2 shows the thermal cycler's profile which was used to carry out the amplification of the Kidd gene. The resultant PCR products were qualitatively checked with 2% agarose gel electrophoresis.

Table 1. PCR components and Master Mix volumes

PCR components	Amount per reaction	Master Mix volumes
2x ReddyMix Master Mix	5 μ L	280 μ L
JK1S forward primer (50 μ M)	0.1 μ L	5.6 μ L
JK2 reverse primer (50 μ M)	0.1 μ L	5.6 μ L
Genomic DNA (50 ng/ μ L)	1 μ L	/
Sterile Water	3.8 μ L	212.8 μ L
<i>Final Volume</i>	<i>10 μL</i>	<i>504 μL</i>

Table 2. PCR Mastercycler® profile for the amplification of the Kidd blood group gene

Number of cycles	PCR step	Temperature	Time
1 cycle	Initial hot start	95°C	5 minutes
28 cycles	Denaturation	95°C	50 seconds
	Annealing	56°C	30 seconds
	Extension	72°C	45 seconds
1 cycle	Final extension	72°C	10 minutes
1 cycle	Incubation period	4°C	∞

2.4 Restriction enzyme digestion

Based on the same published work from which the primers' sequences were obtained, *MnlI* was the restriction enzyme that was chosen to carry out digestion of the PCR products (Denomme, Rios & Reid, 2000). A Master Mix was prepared accordingly, as shown in Table 3, and the

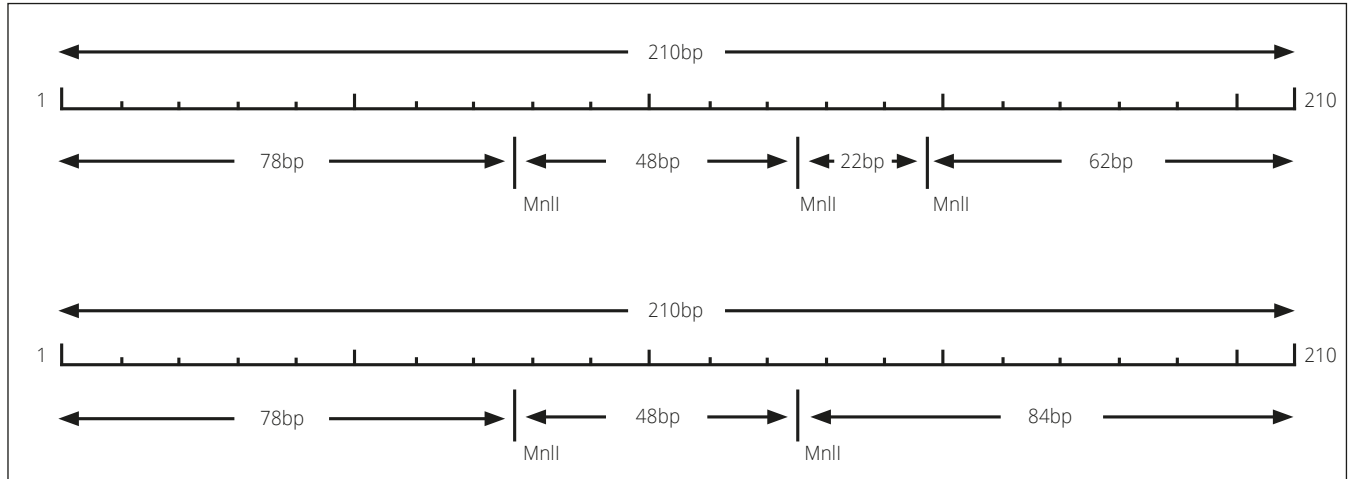


Figure 1. Kidd blood group gene restriction maps; the top image shows a graphical representation of the four DNA fragments obtained from the Jk^a -encoding Jk-1 wildtype allele following digestion by the restriction enzyme *MnlI*. In this case, the enzyme recognised three $5'$ - CCTC (N)₇ ↓ - $3'$ recognition sites. The bottom image shows a graphical representation of the three DNA fragments obtained from the Jk^b -encoding Jk-2 mutant allele which had two recognition sites, with one of the original three recognition sites being abolished due to the 838G → A missense SNP.

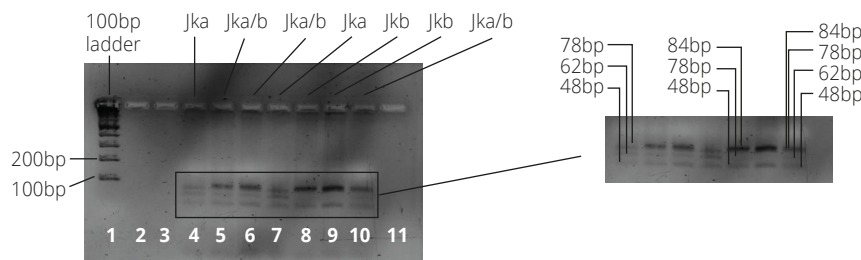


Figure 2. Restriction enzyme digest result; gel image of the *MnlI*-digested Kidd blood group gene when run on 3% Micro ABgarose gel electrophoresis. Lanes 4 and 7 show the homozygous Jk^a genotype, lanes 8 and 9 show the homozygous Jk^b genotype and lanes 5, 6, and 10 show the heterozygous $Jk^{a/b}$ genotype.

PCR products were digested overnight with *MnlI* enzyme (New England Biolabs, Hertfordshire, United Kingdom).

The expected fragment sizes for each genotype were obtained via online analysis with the NEB cutter website (<http://nc2.neb.com/NEBcutter2/>) and are shown in Table 4 and in Figure 1.

Table 3. Restriction enzyme digestion reaction and Master Mix volumes

Digest component	Amount per reaction	Master Mix
NEBuffer 4	2 μ L	112 μ L
<i>MnlI</i> Enzyme (5,000 U/mL)	0.9 μ L	50.4 μ L
Sterile water	12.1 μ L	677.6 μ L
PCR products	5 μ L	/

Table 4. The expected sizes of the DNA fragments (in base pairs) of the three Kidd blood group genotypes

Homozygous Jk^a/Jk^a	Homozygous Jk^b/Jk^b	Heterozygous Jk^a/Jk^b
-	84	84
78	78	78
62	-	62
48	48	48
22	-	22

2.5 Final Micro ABgarose gel electrophoresis

The digested PCR products were separated via 3% Micro ABgarose gel electrophoresis and analysed with a UV-transilluminator Bio-Doc-It® Imaging System.

3 Results

On direct comparison, all PCR-RFLP results matched completely with the haemagglutination results, as shown in Table 5.

Table 5. Haemagglutination results compared with PCR-RFLP results

Number of samples	Haemagglutination	PCR-RFLP	Match percentage
11	Jk ^a + / Jk ^b -	Jk ^a	100%
14	Jk ^a - / Jk ^b +	Jk ^b	100%
25	Jk ^a + / Jk ^b +	Jk ^{a/b}	100%

Additionally, Figure 2 shows the digested PCR fragments and the three Kidd blood group genotypes.

4 Discussion and Conclusion

This study confirmed that the three different Kidd blood group genotypes, homozygous Jk^a, homozygous Jk^b and heterozygous Jk^{a/b}, can be successfully differentiated from one another by PCR-RFLP. This validated PCR-RFLP technique can therefore be applied to blood samples obtained from recently transfused patients, massively transfused patients, chronically transfused patients and individuals who have AIHA, ensuring that these persons who cannot be tested by serology, due to potential interferences from either the donor's transfused red blood cells or their own auto-antibodies respectively, can have their Kidd blood group status genotyped correctly.

Furthermore, given that this study was the first successful undertaking within the field of molecular transfusion, further research is warranted to determine whether this genotyping technique can be used to differentiate the genotypes of other clinically significant blood groups. This is especially relevant in view of the fact that molecular genotyping of the other blood group antigens such as the A, B and O antigens of the ABO blood group system, the C, c, D, E and e antigens of the Rh blood group system, the Fy^a and Fy^b antigens of the Duffy blood group system, the K antigen of the Kell blood group system and the M, N, S and s antigens of the MNS blood group system, amongst others, has never been carried out in Malta.

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7 Conflicts of Interest

The authors report no conflicts of interest.

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