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A new polymerase chain reaction: sequence-specific primer method for the Augustine blood type

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Introduction

The Augustine (At^a) antigen was formerly one of the high incidence antigens in the 900 series (901003 or 901.3)[1] and previously proposed by the International Society of Blood Transfusion as the 36th blood group system. The prevalence of At(a+) is greater than 99% in both African and Caucasian ancestries; the At(a−) phenotype, caused by recessive inheritance, is therefore very rare[2]. Anti-At^a is usually produced by an At(a−) individual after alloimmunisation by transfusion or during a pregnancy, and is associated with immediate or delayed haemolytic transfusion reactions and haemolytic disease of the foetus and newborn[3-6].

In practice, anti-At^a cannot be identified because of the pan-agglutination of all the red blood cell (RBC) antigens that are used in the identification panels. Additional testing with several panels including rare RBC antigens and determining its identity with anti-At^a are recommended[7]. However, anti-At^a is not commercially available and can only be obtained in some reference laboratories[8]. To resolve difficult pre-transfusion testing problems and to provide timely safe blood transfusions, Augustine genotyping can be implemented to identify the At(a−) blood type among patients and donors[9].

Although the genetic basis of the Augustine blood type remains ambiguous, a recent study reported that a non-synonymous single nucleotide polymorphism (SNP) in *SLC29A1* (rs45458701) encoding the equilibrative nucleoside transporter 1 (ENT1) on chromosome 6 is responsible for the At(a−) blood type. This polymorphism is caused by a missense point mutation at c.1171G>A in nm_001078174 resulting in p.Glu391Lys substitution, which encodes for At(a+) and At(a−) blood type[9]. We, therefore, developed a polymerase chain reaction with sequence-specific primer (PCR-SSP) using newly designed primer sets to predict the Augustine blood type.

Material and methods

Known DNA samples consisting of two At(a+) and one At(a−) blood type confirmed by serological testing at the Red Cell Reference Laboratory, Australian Red Cross Blood Service, QLD, Australia[10] and DNA sequencing were used as controls for PCR-SSP development. Of the two At(a+) samples, one sample was c.1171G homozygote and other one was c.1171G/A heterozygote and the At(a−) sample was c.1171A homozygote. In addition, peripheral venous blood was collected in EDTA tubes from 940 unrelated, healthy Thai blood donors at the National Blood Centre, Thai Red Cross Society, Bangkok, Thailand. The donors were from central Thailand and their ages ranged from 19 to 58 years. 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. Genomic DNA, extracted from all samples using the Genomic DNA extraction kit (REAL Genomics, RBCBioscience, Taipei, Taiwan) was then stored at −20 °C until used for genotyping.

Genotyping of the Augustine blood type was performed using an in-house PCR-SSP technique. Individual tests to identify At(a+) and At(a−) included two sets of PCR mixture. Briefly, 1 µL of genomic DNA (50 ng/µL) was amplified in a total volume of 20 µL using 1 µL of 10 µM forward primer (At_1171G_F) and 1 µL of 10 µM reverse primer (At_1171GA_R) to detect c.1171G. To detect c.1171A, 1 µL of 10 µM forward primer (At_1171A_F) was used. Sequences of those primer combinations in the two primer mixtures and the SNP detected by each mixture are shown in Table I. In addition, co-amplification of the human growth

Table I - Sequences of the primers for genotyping the Augustine blood type.

Primer	Primer sequence (5'→3')	SNP	Product size (bp)	Final concentration (µM)
At_1171G_F	GCTACCTGACTGTGGTCTTCG	c.1171G	183	0.5
At_1171GA_R	CCTCTACTGAGTGTCCCCCA			
At_1171A_F	GCTACCTGACTGTGGTCTTCA	c.1171A	183	0.5
At_1171GA_R	CCTCTACTGAGTGTCCCCCA			
HGH-F	TGCCTTCCCAACCATTCCCTTA		434	0.5
HGH-R	CCACTCACGGATTTCTGTTGTGTTTC			

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hormone (*HGH*) gene using 1 µL of 10 µM HGH-F primer and 1 µL of 10 µM HGH-R primer was run as the internal control. The PCR was performed with 10 µL of PCR reaction mixture (OnePCR Plus, GeneDirex, New Taipei City, Taiwan) and 5 µL of sterile distilled water in a G-STORM GS1 thermal cycler (Gene Technologies Ltd., Somerset, UK).

PCR was performed under the conditions described below, i.e., 95 °C for 5 minutes (initial denaturation). The cycle parameters of the PCR programme began with the first step of 9 cycles of 30 seconds at 95 °C and 60 seconds at 69 °C, then 20 cycles of 10 seconds at 95 °C, 50 seconds at 62 °C and 30 seconds at 72 °C. The last step was final extension for 5 minutes at 72 °C. Thereafter, the PCR products were electrophoresed at 100 V with 1.5% agarose gel using 1X Tris borate ethylenediaminetetraacetate (TBE) buffer and were visualised under a blue-light transilluminator. The PCR product size of c.1171G and c.1171A was 183 bp, whereas that of the internal control, the *HGH* gene, was 434 bp (Figure 1). Additionally, to test for reproducibility, 30 DNA samples were randomly selected for repeated genotyping of the Augustine blood type using PCR-SSP.

To validate our in-house PCR-SSP technique for genotyping the Augustine blood type, genomic DNA from 30 genotyped blood donors (c.1171G homozygote) was sequenced. A fragment of 748 bp was obtained from PCR amplification of genomic DNA using the forward primer 5'-GAAAGTATGGTAGTAAGGGGACCA-3' and reverse primer 5'-ACGTGTATGGTGGGGTTGTC-3' and using a PCR programme identical to the PCR-SSP method.

Results

In this study, two primer sets were used to determine the Augustine blood type by in-house PCR-SSP. The first set identified c.1171G while the second set identified c.1171A with the PCR product size of 183 bp. In each set, the *HGH* internal control gave an expected band of 434 bp. The validity of genotyping for the Augustine blood type with the in-house PCR-SSP was checked by using known At(a+), c.1171G and c.1171G/A and At(a−), c.1171A blood types and the results were in agreement. Additionally, this PCR-SSP technique was validated using 33 randomly chosen DNA samples including three DNA controls by DNA sequencing and the results were also in agreement (Figure 2).

Samples from 940 Thai blood donors were genotyped for the Augustine blood type by PCR-SSP. All the donors were identified as c.1171G homozygotes. To test the reproducibility of the PCR-SSP, 30 DNA samples were randomly selected and re-tested by PCR-SSP. The results of the repeated test were similar to those of the first round of testing.

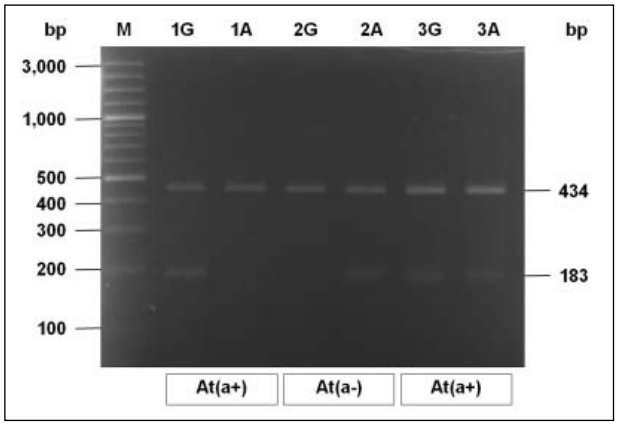

Figure 1 - A representative gel showing genotyping of the Augustine blood type using the PCR-SSP technique. The genotype was deduced from the presence or absence of amplification products specific for the Augustine blood type. From left to right: lanes 1G-1A=At(a+), c.1171G homozygote; 2G-2A=At(a−), c.1171A homozygote and 3G-3A=At(a+), c.1171G/A heterozygote. Arrows indicate the size of gene fragments (right): c.1171G and c.1171A is 183 bp, and the internal control (*HGH*) is 434 bp. M: 100 bp Plus ladder marker (Fermentas, Carlsbad, CA, USA).

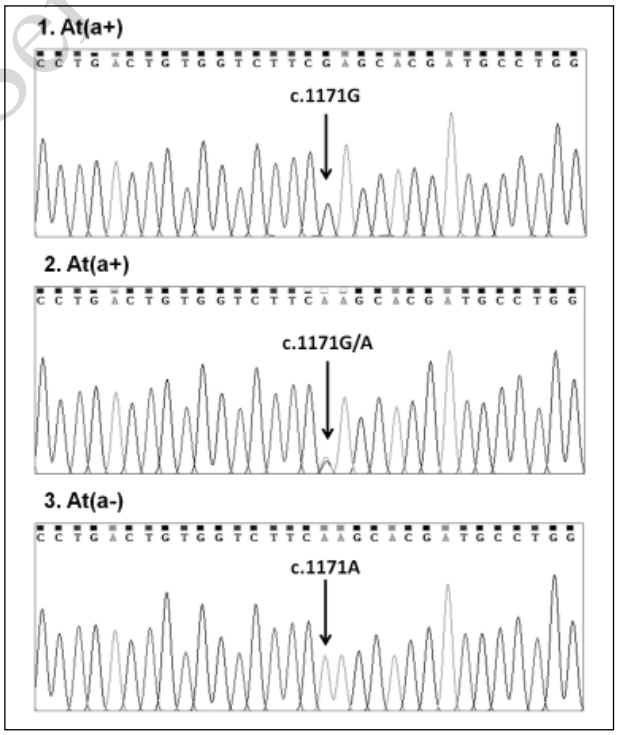

Figure 2 - Electropherograms of the Augustine blood type in the *SLC29A1* (rs45458701) of At(a+) and At(a–) individuals.
DNA sequences of (1) the At(a+), c.1171G homozygote, (2) the At(a+), c.1171G/A heterozygote and (3) the At(a–), c.1171A homozygote are amplified in the *SLC29A1*.

Discussion

Molecular testing is currently used to overcome the limitations of serological testing, such as blood typing in recently transfused patients, short supply, poor quality or unavailable antisera and distinguishing between autoantibodies and alloantibodies[11]. The low-throughput method, PCR-SSP is commonly used for RBC antigen genotyping caused by SNP because of its simplicity, reliability, and cost-effectiveness[12].

Typing c.1171G>A in *SLC29A1* was suggested to identifythe At(a+) and At(a−) blood types[9]. In this study, we established and validated an in-house PCR-SSP method for genotyping the Augustine blood type using two PCR reactions and the results were obtained within 2 hours. After implementing this method, the results of c.1171G homozygote as well as c.1171G/A heterozygote and c.1171A homozygote could be computed to At(a+) phenotypes and At(a−) phenotype, respectively. However, a recent study identified three individuals of European ancestry who had a null mutation in *SLC29A1* (c.589+1G>C) and the At(a−) phenotype[9]. However, this mutation was not found by DNA sequencing in three controls or 30 random donor samples.

This study is the first to report the frequencies of the Augustine blood type in the Thai population; At(a−) was found to be exceedingly rare among Thais. The growing number of inter-ethnic marriages may cause an increase in genetic variation. Further studies with larger sample sizes in different populations would be beneficial for identifying rare blood type donors. PCR-SSP has several advantages over a serological method: first, its ability to identify not only At(a+) but also c.1171G/A heterozygote; second, the cost of the in-house PCR-SSP is approximately € 1.0/test; and third, the results can be obtained within 2 hours.

Conclusions

This study shows that the newly developed PCR-SSP is simple, convenient and cost-effective and can be used as an alternative method to identify the Augustine blood type.

Authorship contributions

ON and KI designed the study and wrote the manuscript; PK and SN contributed materials; ON, KI and WS performed experiments and analysed the data.

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The Authors declare no conflicts of interest.

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