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Serological and Molecular Detection of RhD DEL Phenotype in National Blood Centre, Malaysia

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ABSTRACT

After ABO, Rhesus (Rh) is the second most clinically important blood group regarding transfusion and pregnancy induced alloimmunisation. RhD DEL is a subtype of variant RhD, which is difficult to determine in a routine blood bank, since it expresses an extremely low level of D antigens. Serologically, it can only be detected via adsorption-elution test. To date, there have been limited data available on the Rhd DEL phenotype in Malaysia. Thus, this study was carried out to detect DEL phenotype among RhD negative donors in Malaysia. A total of 43 RhD-negative blood samples were collected from National Blood Centre, Malaysia. Rh phenotype for each sample was tested, followed by adsorption elution technique. Then, identification of DEL carrying RHD1227A allele was performed via SSP-PCR. Rh-phenotype identified were ccee (79.07%), Ccee (13.95%), 4.65% of ccEe phenotype and only 2.33% of CCee phenotype. One (2.3%) out of the 43 samples was identified as DEL phenotype carrying RHD1227A allele when tested using SSP-PCR, but none was identified from adsorption-elution. A larger sample size is recommended to determine the exact prevalence of DEL phenotype, as well as specificity and sensitivity between SSP-PCR as compared with the traditional adsorption elution technique.

Keywords: DEL phenotype, RHD1227A alleles, SSP-PCR

INTRODUCTION

After ABO, Rhesus (Rh) is the second most clinical important and immunogenic blood group in transfusion medicine. In 1940, the Rhesus system was discovered by Landsteiner and Wiener (as cited in Westhoff, 2007). The terms Rh positive or Rh negative are based on the foundation of the D antigen either its presence or not correspondingly. Rhesus proteins encoded by two genes, which are

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RHD and RHCE, are located in a tail-to-tail direction towards the end of the short arm of chromosome 1 (p34–36) (Huang, 2009). RhD encodes D antigen, while RHCE encodes CE antigens in many arrangements such as ce, CE, Ce or cE. Meanwhile, RHD and RHCE are closely related, differing in 36 out of 417 amino acids (Le Van Kim, Colin, & Cartron, 2006). The complex genetic basis of the Rh blood group owes to their large number of antigens (Flegel, 2007).

Rhesus is the largest of all 30 known blood group systems with more than 50 antigens (Kappler-Gratias et al., 2014). Based on their molecular structure and phenotype, these RHD alleles are classified as weak D, partial D, and DEL. DEL, or formerly known as D-elute (Del), was first discovered in 1984 (Okubo et al., 1984). DEL is the most weakly expressed of D antigens. In general, 30 or less copies of the D antigen per RBCs are expressed by DEL phenotype compared with 1500 to 7000 sites for weak D, and 30,000 antigen sites for normal D (Li et al., 2009; Sandler et al., 2014; Wagner, Moulds, & Flegel, 2005). The sensitivity of conventional serological assays is impeded because of the weak expression of the DEL variant that can only be detected using adsorption and elution techniques (Gardener et al., 2012). DEL phenotypes arise from several mechanisms such as deletion, missense, or splice-site mutations. RHD1227A is the most frequent allele reported in Asia, and it also known as K409K alleles (Chen et al., 2004). The aim of this study is to observe the frequency of DEL phenotype among blood donors at the National Blood Centre.

MATERIALS AND METHODS

Sample collection

Forty-three leftover samples in Ethylenediaminetetraacetate (EDTA) tubes were collected from the National Blood Centre. Criteria for samples selection included phenotype as Rh negative and negative results for all the routine screenings for blood donors. This study was approved by the Medical Research and Ethics Committee (MREC) of Malaysia.

Rhesus Phenotyping

The Rh phenotype was determined by testing patients' red blood cells with the five standard antiserums. Serotyping for the RhD, RhC/c, and RhE/e antigens was performed using monoclonal anti-D, monoclonal anti-C, anti-c, anti-E, and anti-e antibodies (DiaCidel, DiaMed, Cressier, Switzerland).

Adsorption Elution Test

For adsorption test, 1000 µl of red cells were incubated with 1000 µl of monoclonal anti- D for 1 hour at 37ºC. The cells were washed six times, while elute was prepared using the heat elution technique.

Genomic DNA Extraction

Genomic DNA was isolated from peripheral blood cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Meanwhile, DNA concentration and purity were determined using Biophotometer (Eppendorf, USA)

Detection of RHD 1227A Polymorphisms

RHD1227A allele analysis was performed based on the method described by Chen et al. (2004). A set of RHD1227A primers (forward primer: 5'-GATGACCAAGTTTTCTGGAAA-3', reverse primer: 5'-GTTCTGTCACCCGCATGTCAG-3') were used in amplifying 348 bp product. Meanwhile, growth hormone gene was used for internal control by using another set of primers (forward primer: 5'- GCCTTCCCAACCATTCCCTTA-3', reverse primer: 5'-TAGACGTTGCTGTCAGAGGC-3') to generate 629 bp fragments. PCR preparation was done using HotStarTaq Mastermix Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total volumes of PCR reactions were 25 µl. Each reaction contains 12.5 µl HotStarTaq Master Mix, 2.5 µl of each primer, 1 µl template DNA and 1.5 µl RNase-free water Afterinitial denaturation for 15 minutes at 95°C, and the samples were subjected to 40 cycles of PCR in the My Cycler (Biorad, USA). Each cycle included 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes. The PCR products were separated by electrophoresis in a 2% agarose gel.

RESULTS AND DISCUSSION

Table 1 summarises the serological results with anti-sera D, C, E, c and e. This resulted in four Rh phenotype patterns. Most samples, i.e. 34 out of 43 samples (79.07%), were identified having ccee phenotype. The incidence of serological phenotype Ccee was the second highest with six samples (13.95%). Then, two out of 43 samples (4.65%) were ccEe phenotype. Lastly, only one sample (2.33%) was detected as the CCee phenotype. Eluates prepared using heat elution from all the samples were tested for indirect antiglobulin tests. However, none of these eluates showed positive reactivity for DEL phenotype. All the 43 RhD negative samples were subjected to RHD1227A polymorphism by Specific Sequence Primer-Polymerase Chain Reaction (SSP-PCR). However, only one sample showed an amplified band of 348bp, which demonstrated the RHD1227A amplification.

Table 1
Possible genotypes for the 43 RhD-negative samples and the Rh phenotype in several terminologies

Apparent Rh phenotypes	Possible genotype	Fisher Race terminology	Rosenfield terminology	Shorthand Notation	Incidence Number (N=43)	Percentage (%)
ccee	ddccee	dce/dce	Rh ^{4,5}	Rr	34	79.07
Ccee	ddCcee	dCe/dce	Rh ^{2,4,5}	r'r	6	13.95
ccEe	ddccEe	dcE/dce	Rh ^{3,4,5}	r''r	2	4.65
CCee	ddCCee	dCe/dCe	Rh ^{2,5}	r'r'	1	2.33

As indicated earlier, DEL is the weakest D positive phenotype that expresses trace amounts of D antigens. It is rare and can be detected serologically using adsorption-elution methods. However, this test is tedious and time-consuming to perform, and it is also not a practical test for screening a large number of samples. Currently, molecular technique has been used to replace the serological technique, which is the easiest and most specific to detect this phenotype. In Asian countries, molecular background of DEL has been intensively investigated since these countries are reported to have high prevalence of DEL phenotype (Gu et al., 2014; Li et al., 2009). Nonetheless, there are limited existing data on DEL phenotypes in Malaysia. Therefore, the objective of this study was to detect DEL phenotype in RhD-negative blood using the adsorption-elution technique and Sequence Primer–Polymerase Chain Reaction (SSP-PCR).

Based on the phenotyping test that had been done, the highest frequency of Rh phenotype was the ccee phenotype (79.09%), followed by the Ccee phenotype (13.93%), while 4.66% of the samples were the ccEe phenotype and the lowest phenotype detected was the CCee phenotype (2.33%). The finding of this study is consistent with other studies on the RhD negative phenotype in other countries, especially in East Asia such as China, Korea, Japan and Thailand, which showed ccee and Ccee phenotypes as the most prevalently reported in the RhD-negative samples (Chen et al., 2004; Moussa et al., 2012; Srijinda et al., 2012). Similarly, a previous study by Kyaw et al. (2014) also showed the same results and discovered the highest RhD negative was in Indians, followed by Malays and Chinese. Nonetheless, no information on the blood donor races was given in this study. The serological heat elution test for the 43 RhD negative samples yielded all negative results, indicating no DEL phenotype was detected. Failure to detect the DEL phenotype might be due to the use of leftover or blood samples and a low number of antigen present on the cells. Hemolysed blood samples reduced the ability of antigen D to be detected. All the samples were tested for the presence RHD1227A allele using the Specific Sequence Primer–Polymerase Chain Reaction (SSP-PCR). Separation of PCR products was done in 2% agarose gel. Data from the present study demonstrated that only one (2.33%) out of 43 samples was positive for DEL carrying RHD1227A allele. The positive sample had Rh phenotype of Ccee. This can be related with the statement that DEL individuals who have intact RHD gene expressed the Cc or CC phenotype but did not show the cc phenotype (Wang et al., 2005). Interestingly, approximately 9% of DEL phenotype was still associated with the ce phenotype (Shao et al., 2002). There were five others sample having the same Ccee phenotypes but not identified as positive for DEL. Even though those samples have the same Rh phenotype, their RH genotypes might be different. In fact, a study proposed that there might be discrepancies between Rh phenotyping and RH genotyping (Musa et al., 2014).

To date, at least 14 DEL phenotype alleles have been described. They are RHDIVS2-2A>G, RHD(A137E), RHD(W408R), RHD(L84P), RHD3G>A, RHD(R10W), RHD(L18P), RHD(Y401X), RD(X418L), RHD(delE×9), RHDIVS3+1G>A, RHDIVS5-38del4, RHD(M295I) and RHD1227A. In East Asia, most of the DEL phenotypes carried RHD1227A allele which can be used as a genetic marker for DEL detection (Gu et al., 2014). A study by Srijinda and colleagues on Thailand RhD-negative donors found that 48 out of 50 DEL samples positive for the adsorption-elution test were also positive for RHD1227 allele (Srijinda et al., 2012). In addition, 154 (96.25%) out of 160 samples that were typed as DEL by the adsorption-elution test have RHD1227A allele in China (Gu et al., 2014). Furthermore, one of the previous

studies in Taiwan discovered the exact same RHD1227A allele found in all 94 (100%) DEL individuals (Chen et al., 2004). It is crucial to note that the frequency of D-negative varies among different ethnic groups. In a multi-ethnic country such as Malaysia, it is important to understand D-negative phenotype distribution to estimate compatible blood unit availability and evaluate the risks of Haemolytic Transfusion Reaction (HTR) and Haemolytic Disease of Newborn (HDN).

CONCLUSION AND RECOMMENDATIONS

Based on the results, one out of the 43 RhD negative samples was positive for DEL phenotype for the RHD1227A polymorphism analysis by SSP-PCR, but not for the heat elution test. In this study, the heat elution technique can be concluded as an ineffective method for detecting the DEL phenotype. The specificity and sensitivity of the molecular technique helped to overcome limitations of the serological study. Herein, a similar study using a bigger sample size or at a different part of the country may give a better outcome.

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