Background: The Rh blood group system is highly polymorphic and next to the ABO system is the most clinically significant in transfusion medicine. The frequency of D- phenotypes and the underlying molecular genetics vary widely in different populations.

Objectives: We determined the prevalence of different D- phenotypes among Malaysian blood donors in a tertiary medical centre and identified the molecular basis of Chinese D- donors in this population.

Materials and methods: A total of 146 D- Chinese donors with various Rh phenotypes were identified from review of blood donor records between January 2003 and September 2008. Fresh blood samples from 36 of these donors were obtained and further characterized by PCR-SSP to determine the molecular basis of these D- individuals.

Results: A total of 86,620 blood donor records were reviewed. Of these, 911 were D-, consisting of 483 Indians, 189 Malays and 146 Chinese. The ccee phenotype was the most common among D- individuals with a prevalence of 91.51% (442/483) in Indians, 74.60% (141/189) in Malays and 55.48% (81/146) in Chinese. D- phenotypes with C and/or E antigens were most common in Chinese {44.52% (65/146)}. In the molecular analysis of the 36 D- Chinese donor samples, 19 samples with ccee phenotype and 5/17 of samples with Ccee phenotype showed no detectable RHD gene. The remaining 12/17 Ccee samples had intact RHD genes with RHD (K409K) mutation.

Conclusion: In our donor population, we found a wide variation in the incidence of D- as well as the distribution of various D- phenotypes among the three major ethnic groups. A significant number of D- Chinese donors with Ccee phenotype were found to be DEL with RHD (K409K) mutation. DEL red cells are known to cause anti-D alloimmunization. Therefore, in clinical practice, it is important to exclude DEL RBCs from D- donor pools.

Keywords: Chinese D- donors, DEL variant, Malaysian blood donors, molecular genotyping, RhD-negative phenotypes

The Rh blood group is the most complex and polymorphic of all human blood group systems, consisting of at least 46 different antigens. Next to the ABO blood group system, it is the most clinically significant in transfusion medicine [1, 2]. The D antigen is a potent immunogen and is responsible for most of the clinical problems, such as haemolytic transfusion reactions (HTR) and haemolytic disease of the foetus and newborn (HDFN). The Rh antigens are encoded by two genes on the short arm of chromosome 1: the RHD and RHCE genes. RHD encodes the RhD protein which carries the D antigen whereas RHCE encodes RhCE protein which carries the CE antigens in various combinations (ce, Ce, cE, or CE) [3-6]. These two genes are highly homologous, having 10 exons each. However, the encoded proteins differ by 32 to 35 amino acids [7]. This degree of difference explains why exposure to D antigen can result in a potent immune response in D-individuals [7].

Wide racial differences are recognized not only in the frequency of RhD-negative (D-) phenotypes but also in the molecular basis of D- phenotypes [8]. The frequent cause of D- in Europeans is the deletion of the entire RHD gene [9] whereas D- phenotypes in Africans and Asians are caused by silent or inactive RHD genes due to the presence of various RHD alleles [7].

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Many Asians who type as D- by routine serologic typing have been found to be D-elute (DEL) [7]. The DEL phenotype is an extreme form of weak D which is only detectable serologically by adsorption and elution techniques [10, 11]. Compared to a normal D antigen which consists of about 30,000 D sites per cell, the number of D sites on a DEL RBC is about 20-40 per cell [12]. In China and Japan, approximately 10-30% of apparent D- individuals are DEL phenotypes whereas its incidence is very low in Europeans [10, 13-17]. DEL phenotypes are caused by many genetic mechanisms. RHD(K409K) is the most frequent allele among DEL variants, occurring with a reported frequency of 1:110 and 1:9091 in Chinese and German populations respectively [13, 15].

The Malaysian blood donor population is composed of three major ethnic groups (Malay, Chinese and Indian) and other minor ethnic groups (e.g. native people from East Malaysia). As the donor population is quite heterogeneous, we expect the distribution of Rh phenotypes and genotypes to vary as well. The aims of this study are; 1) to determine the prevalence of different D- phenotypes among Malaysian blood donors in a tertiary medical centre and 2) to identify the molecular basis of Chinese D- donors in this population.

Materials and methods
Sample selection and collection

We reviewed blood donor records of our centre between January 2003 and September 2008 using the laboratory information system. During this period, D antigen status of blood donors was determined with a monoclonal anti-D (Clones TH-28, IgM, and MS-26, IgG; Diamed, Cressier sur Morat, Switzerland) using the tube typing method. When no agglutination was detected, indirect antiglobulin test was performed to exclude a weak D antigen. Subsequently Rh phenotyping for D, C, c, E and e antigens was performed for all D- samples using column agglutination technique (Diamed, Cressier sur Morat, Switzerland) as per manufacturer’s recommendations. Adsorption-elution tests were not routinely performed for D- donors.

As there was no archived blood samples of donors, D- Chinese donors who donated blood during this period were invited to give blood samples for this study. Out of 126 Chinese D-donors with ccee and Ccee phenotypes, 36 donors volunteered to give blood samples, 19 with ccee phenotype and 17 with Ccee phenotype. Blood was collected into K²-Ethylene diaminetetraacetic acid (EDTA) tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). This study was approved by the Ethics Committee of University Malaya Medical Centre.

PCR-SSP genotyping for RHD and RHCE genes and RHD variants

Genomic DNA extraction was done within 1 week of sample collection using a spin protocol by QIAamp DNA Blood Mini Kit (Qiagen®, Hilden, Germany) according to manufacturer’s instructions. Isolated DNA had a purity index (extinction ratio OD₂₆₀/OD₂₈₀) between 1.5 and 2.0 [measured by the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, USA)].

Polymerase Chain Reaction- Sequence Specific Primers (PCR-SSP) genotyping was performed using the BAGene RH-TYPE kit (BAG Health Care, Lich, Germany) according to manufacturer’s instructions. Each BAGene RH-TYPE kit consists of 10 tests and each test has 13 reactions. Reactions 1 and 2 consist of multiplex-PCR reactions targeting intron 4 and exons 4/7 of the RHD gene in reaction 1 and intron 7 of the RHD gene in reaction 2. The second reaction also has primers for detection of RHD (W16X) and RHD pseudo gene. The primers in reactions 3 to 8 are designed to detect RHD exon 4 for non RHD pseudogene sequence, intron 9 for non RHD (K409K) sequence, RHD (K409K) mutation, RHD (M295I) mutation, RHD (IVS 3+1G>A) mutation and Cde⁴ respectively. Promoter sequence, exon 1, intron 2, exon 2, and exon 5 of RHCE gene were analyzed in reactions 9 to 13 to detect the presence of C, c, e, E, or C⁹ alleles. This PCR strategy can also detect other RHD variants such as RHD-CE (8-9)-D, RHD-CE (3-7)-D, D VI, and D IV type3. Human growth hormone (HGH) gene (generating a band of 434 bp) was used as an internal control in all reactions except in reaction 2. In reaction 2, the control band was of 659 bp (specific for genomic sequence of chromosome 1, 90,000 bp 5’ of the Rhesus box).

PCR master-mix solutions were prepared for each sample and consisted of 10-20 μl of the template DNA (volume adjusted according to the DNA concentration), 124-134 μl of H₂O, 16 μl of PCR buffer and 1.3 μl of Taq polymerase (Qiagen, Hilden, Germany) resulting in a final volume of 160 μl. 10 μl of this prepared master mix was aliquoted into the respective reaction tubes which were coated with
specific primers. PCR amplification was carried out in the Eppendorf MasterCycler® Gradient (Eppendorf, Hamburg, Germany). Thermocycling conditions were applied according to manufacturer’s instructions. PCR products were visualized in 2% agarose gel.

Results

*Rh* serotyping findings

Between the period of January 2003 and September 2008, a total of 86,620 blood donors donated blood at our centre. Of these donors, 44.56% (38,599) were Chinese, 41.34% (35,809) were Malays and 10.12% (8,769) were Indians. The remaining 3.98% (3,443) consisted of donors from minor ethnic groups and foreigners. 911 blood donors during this period were D-.. The incidence of D- was found to be 5.51% (483/8,769) in Indians, 0.38% (146/38,599) in Chinese and 0.53% (189/35,809) in Malays. The commonest D- phenotype was ccee. This was observed in 91.51% of Indians (442/483), 74.60% of Malays (141/189) and 54.87% of Chinese (81/146) and 92.47% of donors of other ethnic groups and foreigners (86/93). D- phenotypes with C and/or E antigens (such as Ccee, CCee, CcEe and ccEe phenotypes) were noted in 44.52% (65/146) of Chinese, 25.40% (48/189) of Malays, 8.49% (41/483) of Indians and 7.53% (7/93) of donors of other ethnic groups and foreigners as shown in Table 1.

**PCR-SSP findings**

Genomic DNA of the 36 Rh-D negative Chinese donors was subjected to PCR-SSP. In 24 D- samples, no PCR products for *RHD* sequences or other *RHD* mutations were obtained. These samples were placed in group I. Of these 24 samples, 19 were of ccee phenotype and five were of Ccee phenotype. In the remaining 12 samples, *RHD* sequences were observed and these were placed in group II. All these 12 samples were of Ccee phenotype and have *RHD* (K409K) mutation. The PCR results for C, c, E, and e alleles of *RHCE* gene were in complete concordance with serological results in all 36 donors of group I and II. PCR results from groups I and II are summarized in Table 2. Gel electrophoresis depicting the above PCR reactions from both groups are illustrated in Figure 1a, 1b, and 1c.

**Table 1.** The prevalence of different Rh phenotypes among 86620 blood donors of various races.

<table>
<thead>
<tr>
<th>Race</th>
<th>RhD+ donors</th>
<th>ccee</th>
<th>Ccee</th>
<th>CCee</th>
<th>CcEe</th>
<th>ccEe</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese</td>
<td>38,453 (99.62%)</td>
<td>81 (0.21%)</td>
<td>45 (0.12%)</td>
<td>14 (0.04%)</td>
<td>2 (0.01%)</td>
<td>4 (0.01%)</td>
<td>38,599</td>
</tr>
<tr>
<td>Indian</td>
<td>8,286 (94.49%)</td>
<td>442 (5.04%)</td>
<td>38 (0.43%)</td>
<td>1 (0.01%)</td>
<td>0</td>
<td>2 (0.02%)</td>
<td>8,769</td>
</tr>
<tr>
<td>Malays</td>
<td>35,620 (99.47%)</td>
<td>141 (0.39%)</td>
<td>38 (0.11%)</td>
<td>5 (0.01%)</td>
<td>2 (0.01%)</td>
<td>3 (0.01%)</td>
<td>35,809</td>
</tr>
<tr>
<td>Others</td>
<td>3,350 (97.30%)</td>
<td>86 (2.50%)</td>
<td>6 (0.17%)</td>
<td>1 (0.03%)</td>
<td>0</td>
<td>0</td>
<td>3,443</td>
</tr>
<tr>
<td>Total</td>
<td>85,709 (98.95%)</td>
<td>750 (0.87%)</td>
<td>127 (0.15%)</td>
<td>21 (0.02%)</td>
<td>4 (0.005%)</td>
<td>9 (0.01%)</td>
<td>86,620</td>
</tr>
</tbody>
</table>

* The phenotyping of D, C, c, E and e antigens were performed for all D- donors after exclusion of weak D by indirect antiglobulin test.

**Table 2.** PCR-SSP results of 36 D- Malaysian Chinese donors of ccee and Ccee phenotypes.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Number</th>
<th>Phenotype</th>
<th><em>RHD</em> Introns 4/7</th>
<th><em>RHD</em> exons 4/7</th>
<th>K409K mutation</th>
<th><em>RHCE</em> gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>19</td>
<td>ccee</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>c, e</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>Ccee</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>C, c, and e</td>
</tr>
</tbody>
</table>

*Group I=the absence of *RHD* gene with no *RHD* K409K mutation, Group II=Intact *RHD* gene with *RHD* K409K mutation
The frequency of D- phenotypes varies widely in different parts of the world and is common in Caucasians (15%) [18]. It is noted in 3 to 7% of Africans [18], 5% of Indians [19] and 0.2 - 0.4% of Chinese individuals [20]. Our study showed that the incidence of D- among Indian and Chinese blood donors was 5.51% and 0.38% respectively which is comparable to previously published data. The incidence of D- in Malay individuals was 0.53%. There is no published data on D- incidence in this ethnic group for comparison. The ccee phenotype was the most

Discussion

The frequency of D- phenotypes varies widely in different parts of the world and is common in Caucasians (15%) [18]. It is noted in 3 to 7% of Africans [18], 5% of Indians [19] and 0.2 - 0.4% of Chinese individuals [20]. Our study showed that the
common among our D- donors but the distribution of this phenotype varied widely among the different races as shown in Table 1. D- phenotypes with C and/or E antigens (such as Ccee, CCee, ccEe and CcEe) were more frequently seen in Chinese donors (44.52%) compared to the other two races. The proportion of these phenotypes was notably lower in Malay (25.40%) and Indian (8.49%) donors. To our knowledge, there are no published data on the prevalence of various RhD- phenotypes in Malay, Chinese and Indian individuals in this region. Understanding the distribution of different D-phenotypes is important in transfusion practice within a multi-ethnic environment and for situations such as estimating the availability of compatible blood as well as evaluating the cases of HTR and HDFN.

The RHD gene is polymorphic in the Chinese population [8, 13, 14, 17, 21-23]. A considerable proportion of apparently D- samples in this Chinese population from China and Taiwan were DEL phenotype with intact RHD gene [13, 14, 17, 24, 25]. RHD (K409K) mutation was found to be the underlying cause of almost all cases of DEL in Asia [2, 13, 24, 26]. In this study, DEL variant was observed in 33.33% (12/36) of D- Malaysian Chinese donors. All these individuals had an intact RHD gene with RHD (K409K) mutation. Although RHD (K409K) mutation is usually observed in DEL, this mutation has also been reported in some weak D phenotypes [13]. As the indirect anti-globulin test is routinely performed in our D- donors, weak D phenotypes were excluded in our donors with RHD (K409K) mutation. Although our sample size for PCR-SSP was small, there was a strong association between D- donors with RhC+ phenotype and DEL. Nearly three-fourths of our D- donors with Ccee phenotype were DEL variant with an intact RHD gene and RHD (K409K) mutation as shown in Table 2. This is in concordance with previous studies done in Chinese populations in China, Hong Kong and Taiwan [13, 24, 25, 27].

Although DEL is the weakest known D positive phenotype in the Rh system, the potential danger that DEL red cells might cause a clinical transfusion reaction cannot be completely excluded. It was noted that recipients who were truly D-negative developed anti-D after transfusion with DEL red blood cells [28, 29]. Therefore, it is important to exclude DEL donors from the D- donor pool particularly in areas with high incidence of DEL. Adsorption and elution technique is the only available serological method for the detection of DEL. This method is difficult and not feasible to be put into practice as a routine screening tool. Alternatively, molecular screening techniques can be used in D- donors particularly those with DcE haplotype for the detection of RHD (K409K) mutation. RhC phenotyping together with molecular screening is simple, reliable and more specific for the detection of this common mutation in D- individuals.

In a large study of 7688 D- Caucasian donors with ccee phenotype conducted by Wagner et al [15], the deletion of RHD gene was the major cause of D- phenotype in these individuals. RHD gene was not detectable in all of our Chinese donors with ccee phenotype. This is in keeping with results observed in a study in China by Ye et al [20]. In other studies where a primer targeting exon 10 was included as a part of PCR strategy in screening for D- donors, RhD alleles were observed in a few individuals. Nearly all of these cases had RhC+ phenotypes and most were found to have RHD-CE (2-9) D2 hybrid allele [13, 23, 30]. As the primer for detection of exon 10 was not included, the presence of such hybrid alleles cannot be completely excluded in our D- donors with Ccee phenotype. However, blood donors carrying RHD-CE (2-9) D2 hybrid allele may safely be overlooked because their RBC units are truly D- [31].

In conclusion, D- phenotype was found predominantly in the Indians with less than 1% noted in Malays and Chinese in our donor population. There was a wide variation in the distribution of different D- phenotypes among our three major races. Understanding the distribution of different D-phenotypes in a multi-ethnic donor population is important for good transfusion medicine practices. Moreover, this study also revealed that a significant number of our D- Chinese donors with Ccee phenotype were in fact DEL with RHD (K409K) mutation. DEL red cells are known to cause anti-D alloimmunization. Therefore, it is important to exclude DEL blood units from D- donor pools particularly in areas with high incidence of DEL.

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Table 1: D- phenotypes and genotypes of Malaysian Chinese donors

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccee</td>
<td>44.52%</td>
</tr>
<tr>
<td>CCee</td>
<td>25.40%</td>
</tr>
<tr>
<td>ccEe</td>
<td>8.49%</td>
</tr>
<tr>
<td>CcEe</td>
<td>10.97%</td>
</tr>
<tr>
<td>CCEE</td>
<td>0.56%</td>
</tr>
</tbody>
</table>

Table 2: Distribution of RHD (K409K) mutation in DEL donors

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>RHD (K409K) Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccee</td>
<td>Present</td>
</tr>
<tr>
<td>CcEe</td>
<td>Present</td>
</tr>
<tr>
<td>CCee</td>
<td>Present</td>
</tr>
</tbody>
</table>

The authors wish to thank all the blood donors who participated in this study. We also thank Mohd. Kamil and Mohd. Ashraf from Molecular and Genetic Analysis Laboratory, Laboratory Medicine Division, University Malaya Medical Centre for their technical assistance, Mrs. Prager, and Mr. Thomas from BAG Health Care for their helpful comments. This work...
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