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Brief communication (Original)

RH genotypes among Malaysian blood donors

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Background: *RH* genotyping studies have been conducted mainly in people of Caucasian and African descent. There is limited information regarding the molecular basis for *RH* genotypes in Malaysia.
Objectives: To investigate the prevalence and characteristics of *RHCE* genotypes among different ethnic groups in Malaysia.
Methods: A total of 1014 whole blood samples were obtained from donors from 4 different ethnic groups (360 Malays, 434 Chinese, 164 Indians, and 56 others). All samples were phenotyped for C, c, D, E, and e using standard serologic methods and genotyped using polymerase chain reaction (PCR)-based analysis.
Results: In the blood samples that we analyzed, the distribution of *RH* genotype antigens was significantly different among the various ethnic groups. Our findings showed that *CCDee* is the most common in Malaysian blood donors; 18.4% (187/1014) compared with other genotypes. The *ccDEE* genotype is more prevalent in the Chinese: 65.6% (82/125), and the *ccee* genotype is more prevalent in Indians: 47.1% (65/138). There were discrepancies between phenotypes and genotypes. There were 17 (1.7%) discrepancies in *RH C/c* genotyping results and of these 47% (8/17) occurred in Malays. Discrepancies in *RH E/e* results occurred in 3 samples (0.3%).
Conclusions: Our study provides a database for the distribution of *RH* genotypes of donors from the major ethnic groups in Malaysia. Methods used in this study are useful for comparing the phenotypes and genotypes. Further investigation should be conducted to study the causes of these discrepancies using other molecular based techniques.

Keywords: Chinese, Indians, Malays, Malaysia, *RH* genotypes

The Rh blood groups are the most complex and polymorphic of all human blood group systems, consisting of at least 45 different antigens, and can be further subdivided into various genotypes. The Rh antigens are also the most clinically significant in transfusion medicine [1, 2]. They are located on two proteins, RhD and RhCE: the former carries the D antigen, while the latter carries C, c, E, and e antigens. The two highly homologous genes encoding these antigens are located on the short arm of chromosome 1 (1p36.13-p34.3) and are inherited together [3].

In the past, it was only possible to determine the Rh phenotype by serologic typing of red blood cells. This serologic approach can be inconclusive in Rh phenotyping of fetuses, in patients who have recently been transfused and those harboring large quantity of donor red blood cells. In all these circumstances, *RH* genotyping is an option [4]. Serologic detection of polymorphic blood group antigens and phenotypes provide valuable sources of samples for molecular studies [5, 6]. Hemolytic disease of the fetus and newborn, autoimmune hemolytic anemia, and transfusion reactions are not only the result of anti Rh-D antibodies, but also sometimes the result of anti-Rh E/e or anti-Rh C/c antibodies [7]. Wide racial differences are recognized not only in frequency of Rh phenotypes, but also at the molecular level. Several *RH D*, *RH C/c*, and *RH E/e* genotyping assays have been developed. A number of studies have been carried out to assess the frequency of these three molecular backgrounds in people of African and Caucasian ancestry [2, 8].

Hyland et al. applied restriction fragment length polymorphism (RFLP) patterns to Southern blots for *RH* genotypes. However, they found a 100% correlation for 102 randomly selected blood donors for the Rh C, Rh e, and Rh D phenotypes, but only 94.8% for the Rh c and 94.3% for the Rh E phenotypes [9]. The sequence of *RH* genes may vary between different ethnic groups. It is important to be aware of the differences in genetic sequences in order to develop genotyping methods that are reliable in a multiracial population [10].

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Malaysia has a multiracial population comprising of Malays, Chinese, and Indians who are the major ethnic groups in Peninsular Malaysia; with other ethnic groups present especially in east Malaysia and in the north of Borneo island. In an effort to gain more insight into the molecular background and frequency of *RH* genotypes, we have undertaken a comprehensive study to determine *RH* genotype distribution among the ethnic groups in the Peninsular Malaysian population. Therefore, the main objective of this study was to determine the prevalence of *RH* genotypes in blood donors from the major ethnic groups in Peninsular Malaysia and to correlate Rh phenotypes with *RH E/e*, *RH C/c* and *RH D* genotyping results. Established *RH* genotyping assays were tested on DNA of phenotyped whole blood samples from donors from different ethnic groups in Malaysia.

Materials and methods

Samples

This study was approved by the Human Research and Ethics Committee of Universiti Sains Malaysia, and the Medical Research and Ethics Committee, Ministry of Health, Malaysia. A total of 1014 whole-blood samples (hemoglobin >12.5 g/dL) collected into a tube containing a potassium salt of ethylene diamine tetra-acetic acid (BD Vacutainer EDTA tube; Becton Dickinson & Co, Franklin Lakes, NJ, USA) was obtained from randomly selected volunteer donors who donated blood at the National Blood Centre, Malaysia or its mobile blood donation facilities between May 2011 and February 2012, and who fulfilled inclusion criteria specified by the National Blood Centre. In brief, donors were between 17 and 65 years old, in good health (as certified by a physician where the donor was 60–65 years old) with a minimum weight >45 kg, no history of recent illness nor a history of taking any medication. In addition, they must not have a history of any inherited bleeding disorder or conditions mentioned in the Guidelines for Donor Deferral. Written informed consent was obtained from eligible donors, or their parent or guardian where the donor was 17 years old. The donors included 360 Malays, 434 Chinese, 164 Indians, and 56 from other minority ethnic groups.

Serology

Red blood cells of all donor samples were phenotyped for C, c, D, E, and e by standard serologic methods using an automated Olympus PK7200 Blood Grouping System in accordance with validated protocols and the manufacturer's instructions. The commercial monoclonal antibody reagents used were from CSL (Parkville, Victoria, Australia), Bio-Rad Laboratories (Glattbrugg, Switzerland), and Millipore (Livingston, West Lothian, UK). CSL reagents were used to test the following specificities: monoclonal Epiclone-2 anti-D (RUM 1 and MCAD6), monoclonal Epiclone anti-c (MS33) and monoclonal anti-E (MS30 and MS258). Bio-Rad reagents were used to test the following specificities: monoclonal DiaClon anti-C (MS24) and monoclonal DiaClon anti-e (MS16, MS21, and MS63). The Millipore reagents were used to test monoclonal anti-D (TH28 and MS26).

Genomic DNA extraction

Genomic DNA was isolated from 200 μL of whole blood using a DNA isolation kit (NucleoSpin Blood, Macherey-Nagel, Germany). Oligonucleotide sequences of all primers used in this study are listed in **Table 1**. All primer used were those published and synthesized by Sigma-Proligo (Singapore).

RHC and RHc multiplex PCR

The *RHC* and *RHc* multiplex assays have been previously described and were optimized to suit local conditions [8]. An 8-primer multiplex PCR-based method was used to detect the presence of *RHD* exon 7 and intron 4, and the *RHCE C* and *c* alleles. The list of the primers is shown in **Table 1**. Amplification was conducted in a final volume of 25 μL, containing 5 μL of template DNA, 4.0 μL (1.25 mM) dNTP mix, 2.5 L multiprimer mix, 3.0 (50 mM) $MgCl_2$, 2.5 μL (10×) buffer, 0.125 (5U) Taq Polymerase (Bio-Rad, CA, USA). Thirty-two cycles of PCR were performed at 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min 30 s. PCR were performed in a thermal cycler (Veriti, Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands).

RHE and Rhe allele-specific primer amplification (ASPA) assays

Similarly, the *RHE* and *RHe* specific ASPA assays have also been previously described and were optimized to suit local conditions [11]. Three sets of primers were used and are shown in **Table 1**. For *RHE*, a combination of primers from Set A and Set B, and *RHe* from Set B and Set C were used. ASPA reactions were performed with 1.5 μL of template DNA, 3.2 μL primer mix and 10 μL 2× Exprime Taq

Premix (Genet Bio, Korea). PCRs were performed in a thermal cycler (Veriti). The conditions for RHE were 94°C for 30 s, 63°C for 30 s, and 72°C for 12 s while for *RHe* the conditions were 94°C for 30 s, 54°C for 30 s, and 72°C for 18 s. Both conditions were performed for 35 cycles.

Gel electrophoresis

PCR products were separated by size in a 2% agarose gel containing 0.1 g per mL of SybrSafe DNA gel stain (Invitrogen, CA, USA.) was used for all electrophoresis procedures. The results were visualized using a gel imaging system (U:Genius, Syngene, Frederick, MD, USA). A low range DNA ladder (Jena Bioscience, Jena, Germany) was used to determine level of PCR product present.

Results

Serology and genotyping findings

DNA from 1014 blood donors from 4 major different ethnic groups with various Rh phenotypes and genotypes were tested. Of these donors, 434 (42.8%) were Chinese, 360 (35.5%) were Malays, 164 (16.2%) were Indians, and the remaining 56 (5.5%) were from minor ethnic groups, grouped together as 'others'. We found that *RH* genotypes had a significantly different distribution among the ethnic groups based on *RH* genotypes. However, no significant associations were noted between discrepancies results in allele *C/c* and *E/e* and the ethnic groups (p = 0.05). A total of 187 blood donors were found to be *CCDee*, which is common in Malaysia, and 827 blood donors have rare and very rare phenotypes. The results showed that *ccDEE* was more prevalent in Chinese (8.1%) as compared with Malays, Indians, and others (3.4%, 0.5%, and 0.4% respectively). The prevalence of *ccee* was very low in all ethnic groups, but was relatively high in Indian donors. The distribution of *RH* genotypes among the 4 major ethnic groups in Malaysia is shown in **Table 2**. There were discrepancies found in serological and genotyping results as shown in **Table 3**.

RHC and RHc multiplex PCR analysis

We performed *RHC* and *RHc* multiplex-PCR analysis of all blood samples. Discrepancies between phenotyping and genotyping results were occasionally observed. This is mainly because the phenotype results fail to detect heterozygous genes. We found that 10 samples (8 Malays, 1 Chinese, and 1 other) phenotyped as Rh CC were shown to be of genotype *RH Cc* by multiplex-PCR analysis. Seven donor blood samples (3 Chinese, 3 Indians, and 1 other) were phenotyped as Rh cc, but genotyped as *RH Cc*. From the outcome of this study, *RHC+* showed false positive, mainly in Malays (47%), which were 8 out of 10 donors. In this study, no significant association was found between discrepancies in allele *C/c* results and any ethnic group.

Table 1. Summary of primers used for *RH C/c* and *RH E/e* genotyping

Primer Name		Direction	Sequence (5′ to 3′)	Product Size (bp)
RH C/c				
Exon 7		Forward	AGCTCCATCATGGGCTACAA	95
Exon 7		Reverse	ATTGCCGGCTCCGACGGTATC	
Intron 3		Forward	GGGTTGGGCTGGGTAAGCTCT	498 or 535
Intron 4		Reverse	GAACCTGCTCTGTGAAGTGCT	
RHC		Forward	CAGGGCCACCACCATTTGAA	320
RHC		Reverse	GAACATGCCACTTCACTCCAG	
RHc		Forward	TCGGCCAAGATCTGACCG	177
RHc		Reverse	TGATGACCACCTTCCCAGG	
RH E/e				
RHE	Set A	Forward	CCAAGTGTCAACTCTC	108
RHE	Set A	Reverse	TGACCCTGAGATGGCTGT	
RHD	Set B	Forward	ACAGACTACCACATGAAC	94
RHD	Set B	Reverse	GCTTTGGCAGGCACCAGGCCAC	
RHe	Set C	Forward	CCAAGTGTCAACTCTG	141
RHe	Set C	Reverse	CATGCTGATCTTCCT	

Table 2. The distribution of the *RH* genotype among 1014 whole blood donors at the National Blood Centre, Kuala Lumpur, Malaysia

	Ethnic group				
***RH* Genotype**	**Malay**	**Chinese**	**Indian**	**Others**	**Total**
CCDee	92 (25.6%)	80 (18.4%)	4 (2.4%)	4 (7.1%)	180
ccDEE	34 (9.4%)	82 (18.9%)	5 (3.1%)	4 (7.1%)	125
CcDeE	41 (11.4%)	62 (14.3%)	16 (9.8%)	2 (3.6%)	121
CCDeE	45 (12.5%)	32 (7.4%)	3 (1.8%)	5 (9.0%)	85
cCDEE	2 (0.5%)	9 (2.1%)	0 (0%)	0 (0%)	11
CcD^{weak}ee	54 (15.0%)	66 (15.2%)	27 (16.5%)	10 (17.9%)	157
ccDEe	25 (6.9%)	61 (14.1%)	20 (12.2%)	4 (7.1%)	110
ccDee	8 (2.2%)	17 (3.9%)	13 (7.9%)	4 (7.1%)	42
ccee	38 (10.6%)	14 (3.2%)	65 (39.6%)	21 (37.5%)	138
Ccee	16 (4.4%)	8 (1.8%)	8 (4.9%)	1 (1.8%)	33
ccEe	1 (0.3%)	1 (0.2%)	3 (1.8%)	0 (0%)	5
CceE	2 (0.6%)	0 (0%)	0 (0%)	1 (1.8%)	3
Ccee	2 (0.6%)	2 (0.5%)	0 (0%)	0 (0%)	4
Total	360 (100%)	434 (100%)	164 (100%)	56 (100%)	1014

Table 3. Discrepancies of serological phenotype and genotype results

Bil	Race	Serology phenotype result	Genotype results
1	Chinese	CCDee	*C**c**Dee*
2	Malay	CCDee	*C**c**Dee*
3	Malay	CCDee	*C**c**Dee*
4	Malay	CCDee	*C**c**Dee*
5	Malay	CCDee	*C**c**Dee*
6	Malay	CCDee	*C**c**Dee*
7	Malay	CCDee	*C**c**Dee*
8	Malay	CCDee	*C**c**Dee*
9	Malay	CCDee	*C**c**D**E**e*
10	Chinese	ccDEE	***C**cDEE*
11	Malay	CcDee	*CCD**E**e*
12	Chinese	ccDEe	***C**cDEe*
13	Indian	ccDEe	***C**cD**e**e*
14	Chinese	ccDee	***C**cDee*
15	Indian	ccDee	***C**cDee*
16	Indian	ccee	***C**cee*
17	Others	Ccee	***c**cee*
18	Others	ccEe	***C**cEe*

*Discrepancy shown in bold indicating the change of a single allele

RHE and Rhe allele-specific primer amplification assays

RHE and *RHe* allele-specific primer amplification assays of the 1,014 blood samples were performed. We found only 2 Malay donor samples phenotyped as Rh ee, but they appeared as *RH Ee* by genotyping. Only 1 Indian donor sample was phenotyped as Rh Ee, but this sample appeared as *RH ee* by genotyping. The discrepancies were observed in heterozygous genes. However, no significant association was noted between discrepancies in allele *E/e* results and any ethnic group.

Discussion

While the molecular background of blood groups are well characterized in populations of Caucasian descent and the differences between people of Caucasian and African ancestry are well established, there are limited data regarding Asian populations. Little is known of the molecular basis of the Rh and other blood group systems among the various ethnic groups that live in Peninsular Malaysia.

We have previously reported that the Rh blood group system has a significantly different distribution among ethnic groups [12]. In this study, Rh phenotypes and *RH* genotypes showed heterogeneity and significant association between all the ethnic groups among blood donors ($P < 0.05$). We also compared phenotypes with some Asian populations and they showed some similarity. For example, the *cDE/cDE* (*R2R2*) was more prevalent in Chinese donors than it was in other ethnic groups, with a distribution similar to that in the Chinese population in Hong Kong [13, 14].

We have conducted molecular analysis for Rh genotypes in 1014 donors, including 360 Malays, 434 Chinese, 164 Indians, and 56 from other minority ethnic groups in Malaysia using previously described PCR assays for *RH C/c* and *RH E/e* genotyping adapted for local conditions [8, 11]. We observed discrepancies in both *RH C/c* and *RH E/e* on phenotypes and genotypes. The *RHC* and *RHc* alleles have been reported to differ by a single nucleotide substitution in exon 1 and five base changes in exon 2 [15].

Here, we used *RHC* and *RHc* multiplex-PCR analysis for *RHC/c* genotyping in which the primers to detect the *RHC* allele were from intron 2, and for the *RHc* allele were from exon 2. We used this method to examine our 1,014 samples and found that there was a correlation between *RHC/c* genotype and phenotype in 997 samples, whereas there was a discrepancy in 10 samples being from 8 Malays, 1 Chinese, and 1 other who apparently had a CC phenotype, but were found to have a *Cc* genotype, and 7 samples being from 3 Chinese, 3 Indians, and 1 other who apparently had a cc phenotype, but were found to have a *Cc* genotype. All donors were *RHD* positive. This was also reported by Tanaka et al. who also showed discrepancy in 17 cases of 656 samples from among those who were phenotyped as cc, but were actually of the *Cc* genotype [16, 17].

Hyland et al. [9] used *Msp*I RFLP digestion patterns of the 3′ noncoding regions of the genes to determine *RH E/e* genotypes. For *E* genotype they showed a 100% concordance between the results of phenotyping and genotyping based on RFLP patterns, but for the *E* genotype the concordance was only 94.3%. The discrepancies they found between the results of phenotyping and genotyping appeared to be associated with the *cE* allele in D-negative subjects. The *cE* alleles in D-negative donors whose DNA was tested were all genotyped as *ce*. In this study, we used *RHE* and *Rhe* allele-specific primer amplification assays to determine the *RH E/e* genotype. We used this method for the same 1,014 blood donors as *RH C/c* genotype. We found a correlation between *RH E/e* genotype and phenotype in 1,011 samples, whereas there was discrepancy in 2 samples from Malay donors who had an ee phenotype, but an *Ee* genotype, and 1 sample from an Indian donor who had an Ee phenotype, but *ee* genotype. All cases were from RhD positive donors.

From studies of both *RH C/c* and *RH E/e* genotyping, we showed that discrepancy only occurs in RhD positive donors and samples with heterozygous alleles that cannot be detected by the phenotyping method. This may be the result of transmission of silent alleles at the Rh locus [11]. Different methods used in various laboratories and different monoclonal antibodies in FDA-licensed reagents may react differently in our population. The large number of different *RHD* and *RHCE* genes, can affect both the level of expression and, potentially, the structure of the molecule and D-epitopes.

In summary, we performed a PCR-based method to determine the *RH C/c* and *RH E/e* genotypes in the Malaysian population. We provide a basic database for the distribution of *RH* genotypes in blood donors of major ethnic groups of Malaysia. We also demonstrated that there may be discrepancies between Rh phenotyping and *RH* genotyping. This method has been shown to be useful to determine *RH* genotypes in recently transfused patients with a large amount of circulating donor cells and to identify compatible donors for patients with antibody to high incidence Rh antigen. The understanding of the molecular basis associated with Rh blood group antigens and phenotypes enables us to identify blood group antigens and antibodies using molecular approaches. Screening donors by DNA testing would conserve antibodies for confirmation by hemagglutination of predicted antigen negativity. Our findings may be used to devise future molecular analyses for mutation determination of *RH* genotypes in the Malaysian population.

The authors have no conflicts of interest to declare.

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