Original Article

Molecular basis of Rh blood group system in the Malaysian population

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Abstract:

Background: Rh molecular studies have been previously mainly conducted in Caucasians and African population. There is a limited data on the molecular basis for Rh genotypes among Asians. **Aims:** This study aims to characterize the Rh genes and frequency of the various RH genotypes among blood donors in National Blood Centre (NBC), Kuala Lumpur. **Materials and Methods:** A total of 1014 blood samples were obtained from blood donors from four different ethnic groups (360 Malays, 434 Chinese, 164 Indians and 56 others). Serological and molecular analysis of all 1014 blood samples were performed. An automated deoxyribonucleic acid sequencing analysis was performed. **Results:** Rh phenotypes and RH genotypes showed heterogeneity and significant association with ethnicities. Discrepancies in allele D, C/c and E/e between phenotypes and genotypes results were observed. Discrepancy results in allele D showed significant association with the ethnic groups of the blood donors in NBC. There were multiple novel mutations (23) and published mutations (5) found in this study. Significant associations between discrepancy results and mutations were found in allele D and C/c. **Conclusion:** Performing RH molecular analysis in Malaysian population provided the basic database for the distribution of Rh genotypes of donors from major ethnic groups in Malaysia.

Key words:

Allele, Rh genotypes, Rh molecular, Rh phenotypes

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Introduction

The Rh system is the most important of the commonly utilized blood grouping system. Two genes (RHD, RHCE) in close proximity on chromosome 1 encode the erythrocyte Rh proteins, RhD and RhCE in which one carries the D antigen and the other carries CE antigens. The genes have ten exons each and are 97% identical.[1] RHD and RHCE proteins differ by 32-35 of 416 amino acids. Individuals who lack RhD protein often have a complete deletion of the RHD gene. An important consideration in the immunogenenicity of a protein is the degree of foreignness to the host. The large number of amino acid changes explain why exposure to RhD can result in a potent immune response in a D-negative individual.[1]

Many RH genes carry point mutations, or have rearrangements and exchanges between RHD and RHCE that result from gene conversion events.[2] The latter encode hybrid proteins that have RHCE-specific amino acids in RHD or RHD-specific residues in RHCE. These can generate new antigens in the Rh blood group system and alter or weaken the expression of the conventional antigens.[1]

This serologic typing can be inconclusive, e.g., in patients who have recently been transfused and those harboring large quantity of donor red blood cells (RBC). In all these cases, Rh genotyping is an option.[3] Serologic detection of polymorphic blood group antigens and phenotypes provide valuable sources of appropriate blood samples for molecular studies.[4]

Molecular investigation of D variants has revealed that there are numerous different phenotypes. Nucleotide mutations that encode amino acid changes in the D protein are a common cause of variant phenotypes. The position of substitution is thought to be important in determining the D epitope and hence whether the variant can make anti-D.[5] The mutations could interfere with membrane integration of D protein, possibly by influencing the interaction of RhD protein with RhAG glycoprotein.[6]

Until date, no previous studies of molecular analysis in blood transfusion medicine in the National Blood Centre (NBC), Kuala Lumpur, Malaysia have been conducted. Malaysia has a multiracial population comprising of Malays, Chinese, Indians being the major races in Peninsular Malaysia. Other ethnic groups especially in East Malaysia in the north of Borneo island are also associated. It is therefore, timely to conduct a molecular and structural characteristics study and establish our local database on Rh blood group system for the Malaysian population.

Materials and Methods

Study population

A cross-sectional study was conducted from May 2011 to February 2012. A total of 1014 voluntary

blood donors who fulfilled the eligibility criteria according to the national guidelines for blood donation in Malaysia were recruited from NBC and mobile blood donation sessions of this study. Informed consent was obtained from the eligible donors. This study was approved by Medical Research and Ethics Committee, Ministry of Health Malaysia and Research and Ethics Committee of Universiti Sains Malaysia (USM).

Blood sampling

A volume of 6 ml of peripheral blood was collected in an ethylenediaminetetraacetic acid tube. Serological testing and molecular analysis (polymerase chain reaction, sequence specific primers [PCR-SSP]) of all 1014 blood samples were performed. 20 blood samples were sequenced by automated analysis.

Serological testing

RBC of all samples were phenotyped for C, c, D, E and e by standard serologic methods using the automated machine Olympus PK7200 in accordance with validated protocols and manufacturer's instructions. The commercial monoclonal antibody reagents used were from CSL (Australia), Millipore (United Kingdom) and Bio-Rad (Switzerland). 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). Millipore reagent was used to test monoclonal anti-D (TH28 and MS26). All antisera were prepared and diluted before running the machine. One part of each anti-C, c, E and e were diluted with 16 parts of 0.3% Bromelin and one part of anti-D was diluted with 64 parts of 0.9% normal saline. All the results were interpreted and printed by the machine.

Molecular testing

Genomic deoxyribonucleic acid (DNA) was isolated from 200 µl of whole blood using the DNA isolation kit (NucleoSpin® Blood, Macherey-Nagel GmbH and Co., KG, Germany).

PCR-SSP were performed for 10 exons of RHD and RHCE. Oligonucleotide sequences of all 26 primers used in the RH typing system and their combinations, amplification product lengths and specificities are listed in Table 1. In each PCR, control human growth hormone (HGH) oligonucleotides were used to amplify a 434 bp PCR fragment from the HGH locus position 5559 to 5992, which served as a positive amplification control.

Amplification was carried out in a final volume of 50 µl, containing 33.8 µl of H_2O, 5.0 µl (×10) buffer, 0.6 µl (10 mM) dNTP mix, 3.0 µl (25 mM) $MgCl_2$, 0.4 µl (25 µM) forward and reverse for all Rh primers [refer to Table 1], 0.2 µl (25 µM forward and reverse control primers) [Table 1], 0.4 µl (5U) Taq Polymerase (Exprime Taq, Genet Bio, Korea), 6 µl (50 – used were published and synthesized by Sigma-Proligo Singapore). 100 ng/µl of template DNA. PCR conditions were an initial denaturation step of 120 s at 94°C, 10 incubation cycles for 10 s at 94°C and 60 s at 65°C; and 20 incubation cycles for 30 s at 94°C, 60 s at 61°C and 30 s at 72°C. PCR was performed in a thermal cycler (Veriti, Applied Biosystem, Netherlands).

The PCR product was purified using Expin™ Kit (GeneAll, Korea). PCR products were separated by size in a 2% agarose gel containing 0.1 µg/ml of Sybr Safe DNA gel stain (invitrogen, U.S.A) and was used for all electrophoresis procedures. The results were visualized using the gel documentation system (U: Genius, Syngene, U.S.A). Low range DNA ladder (Jena Bioscience, Germany) was used to determine the levels of PCR product present.

Table 1: Primers used for RH PCR-SSP*

Exon	Specificity	Direction	DNA sequence of primers (5'-3')	Product size (bp)
2	RHD/C	Forward	GCT TGG GCT TCC TCA CCT CG	148
	RHD/C	Reverse	CAG TGT GAT GAC CAC CTT CCC AGA	
3	RHD	Forward	TTG TCG GTG CTG ATC TCA GTG GA	113
	RH^{all}†	Reverse	ACT GAT GAC CAT CCT CAG GTT GCC	
4	RH^{all}†	Forward	ACA TGA TGC ACA TCT ACG TGT TCG C	122
	RHD	Reverse	CAG ACA AAC TGG GTA TCG TTG CTG	
5	RHD/e	Forward	ATG TTC TGG CCA AGT GTC AAC TCT G	157
	RHD	Reverse	ctg ctc acC TTG CTG ATC TTC CC	
6	RH^{all}†	Forward	TTA TGT GCA CAG TGC GGT GTT GG	132
	RHD	Reverse	CAG GTA CTT GGC TCC CCC GAC	
7	RH^{all}†	Forward	GTT GTA ACC GAG TGC TGG GGA TTC	122
	RHD	Reverse	TGC CGG CTC CGA CGG TAT C	
9	RH^{all}†	Forward	tat gca ttt aaa cag GTT TGC TCC TAA ATC	83
	RHD	Reverse	AGA AAA CTT GGT CAT CAA AAT ATT TAG CCT	
10	RH^{all}†	Forward	TCC TCA TTT GGC TGT TGG ATT TTA AG	147
	RHD	Reverse	CAG TGC CTG CGC GAA CAT TG	
1	RH^{all}†	Forward	GAT GCC TGG TGC TGC TGG TGG AAC	112
	RHC/c (cyt48)	Reverse	GCT GCT TCC AGT GTT AGG GCG	
2	RHc/c (cyt48)	Forward	GGC TTG GGC TTC CTC ACC TCA	149
	RHc/c (cyt48)	Reverse	AG TGT GAT GAC CAC CTT CCC AGG	
5	RHE	Forward	GAT GTT CTG GCC AAG TGT CAA CTC TC	158
	RHE/e	Reverse	ct gct cac CAT GCT GAT CTT CCT	
5	RHD/e	Forward	ATG TTC TGG CCA AGT GTC AAC TCT G	158
	RHE/e	Reverse	ct gct cac CAT GCT GAT CTT CCT	
Control	HGH‡	Forward	TGC CTT CCC AAC CAT CTT A	434
	HGH‡	Reverse	CCA CTC ACG GAT TTC TGT TGT GTT TC	

*For all primers; lower-case letters are sequences occurring in introns; upper-case letters represent those in exons; †RH^{all} denotes primers that are specific for either RHD; RHC, RHc, RHc (cyt^{48}); RHE, or Rhe; ‡As control for amplification; primers for the HGH are used in all PCRs; HGH: Human growth hormone; PCR: Polymerase chain reaction; SSP: Sequence specific primers; DNA: Deoxyribonucleic acid

Automated sequencing

Purified PCR products were sequenced in both directions using applied biosystems 3730xl DNA analyzer and the reagent used was the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystem, U.S.A).

Result sequences were aligned using molecular evolutionary genetics analysis (MEGA) software version 4.0.2. (MEGA, USA) and compared with normal sequences for all 10 exons of RHD (ENSG00000187010) and RHCE (ENSG00000188672).

Statistical analysis

Statistical analyses in this study were analyzed using Statistical Package for Social Sciences (SPSS version 20.0, IBM Corporation, USA). Basic characteristics and prevalence of Rh phenotype and RH genotypes were described by using descriptive statistics. Association for categorical variables was carried out using Pearson Chi-square and Kruskal-Wallis test where the significant value is considered $P < 0.05$.

Results

Basic characteristics for this study showed that 68.6% (696) were male and 31.4% (318) were female. Of these donors, 42.8% (434) were Chinese, 35.5% (360) were Malays and 16.2% (164) were Indians. The remaining 5.5% (56) consisted of donors from other minority ethnic groups. More than 82% (834) of the donors in this study were RhD positive and 17.8% (180) were RhD negative. Apart from that, the biggest donor category was blood type O RhD positive, which consisted of 32.1% (325) followed by B RhD positive 27% (274), A RhD positive 23% (233) and AB RhD positive 0.2% (2).

Serogical testing and molecular analysis

The results showed that there was a significant association between Rh phenotypes and ethnic groups in blood donors with $P < 0.001$ and $\chi^2 = 148.286$. Rh phenotype showed that CCDDee (R_1R_1) was highest in Malays (52.7%), Chinese (43.1%), Indians (2.1%) and others (2.1%). This study also showed that ccDDEE (R_2R_2) was more prevalent in Chinese (65.9%) when compared to Malays, Indians and others (27.0%, 4.0% and 3.2% respectively) and CcDDEE (R_2R_Z) was found only in two Malays and eight Chinese donors. The ccee (rr) phenotype was very low in all ethnic groups, but was relatively high in Indian donors (47.8%).

The results also showed that there was a significant association between Rh genotypes and ethnic groups in blood donors with $P < 0.001$ and $\chi^2 = 141.836$. The findings of Rh genotypes were similar to Rh phenotypes, CCDDee was highest in Malays (51.1%), ccDDEE in Chinese (65.6%) and ccee in Indians (47.9%). D variants results showed that in all the donors, (C/c/cyt48) cDvaree was found in 33.3% of Malays and Indians, 27.8% in Chinese and 5.6% in others as compared to CCDvaree which was found only in 2 Malays and 1 Chinese.

The association between the Rh phenotypes and Rh genotypes with the ethnic groups is presented in Tables 2 and 3.

The donor's genotype results were interpreted based on the RH PCR-SSP analysis, which showed the presence and specificity of RHD, RH C/c and/or RH E/e alleles. The results were categorized as discrepancies in allele D, allele C/c and allele E/e. Discrepancy results in allele D showed significant association with the ethnic groups of the blood donors in NBC ($P > 0.05$). The results are shown in Table 4.

Table 2: Association between Rh phenotypes with ethnic groups among respondents

Rh phenotypes	Race ($N = 1014$)			
	Malay (%) ($n = 360$)	Chinese (%) ($n = 434$)	Indian (%) ($n = 164$)	Others (%) ($n = 56$)
CCDDee (R1R1)	99 (52.7)	81 (43.1)	4 (2.1)	4 (2.1)
ccDDEE (R2R2)	34 (27.0)	83 (65.9)	5 (4.0)	4 (3.2)
CcDDEe (R1R2)	40 (33.6)	61 (51.3)	16 (13.4)	2 (1.7)
CCDDEe (R1RZ)	45 (52.9)	32 (37.6)	3 (3.5)	5 (5.9)
CcDDEE (R2RZ)	2 (20.0)	8 (80.0)	0 (0)	0 (0)
CcDee (R1r)	49 (32.9)	64 (43.0)	26 (17.4)	10 (6.7)
ccDEe (R2r)	25 (22.3)	62 (55.4)	21(18.8)	4 (3.6)
ccDee (R0r)	8 (18.2)	18 (40.9)	14 (31.8)	4 (9.1)
ccee (rr)	38 (27.5)	14 (10.1)	66 (47.8)	20 (14.5)
Ccee (r'r)	15 (48.4)	8 (25.8)	6 (19.4)	2 (6.5)
ccEe (r"r)	1 (16.7)	1 (16.7)	3 (50.0)	1 (16.7)
CcEe (r'r")	2 (100)	0 (0)	0	0
CCee (r'r')	2 (50.0)	2 (50.0)	0	0

Significant association ($P < 0.001$) in Rh phenotypes ($\chi^2 = 148.286$) among ethnic groups

Table 3: Association between RH genotypes with ethnic groups among respondents

RH genotypes	Race ($N = 1014$)			
	Malay (%) ($n = 360$)	Chinese (%) ($n = 434$)	Indian (%) ($n = 164$)	Others (%) ($n = 56$)
CCDDee	91 (51.1)	79 (44.4)	4 (2.2)	4 (2.2)
ccDDEE	34 (27.2)	82 (65.6)	5 (4.0)	4 (3.2)
CcDDEe	42 (34.4)	62 (50.8)	16 (13.1)	2 (1.6)
CCDDEe	45 (52.9)	32 (37.6)	3 (3.5)	5 (5.9)
CcDDEE	2 (18.2)	9 (81.8)	0	0
CcDee	49 (32.2)	66 (43.4)	27 (17.8)	10 (6.6)
ccDEe	25 (22.7)	61 (55.5)	20(18.2)	4 (3.6)
ccDee	8 (19.0)	17 (40.5)	13 (31.0)	4 (9.5)
ccee	33 (26.9)	13 (10.9)	57 (47.9)	17 (14.3)
Ccee	12 (52.4)	4 (19.0)	5 (23.8)	1 (4.8)
ccEe	1 (33.3)	1 (33.3)	1 (33.3)	0 (0)
CcEe	1 (50.0)	0	0	1 (50.0)
CCee	1 (100.0)	0	0	0
(C/c/cyt48)cDDee	5 (71.4)	1 (14.3)	1 (14.3)	0
(C/c/cyt48)cDvaree	6 (33.3)	5 (27.8)	6 (33.3)	1 (5.6)
CCDvaree	2 (66.7)	1 (33.3)	0	0
ccDvaree	4 (36.4)	1 (9.1)	3 (27.3)	3 (27.3)
ccDvarEe	1 (25.0)	0	3 (75.0)	0

Significant association ($P < 0.001$) in Rh genotypes ($\chi^2 = 141.836$) among ethnic groups

DNA sequencing analysis

There were multiple novel mutations (23) and published mutations (5) found in our study. Table 5 shows point mutations, deletion, insertion and frameshift mutations. Out of 20 samples, 13 samples showed mutations and most of the donors were phenotyped as RhD negative, but genotyped as RHD variants (9 donors). Donor 3 with RhD positive and Donor 11 with RhD negative results showed nonsense mutations in exon 3 which had specificity for RHD. Most of the mutations occured in the RHD specificity, which were in exon 3, 4 and 7, while for RH C/c specificity, mutations occurred only in exon 9, which is specific for C/c/cyt48. There was no mutation observed in RH E/e specificity, which was in exon 11 and 12.

Table 4: Association between the discrepancy in results for allele D, C/c and E/e with the ethnicities of the respondents

Discrepancy results	Race (*N* = 1014)				Total (%)	χ^2	*P* value
	Malay (%) (*n* = 360)	Chinese (%) (*n* = 434)	Indian (%) (*n* = 164)	Others (%) (*n* = 56)			
Allele D							
Present	16 (39.0)	9 (22.0)	12 (29.3)	4 (9.8)	41 (4.0)	10.395	0.015*
Absent	344 (35.4)	425 (43.7)	152 (15.6)	52 (5.3)	973 (96.0)		
Allele C/c							
Present	21 (42.0)	17 (34.0)	9 (18.0)	3 (6.0)	50 (4.9)	1.706	0.636
Absent	339 (35.2)	417 (43.3)	155 (16.1)	53 (5.5)	964 (95.1)		
Allele E/e							
Present	2 (50.0)	0	2 (50)	0	4 (0.4)	5.014	0.171
Absent	358 (35.4)	434 (43.0)	162 (16.0)	56 (5.5)	1010 (99.6)		

*Significant association ($P < 0.05$) in allele D compared to allele C/c and E/e ($P = 0.05$)

Significant association between discrepancy results and mutation were found in allele D and C/c with $P < 0.001$.

Discussion

In this study, Rh phenotypes and RH genotypes showed heterogeneity and significant association between all the ethnic groups in blood donors. All Rh phenotypes (N = 1,014) were properly characterized for D, C, c, E and e. The Rh phenotype showed that CCDDee was more prevalent phenotype (18.5%) and genotype (17.6%) in blood donors with highest in Malay population[phenotype (52.7%) and genotype (51.1%)]. These findings were comparable to prevalence found in another study on Asian subjects but higher than the prevalence in Caucasians (42%), Blacks (17%) and Native Americans (44%).[7] The Thai population showed the frequency of CCDDee (R1R1) to be at 51.5%, which is similar to our Malay participants.[8]

This study showed that ccDDEE (R2R2) was more prevalent phenotype (65.9%) and genotype (65.6%) in Chinese population when compared to Malays, Indians and others (27.0%, 4.0% and 3.2% respectively). The Chinese population in Hong Kong showed 19% prevalence of ccDDEE.[9] CcDDEE (R2RZ) is considered a rare phenotype[7] and CDE is usually found in people from Southeast Asia,[10] but we found it in two Malays and nine Chinese.

The ccee (rr) was very low in all ethnic groups but was relatively high in Indian donors. On the other hand, this genotype was found to be on a higher side in Caucasians (15.0%) and Africans (3-7%).[11] The Chinese population in Shenzhen Blood Centre, China showed only 1.2%[12] and Hong Kong only showed (2.0%) with ccee.[9]

Ethnic origin has been suggested to influence deductions of genotypes as the incidence of Rh antigen and genes differs from one geographic group to another. For example, a white person with the phenotype cDe would probably be ccDdee, but in a Black person, the genotype could be either ccDDee or ccDdee.[13] The present study results contribute to increasing knowledge of the differences in the molecular genetics and expression of the D antigen among Asians, European and African population. Although these differences are of interest from the perspective of evolution and development of the Rh blood group system, this also has practical implications for transfusion services serving the Malaysian population, because the population frequencies of D antigen and genes are two of three major factors contributing to an improved transfusion strategy as proposed by Wagner *et al*.[14] Recognizing the different RHD alleles and their frequencies in different populations in Malaysia and other Asian populations supports the development of pertinent Rh-related transfusion and obstetric practices.

Discrepancy results in allele D, showed significant association with the ethnic groups of the blood donors in NBC ($P > 0.05$). However, no significant association was noted between discrepancy results in allele C/c and E/e with the ethnic groups ($P = 0.05$). Variant RHD has different gene frequency among different ethnic groups. RhCE, however, seems to be less variables because C and c cover fewer epitopes, it might be complex to design reliable genotyping assays for RHCand RHc.[15]

Among the 1014 respondent's blood samples, the study detected 41 (4.0%) discrepancy results in allele D with 16 Malays (39.0%), 12 Indians (29.3%), 9 Chinese (22.0%) and 4 others (9.8%). From these results, it was observed that 35 out of 41 (85.4%) were from RhD negative phenotypes and only remaining 6 (14.6%) were RhD positive phenotypes [Table 5]. The RhD negative were phenotyped as D negative, but genotyping showed RHD variants results. This finding was comparable with what was found in another study; the Shanghai population showed 13.8%[11] and Denmark only 2.7%.[16]

The serological analysis failed to detect allele D due to multiple factors complicating the determination of the D status. This includes the different monoclonal antibodies in Food and Drug Administration-licensed reagents that can react differently with variant D antigens. The large number of different RHD genes, which can affect both the level of expression and potentially, the structure of the molecule and D-epitopes.[1]

The discrepancy results in allele C/c were observed in 50 out of 1014 (4.9%) with 21 Malays (42.0%), 17 Chinese (34.0%), 9 Indians (18.0%) and 3 others (6/0%). Out of 50, 25 showed the RH (C/c/cyt48) genotype, which cannot discriminate between RHC and RHc. This can be explained by an infrequent RHc variant, denoted Rhc (cyt48) that shares cytosine 48 in exon 1 with RHC.[17] The phenotypes that showed this were genotype as CCDDee (6), CcDee (1), ccee (8), Ccee (8) and CCee (2). These results were compared with a study done in Caucasian from Austria and Germany which observed CcDee (68), ccDEe (1), ccDee (1) and CcDEe (24).[17] This study also detected four blood samples with CC phenotype that actually was Cc by genotype and 5 were cc phenotype, but were Cc on genotype on molecular testing. This was also reported by

Table 5: List of alleles mutations found from exon sequencing in respondents

Donors	Exon	Nucleotide change*	Amino acid substitution	Mutation	Genotype
Novel mutation					
7	1	49 G>C	Ala17Pro	Missense	ccDvarEe
3	3	428 T>C	Val143Ala	Missense	(C/c/cyt48)cDDee
		429 G>A			
		429 ins AC	Leu144Thr		
		433 G>A	Val145Stop	Nonsense	
11	3	387 del T	—	Silent	CcEe
		388 G>A			
		390 C>G	Val130Cys	Missense	
		394 G>C	Leu 131Trp	Missense	
		411 G>A	Ala137His	Missense	
		415 T>C	Leu 139>Ser138	Frameshift	
		421 G>A	Val141>Stop140	Nonsense	
9	4	505 A/C>T	Met/Leu169 Leu	Missense/silent	ccDvaree
		527 C>A	Ala176Glu	Missense	
		530 C>G	Ala177Gly	Missense	
14/19	4	533 A>T	Tyr178Phe	Missense	CCDvaree/ccDvaree
		566 del A	Lys189Ser	Missense	
8/13	7	992 A/T>C	Asn/Ile331Thr	Missense	(C/c/cyt48)cDvarEe/CCDvaree
13	7	974 G/T>A	Ser/Ile325Asn	Missense	CCDvaree
		985 G/C>A	Gly/Arg329Ser	Missense	
14/15/19	7	1025 T/C>A	Ile/Thr342Asn	Missense	CCDvaree/(C/c/cyt48)cDvaree/ccDvaree
5	9	1175 T>A	Ile392Asn	Missense	(C/c/cyt48)cDDee
		1176 A>T			
13	9	1200 del A	Lys400Asn	Missense	CCDvaree
		1222 ins TCT	Trp408Phe	Frameshift	
14	9	1168 C>T	Leu390Phe	Missense	CCDVaree
		1171 del A	—	Frameshift	
		1172 del A			
		1173 del A			
		1174 del A			
		1176 A>T	Ile392>391Tyr	Frameshift	
		1181 A>T	Lys394>Asn393	Frameshift	
Published mutation					
10/17	3	410 C>T	Ala137Val	Missense	(C/c/cyt48)cDvarEe/(C/c/cyt48)cDvaree
6/10	3	455 A>C	Asn152Thr	Missense	(C/c/cyt48)cDDee/(C/c/cyt48)cDvarEe
14	3	361 T/A>C	—	Silent	CCDvaree
		365 C>T	Ser122 Leu	Missense	
9/10	7	1048 G/C>T	Asp/His350Tyr	Missense	ccDvaree/(C/c/cyt48)cDvarEe

*RHD/RHCE normal sequence

Tanaka, *et al*., 1997, also showed a discrepancy in 17 blood samples who were phenotyped as cc but were actually of Cc genotype.[18]

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.[19] In those with African ancestry, complete correlation was observed and no false negatives were detected among all serologically positive RHC samples with RHC-associated with 48 cytosine (cyt48) in exon 1. However, among 55 Rhcc phenotyped Caucasian samples, four false positives were observed and among 80 RhCc African American samples, 45 false positives were observed.[20] This was comparable with the finding of this study that showed 25 out of 50 samples showed RH (C/c/cyt48) genotype, which cannot discriminate between RHC and RHc serologically.

A study by Hyland *et al*. used Msp I restriction fragment length polymorphism (RFLP) digestion patterns of the 3' non-coding regions of the genes to determine Rh E/e genotypes. A100% concordance was seen between the results of phenotyping and genotyping based on RFLP patterns, but for E, the concordance was only 94.3%. The discrepancies found between the results of phenotype and genotype testing 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.[21] In Netherlands, allele-specific primer amplification for RH E/e genotyping was used and full concordance between Rh E/e genotyping and serology phenotype results.[22] Although some studies showed the full concordance between RH E/e genotyping and phenotyping, but one must be aware of the fact that discrepancies may occur in rare cases, such as where there is the transmission of silent alleles at the Rh locus.

In this study, RH PCR-SSP analysis was used and the results showed discrepancy in only 4 (0.4%) of which 2 were Malays, which phenotyped as ee but Ee on genotype testing and 2 were Indians of which one was of Ee phenotype, but was ee on genotype testing and the other was vice versa. Three cases were from RhD positive donors and only one from RhD negative donor.

These discrepancies were observed, because of weak reaction on phenotype testing and false results by Olympus PK7200 machine .

Though this study involved a small number of samples; despite of this, a variety of different variant D genotypes and mutations were found. Sequencing of the samples revealed a novel mutation with detected published mutations. In this study, significant association between discrepancy results in allele D with the presence of mutation due to nucleotide changes and amino acid substitution ($P > 0.05$) was seen. Many studies have focused on the molecular causes of the involved weak D and DEL phenotypes. Some of these alleles express the D antigen so weakly that they may not be detected by routine serology. Frequencies of RHD alleles may differ significantly among ethnic groups even within one population.

This study showed that the discrepancy results in allele D varied among the ethnic groups such as Malays (39.0%), Indians (29.3%), Chinese (22.0%) and others (9.8%). Table 6 shows comparable results to those found in populations of China, Japan and South Korea.[23] The findings of the present study along with other studies, indicate that the prevalence and molecular basis of D variants in Asians are significantly different from those in European and African populations.

Novel mutations were found in 10 donors out of 13 donors with mutations. Donors 3 and 11 had a nonsense mutation in exon 3, which indicates RHD specificity. Donor 3 had an AC insertion after nucleotide position 429 and donor 11 had a T insertion after nucleotide position 387 resulting in a frameshift mutation. This mutation leads to a premature termination and causes a non-functional protein leading to deletion of the D antigen. Interestingly, this study also found one of the samples was RhD positive with genotype (C/c/cyt48)cDDee. In exon 7, the common mutation was RHD/RHCE (1025 T/C>A) resulting in amino acid substitution (Ile/Thr432Asn) and was present in donor 14, 15 and 19. All these 3 donors were of RhD variants genotype.

Exon 9, which is specific for RH C/c/cyt48, showed 3 RhD positive donors (5, 13 and 14) with mutations. Donor 13 and 14 were CCDvaree and donor 5 with (C/c/cyt48) cDDee genotype. Donor 13 had an A deletion at nucleotide position 1200 and codon insertion (TCT) after nucleotide position 1222 resulting in a frameshift mutation while donor 14 had a AAAA deletion at nucleotide position from 1171 to 1174 and resulting in frameshift mutation. Previous study had also showed that mutations were found among samples of partial RHD, which expanded the genetic heterogeneity of RHD^{VI} and RHDFR, which were caused by a single point mutations.[17]

In all ethnic groups, RHCe and RHcE alleles seems to be strongly preserved for mutations in exon 2 that are specific for c expression. This was expected for P103, as the amino acid is essential for c expression, but it also seems to be the case for L60.[15] For this study, no mutation was found in exon 2. This finding is similar to that found in the Netherlands.[15]

RH C/c polymorphisms were first described by Mouro *et al*. and they found a W at amino acid position 16 (G48) on the Rhc allele derived from white donors of which RHC with G48 is the predominant genotype. Results showed that 48 G > C mutation in exon 1 of the RHC allele in absence of a RHC allele is highly frequent in blacks (67.3% in South Africans, 47.9% in Ethiopians and 41.9% in Curacao).[24] However, no mutation at 48 G > C was found, but one mutation was found at 49 G > C which was atamino acid position 17 on the RHc allele derived from Malay donor. This was a novel mutation; no published data was found with this mutation.

In this study, novel mutations were found at exon 4 in 3 donors (9, 14 and 19), which involved missense mutations. All donors were RhD variants with donors 9 and 19 were RHccee and donor 14 was RHCCee. Donor 9 (Indian) had 3 mutations at 505 A/C > T, 527 C > A and 530 C > G and led to Met/Leu169 Leu, Ala176Glu and Ala177Gly. Donor 14 and 19 (Malays) had same mutation at 533 A > T and had a deletion at nucleotide position 566 and led to Tyr178Phe and Lys189Ser. From these findings, it showed that different ethnic groups likely have different mutations as results showed that donors 14 and 19 were Malays and had same mutations compared to Donor 9 (an Indian) who had a different mutation. In Korea, a novel mutation at exon 4 in Weak D donor was found which was missense mutation at 605 C > T that led to Ala202Val.[23] In China, they also found 2 missense mutations in exon 4 from one sample weak D donor. The mutations occur at 594 A > T and 602 C > G that led to Lys198Asn and Thr201Arg.[25] Recognizing the different RHD molecular mechanisms and their frequencies in different populations, additional studies including other ethnic groups, especially in East Malaysia, should be carried out to optimize Malaysian population regional data. In conclusion, performing RH molecular analysis in Malaysian population provided the basic database for the distribution of Rh genotypes of donors from major ethnic groups in Malaysia. The understanding of the molecular bases associated with Rh blood group antigens and phenotypes enables consideration of the identification of blood group antigens and antibodies using molecular approaches.

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Table 6: Comparison of discrepancy in results for allele D and their frequencies in various populations in Asian studies

Population	Japanese	Han Chinese							Korean		Malay	Chinese	Indian	Others
Location	Osaka	Mainland China			Taiwan				Seoul	Gwangju	Malaysia			
First author	Hiroshzi	Lan	Shao	Qun	Sun	Peng	Lee	Chen	Kim	Timo	Present study			
Year	1997	2000	2002	2005	1998	2003	2003	2004	2005	2006	2013			
Discrepancy results in allele D	28.0	25.0	19.0	31.0	33.0	20.0	22.0	32.0	17.0	15.0	39.0	22.0	29.3	9.8

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