Analysis of *RHD* Genes in Taiwanese RhD-Negative Donors by the Multiplex PCR Method

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The determination of the RhD phenotype is important in transfusion medicine. However, due to the complexity of D antigen expression, the routine serological method cannot differentiate all RhD variants. In addition, the induction of the anti-D antibody is still the major cause of severe hemolytic disease of the newborn (HDN). Therefore, it is important to understand *RHD* gene profiles. To analyze the *RHD* gene profiles of Taiwanese RhD-negative donors, the multiplex PCR method was applied to amplify *RHD* specific exons 3, 4, 5, 7, and 9. Based on the PCR results, the 156 RhD-negative donors were divided into

12 groups according to the different expression patterns of the *RHD* gene. These 12 groups were further divided into three categories: type I = Rh D_{el} (21.8%); type II = partial D, containing some exons (9.0%); and type III = true RhD-negative (69.2%). The results indicated that 21.8% of RhD-negative donors in Taiwan were RhD_{el}, and 9% carried a part of the *RHD* gene. Six defined RhD variants were found in this study: four R_O^{Har}, one D^{Va}, and two D^{Iv}. However, no true RhD-negative or RhD_{el} donor with the CcdEe phenotype was found in this analysis. J. Clin. Lab. Anal. 17:80–84, 2003. © 2003 Wiley-Liss, Inc.

Key words: RhD-negative; Del; partial D; multiplex PCR; Chinese

INTRODUCTION

The Rh blood group system has been known for more than 60 years, since a hemolytic transfusion reaction was first described by Levine and Stetson (1). Thanks to improvements in medical knowledge and technology, Rh-incompatible blood transfusion reactions and pregnancy-induced hemolytic disease of the newborn (HDN) can now be prevented and treated (2). To prevent RhD-incompatible HDN, pregnant women and children of RhD-negative mothers should be typed for RhD. Once the phenotype of the fetus of an RhD-negative mother is identified as positive, a prevention program should be undertaken. Usually a blood product called Rh immunoglobulin (RhIg; also known as RhoGAM) is given to the mother to suppress antibodies reacting to the Rh-positive cells. In recently-transfused pregnant women, it can be difficult to obtain a serological determination; therefore, it is important to determine the Rh phenotype carefully and correctly (3). The determination of whether a subject is RhD-positive or -negative is based on whether or not the D antigen is present on the red cell membrane. In the conventional serological method, anti-D antibody is used to react with blood cells of the tested subject. However, apparent low expression of D antigen, or partial D resulting from

aberrant expression of one or more antigens cannot be detected by the routine serological method (4,5).

In 1993, DNA typing was first applied for classifying RhD blood groups and preventing HDN (6). Since then, this method has been widely used to analyze the *RHD* gene (7–9). Because the structure of *RHD* gene is so complex and highly homologous to the *RHCE* gene (5,10,11), and the genetic diversity has been evidenced underlying RhD-negative people (12,13). Thus, it is necessary to use more than one pair of primers to prevent false results. A previous study (10) found that while no *RHD*-specific gene was detected among RhD-negative Europeans, most RhD-negative Africans have an *RHD* gene. About the same time, Okuda et al. (14), using the PCR method to amplify *RHD*-specific exons 4 and 10, also found the *RHD*-specific gene in RhD-negative Japanese and Africans. These data suggest that

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the *RHD* genes vary among people of different origins and ethnicities. We used the multiplex PCR method amplifying the specific exons 3, 4, 5, 7, and 9 of the *RHD* gene to examine the *RHD* gene profiles of Taiwanese RhD-negative donors. It is hoped that these analyses will provide valuable information to help minimize the occurrence of RhD-induced transfusion reactions and HDN.

MATERIALS AND METHODS

Serological Rh Phenotyping

Blood samples from 156 RhD-negative and 30 RhD-positive donors were kindly provided by Taichung Blood Center, Chinese Blood Service Foundation, Taichung. The presence of D antigen in these samples was determined by the use of monoclonal anti-D (Gamma Biologicals, Inc., Houston, TX) applied on an automatic analyzer (TK 7200; Olympus, Tokyo, Japan). Bromelain digestion was included in this method. An antihuman globulin test was used to recheck the D antigen in all RhD-positive and -negative samples. Further Rh phenotyping for the five major antigens (C, c, D, E, and e) was carried out with a commercially available antibody (DiaMed, Cressier, Switzerland) according to the conventional serological method.

Detection of D_{el} Phenotype

Adsorption and elution tests were performed on all 156 RhD-negative samples, and the eluate was prepared by the chloroform elution method according to the AABB technical manual (15).

Multiplex PCR Analysis

Genomic DNA prepared from whole blood was subjected to multiplex PCR analysis. The sequences of the primer set were adopted from Maaskant-van Wijk et al. (16). Five sets of primers were used to amplify *RHD*-specific exons 3, 4, 5, 7, and 9. The lengths of the product of each primer set specific for exons 3, 4, 5, 7,

and 9 were 111, 126, 157, 96, and 71 bps, respectively. A pair of nucleotides were used to generate a 200-bp PCR fragment from the β -actin gene, which was included as an internal control. The five RhD exons were amplified together in a reaction tube. The multiplex PCR conditions used in this study were slightly modified from those described by Maaskant-van Wijk et al. (16), as follows: 5 min at 95°C for denaturation, followed by 1 min at 95°C, 1 min at 55°C, and 45 sec at 72°C for 32 cycles, and one cycle of 5 min at 72°C. Each 50- μ L PCR reaction mixture contained 1 μ g of DNA, 0.6 mM of dNTPs, 2 U of Taq DNA polymerase, 1.5 mM of $MgCl_2$, and primers. The PCR products were separated by electrophoresis in a 12% acrylamide gel.

RESULTS

The Rh phenotyping of all 156 RhD-negative individuals was determined by the routine serological method. In this group the incidence for the ccdee phenotype was 77 in 156 (49.3%); for the Ccdee phenotype, 59 in 156 (37.8%); for the CCdee phenotype, 14 in 156 (9.0%); for the ccdEe phenotype, 5 in 156 (3.2%); and for the CcdEe phenotype, only one (Table 1). However, by the adsorption and elution method, 34 donors were determined as D_{el} (22.3%) among these serologically RhD-negative individuals. As shown in Table 1, 27 individuals (79.4%) had the Ccdee phenotype, six (17.6%) had the Ccdee phenotype, and one (3%) had the ccdee phenotype.

Based on multiplex PCR analysis, *RHD* exons (including 3, 4, 5, 7, and 9) were present in all 30 serologically RhD-positive individuals, as visualized on the acrylamide gels (at size 111, 126, 157, 96, and 71 bp, respectively). Of the 156 RhD-negative individuals, only 27 individuals showed all exons examined; 108 donors lacked all exons examined (these were classified as true RhD-negative), and in the remaining 21 individuals certain exons of these five exons were amplified. These 156 RhD-negative donors were divided into 12 groups according to the different expression patterns (Table 2) with the exception of group L, all groups consisting of 48 individuals carried at least one exon.

TABLE 1. The Rh phenotypes of serological D negative, true D negative, D_{el} and partial Ds donors

Phenotype	Serological D negative	True D negative	D_{el}	Partial D
	No. (%)	No. (%)	No. (%)	No. (%)
ccdee	77 (49.3)	76 (70.4)	1 (3.0)	0 (0)
Ccdee	59 (37.8)	25 (23.1)	27 (79.4)	7 (50)
CCdee	14 (9.0)	2 (1.9)	6 (17.6)	6 (42.9)
ccdEe	5 (3.2)	5 (4.6)	0 (0)	0 (0)
CcdEe	1 (0.6)	0 (0)	0 (0)	1 (7.1)
Total	156 (100)	108 (100)	34 (100)	14 (100)

TABLE 2. Multiplex PCR results of 156 serological RhD-negative donors and their Rh phenotypes

Group	<i>RhD</i> gene					D(-)C(+)c(+/-)			D(-)C(-)c(+)			
	Exon3	Exon4	Exon5	Exon7	Exon9	CCdee	Ccdee	CcdEe	Total	ccdee	CcdEe	Total
A	+	-	-	-	-	1	0	0	1	0	0	0
B ^a	-	-	+	-	-	2	2	0	4	0	0	0
C	+	-	+	-	-	0	1	1	2	0	0	0
D	-	-	-	+	+	0	1	0	1	0	0	0
E ^b	+	+	+	-	-	1	1	0	2	0	0	0
F	+	+	-	+	-	1	0	0	1	0	0	0
G	-	+	+	+	-	0	1	0	1	0	0	0
H	-	-	+	+	+	0	1	0	1	0	0	0
I	+	+	+	+	-	0	7	0	7	0	0	0
J ^c	+	+	-	+	+	1	0	0	1	0	0	0
K	+	+	+	+	+	6	20	0	26	1	0	1
L	-	-	-	-	-	2	25	0	27	76	5	81
Total						14	59	1	74	77	5	82

^aThis group was most likely classified as Ro^{Har}.

^bThis group was most likely classified as D^{IV}.

^cThis group was most likely classified as D^{Va}.

It was noted that the genotypes of these 34 D_{el} individuals either contained all exons examined (27 out of 34, group K) or lacked only exon 9 (7 out of 34, group I), and the latter all had the Ccdee phenotype. Our data indicated that in addition to D_{el}, 9.0% of the RhD-negative individuals also possessed a part of the *RHD* gene. Antigen C (either Ce/Ce or Ce/ce) was expressed in all of the individuals who carried at least one exon, except for one who had the ccdee phenotype and carried all five exons.

Rearranged *RHD* genes are responsible for some partial D phenotypes. Based on classification criteria of Maaskant-van Wijk et al. (16), in our test group four samples expressed only *RHD* exon 5 and were classified as Ro^{Har}; two individuals lacking *RHD* exons 7 and 9 were designated as D^{IVb}, and one subject lacking exon 5 (but with other exons intact) was classified as D^{Va}.

DISCUSSION

The most common phenotypes of serological RhD-negative individuals in our group were ccdee (49.3%; estimated haplotype cde/cde), Ccdee (37.8%; estimated haplotype Cde/cde), and CCdee (9.0%; estimated haplotype Cde/Cde). This was in accordance with a previous study performed on another Taiwan Chinese group (17). The results also indicated that in Taiwan Chinese, cde was the most prevalent haplotype among RhD-negative individuals, followed by Cde. This distribution pattern was consistent with the data reported by Lan et al. (18), who used gene counting to estimate the number of RhD-negative Chinese living in mainland China. Using a *z*-test to check the proportionate difference between two groups, we compared the

distribution patterns of RhD-negative individuals in our test population and a Japanese population (19). The results were statistically significant ($P < 0.01$, Table 3) for ccdee, Ccdee, CCdee, ccdEe, and CCdEe. For three other phenotypes (ccdEE, CcdEE, and CcdEe), which were not detected in our test population, the differences were not significant. This may have been due to the low incidence of either phenotype in both populations.

In this study, we adopted a multiplex PCR method to analyze the *RHD* gene profiles of Taiwanese serologically RhD-negative individuals. *RHD*-specific exons 3, 4, 5, 7, and 9 were amplified simultaneously. All five exons were amplified from 30 serologically RhD-positive individuals, whereas the 156 serologically RhD-negative individuals showed different expression patterns. Among these serologically RhD-negative individuals, most of the D_{el} individuals (who expressed D antigen on red blood cells, albeit very weakly) contained all five exons (Table 2, group K). However, about 20% of the D_{el} individuals lacked exon 9 (group I). The lack of exon 9, but not other exons, for some D_{el} individuals was in accordance with one previous study that pointed out human Rh D_{el} was caused by a gene deletion including the exon 9 of *RHD* gene (20). However, our study indicates that only about 20.6% of the D_{el} individuals had this kind of mutation. All of the D_{el} individuals lacking exon 9 were of the Ccdee phenotype. In addition to D_{el}, 9.0% (14 individuals) also carried a part of the *RHD* gene (partial D); the rest (108 individuals; 69.2%) had no exons and were classified as the true D negative. Within the D_{el} group, the most prevalent Rh phenotypes were Ccdee (79.4%) and CCdee (17.6%). Both Ccdee and CCdee were also prevalent in D_{el} individuals of the Japanese and

TABLE 3. The phenotype distributions of serological RhD-negative individuals in Taiwan Chinese and Japanese.

Rh phenotype	Taiwan Chinese ^a No. (%)	Japanese ^b No. (%)	<i>P</i> value ^c
ccdee	77 (49.3)	59 (19.3)	<0.001
Ccdee	59 (37.8)	86 (28.1)	<0.01
CCdee	14 (9.0)	5 (1.6)	<0.001
ccdEe	5 (3.2)	59 (19.3)	<0.001
CcdEe	1 (0.6)	66 (21.6)	<0.001
ccdEE	0 (0.0)	29 (9.5)	0.549
CcdEE	0 (0.0)	1 (0.3)	0.675
CCdEe	0 (0.0)	1 (0.3)	0.675
Total	156 (100)	306 (100)	

^aData obtained from this study.^bData obtained from Fukumori et al. (19).^cStatistic differences between this two populations were calculated from *z* test.**TABLE 4. The incidences of D_{el} in different populations with various serological RhD-negative phenotypes**

Rh phenotype	Taiwan Chinese ^a %	Taiwan Chinese ^b %	Hong Kong Chinese ^c %	Japanese ^d %	Japanese ^e %
Ccdee	45.8 (27/59)	68 (13/19)	68.2 (105/157)	61.6 (53/86)	50.2
CCdee	42.9 (6/14)	93 (14/15)	81.3 (26/32)	80 (4/5)	64.7
CcdEe	0.0 (0/1)	0.0 (0/2)	60.0 (3/5)	68.2 (45/66)	56.1
ccdee	1.3 (1/77)	4.5 (1/22)	0.0 (0/264)	0.0 (0/59)	0.3
ccdEe	0.0 (0/5)	0.0 (0/2)	0.0 (0/7)	0.0 (0/59)	0.4
CCdEe	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	0.0 (0/1)	0.0
CcdEE	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	0.0 (0/1)	0.0
ccdEE	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	0.0 (0/29)	0.0
Total	(34/156)	(28/60)	(136/465)	(102/306)	0.0

^aData obtained from this study.^bData obtained from Lin-Chu et al. (21).^cData obtained from Mak et al. (24).^dData obtained from Fukumori et al. (19).^eData obtained from Okubo et al. (28).

Hong Kong Chinese populations (Table 4). It is interesting that although the prevalence of the CcdEe phenotype was about 44% (45 out of 102) in the D_{el} group of the Japanese population (19), we did not find any D_{el} individual with this phenotype in our test group or in another population of Taiwan Chinese (21).

As regards D antigen density, Wagner (22) demonstrated, using flow cytometry, a suppressive effect induced by C antigen density. Okuda et al. (14) also pointed out that all the serologically RhD-negative donors carrying parts of the *RHD* gene under analysis showed either the CC or the Cc phenotype. Only one exception was found: a Japanese subject had a ccdee phenotype (23). Forty-eight RhD-negative (including 34 D_{el}) individuals carrying at least one exon were detected in this study. Of these, 47 individuals had the C antigen, and one possessed the ccdee phenotype (Table 2). A previous study of another Taiwan Chinese population also identified one D_{el} individual with the same ccdee

phenotype (21). The incidence of D_{el} in different populations with various serological Rh phenotypes is shown in Table 4. The differences between the two Japanese populations, and between the two Taiwan Chinese populations were not statistically significant (a *z*-test was used to analyze the proportionate difference between the two populations (data not shown), *P*>0.05), nor was it significant (*P*>0.05) between the Taiwan Chinese and Japanese populations. Fukumori et al. (19) also found that the difference between the Japanese and Hong Kong Chinese populations was not significant.

Flegel et al. (8) recommended that three “diagnostic sites” at exons 4, 5, and 7 be tested to examine most relevant partial Ds. The partial D categories have been defined (D^{II}-D^{VII}, R_o^{Har}, DFR, and DBT), and each category has a different number of D epitopes (4). The rearranged gene responsible for R_o^{Har} has exon 5 but lacks other exons. Four individuals in the current study

showed this pattern. On the other hand, one individual (probable phenotype: D^{Va}) lacked exon 5 but possessed other exons intact, while other two individuals (probable phenotype: D^{IVb}) lacked exons 7 and 9. Approximately 15% of Caucasians are RhD-negative, whereas only about 0.1–0.4% of Chinese are classified as RhD-negative (17,18,24). Therefore, the estimated frequencies of these three partial Ds (R_o^{Har} , D^{Va} , and D^{IVb}) in the RhD-negative Taiwan Chinese were about 0.0026% (4/156,000), 0.0006% (1/156,000), and 0.0013% (2/156,000), respectively. Flegel and Wagner (25) noted that most R_o^{Har} variants were found in Caucasians (0.002%; 1/60,000). In this study, we found no difference in the frequency of R_o^{Har} variants between Chinese and Caucasian populations. Moreover, because *RHD* genes were detected and showed high polymorphism in RhD-negative individuals of different ethnic groups, we suggest that further investigations into these complex genes are needed in oriental countries to elucidate the different gene profiles of various populations.

It is unlikely that DNA typing will replace conventional serological methods for RhD typing in the near future. However, previous reports (26,27) have indicated that antibody formation may be induced in individuals of the partial D phenotype. Therefore, the PCR multiplex method may have substantial clinical applications, and may help to ensure safety in blood transfusions and reduce the rate of HDN occurrences.

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REFERENCES

- Levine P, Stetson RE. An unusual case of intragroup agglutination. *J Am Med Assoc* 1939;113:126–127.
- Urbaniak SJ, Greiss MA. RhD hemolytic disease of the fetus and the newborn. *Blood Rev* 2000;14:44–61.
- Merilyn W. The Rh blood group system. In: Harmening DM, editor. *Modern blood banking and transfusion reactions*. Philadelphia: F.A. Davis; 1999. p 136–137.
- Tippett P, Lomas-Francis C, Wallace M. The Rh antigen D: partial D antigens and associated low incidence antigens. *Vox Sang* 1996;70:123–131.
- Avent ND. Human erythrocyte antigen expression: its molecular bases. *Br J Biomed Sci* 1997;54:6–37.
- Bennett PR, Le Van Kim C, Colin Y, et al. Prenatal determination of fetal RhD type by DNA amplification. *N Engl J Med* 1993;329:607–610.
- Aubin J-T, Kim CLV, Mouro I, et al. Specificity and sensitivity of RHD genotyping methods by PCR-based DNA amplification. *Br J Hematol* 1997;98:356–364.
- Flegel WA, Wagner FF, Muller TH, Gassner C. Rh phenotype prediction by DNA typing and its application to practice. *Transfus Med* 1998;8:281–302.
- Chan FY, Cowley NM, Wolter L, et al. Prenatal RHD gene determination and dosage analysis by PCR: clinical evaluation. *Prenat Diagn* 2001;21:321–326.
- Daniels G, Green C, Smart E. Differences between RhD-negative Africans and RhD-negative Europeans. *Lancet* 1997;350:862–863.
- Gassner C, Schmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequence-specific primers. *Transfusion* 1997;37:1020–1026.
- Avent ND, Martin PG, Armstrong-Fisher SS, et al. Evidence of genetic diversity underlying RhD-, weak D (D^U), and partial D phenotypes as determined by multiplex PCR analysis of the RHD gene. *Blood* 1997;89:2568–2577.
- Sun CF, Chou CS, Lai NC, Wang WT. RHD gene polymorphisms among RhD-negative Chinese in Taiwan. *Vox Sang* 1998;75:52–57.
- Okuda H, Kawano M, Iwamoto S, et al. The RHD gene is highly detectable in RhD-negative Japanese donors. *J Clin Invest* 1997;100:373–379.
- Vengelen-Tyler V. Confirmation of weak A or B subgroup by adsorption and elution. In: American Association of Blood Banks, editor. *Technical manual*. 14th ed. Bethesda: American Association of Blood Banks; 2002. p 673–674.
- Maaskant-van Wijk PA, Faas BHW, de Ruijter JAM, et al. Genotyping of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. *Transfusion* 1998;38:1015–1021.
- Chu ML. The Rhesus blood group in Taiwan. *J Formos Med Assoc* 1984;83:796–799.
- Lan JC, Chen Q, Wu LD, Ding H, Pong DB, Zhao T. Genetic polymorphism of RhD-negative associated haplotypes in the Chinese. *J Hum Genet* 2000;45:224–227.
- Fukumori Y, Hori Y, Ohnoki S, et al. Further analysis of Del (D_{elute}) using polymerase chain reaction (PCR) with RHD gene-specific primers. *Transfus Med* 1997;7:227–231.
- Chang JG, Peng CT, Tsai CH. Human RhD_{el} is caused by a deletion of 1,013bp between intron 8 and 9 including exon 9 of RHD gene. *Blood* 1998;92:2602–2604.
- Lin-Chu M, Broadberry RE, Yen L-S. An appraisal of RH(D) typing in Chinese. *J Formos Med Assoc* 1986;85:196–198.
- Wagner FF. Influence of Rh phenotype on the antigen density of C, c, and D: flow cytometric study using a frozen standard red cell. *Transfusion* 1994;34:671–676.
- Umenishi F, Kajii E, Ikemoto S. Molecular analysis of Rh polypeptides in a family with RhD-positive and RhD-negative phenotypes. *Biochem J* 1994;299:207–211.
- Mak KH, Yan KF, Cheng SS, Yuen MY. Rh phenotypes of Chinese blood donors in Hong Kong, with special reference to weak D antigens. *Transfusion* 1993;33:348–351.
- Flegel WA, Wagner FF. The frequency of RHD protein variants in Caucasians [Abstract]. *Transfus Clin Biol* 1996;3:10.
- Beckers EAM, Geraedts JPM, Van Rhenen DJ. A study of the prevalence and patterns of inheritance of partial D antigen category VI in a white donor population. *Transfusion* 1994;34:455.
- Mayne K, Howell P, Woodward T, Sibley C, Lomas C, Tippet P. Rh immunization by the partial D antigen category D^{Va} . *Br J Hematol* 1990;76:537–539.
- Okubo Y, Yamaguchi H, Tomita T, Nagao N. A D variant D_{el} ? *Transfusion* 1984;24:542.