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RHD Genotyping of Rh-Negative and Weak D Phenotype among Blood Donors in Southeast Iran

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ABSTRACT

Background: The D antigen is a subset of Rh blood group antigens involved in the hemolytic disease of the newborn [HDFN] and hemolytic transfusion reaction [HTR]. The hybrid Rhesus box that was created after RH gene deletion, was known as a mechanism of the Rh-negative phenotype. Hybrid marker identification is used to confirm the deletion of the RHD gene and to determine zygosity. This study aims to detect this marker in Rh-negative and weak D phenotype blood donors of the southeast of Iran.

Materials and Methods: The molecular analysis of the hybrid Rhesus box was performed on the 200 Rh-negative blood donors in Sistan and Baluchestan province, southeast Iran. The presence of alleles responsible for the D variants was assessed by DNA sequencing in 26 weak D phenotype donors.

Results: Of the 200 Rh-negative blood samples, 198 samples were homozygous (99%), and two samples were heterozygous (1%). Heterozygous samples had RHD*01N.73 allele and the RHD*01N.18 allele. Of the 26 samples with weak D phenotype, 16 partial DLO (61%), 4 partial DBT1 (15.3%), 2 partial DV type 2 (7.7%), 1 weak D type 1, 1 weak D type 4.2.3, 1weak D type 105 and 1 RHD (S103P) (4%) were determined.

Conclusion: Since RHD gene deletion is the main mechanism of the Rh-negativity in Sistan and Baluchestan provinces, a hybrid Rhesus box marker can be used in resolving RhD typing discrepancies by RHD genotyping methods.

Keywords: Hybrid Rhesus box; D variant;RHD gene deletion; RhD-negative phenotype; Weak D

INTRODUCTION

The Rh blood group system, located on the short arm of chromosome number 1 (1p34/1-1p36), is the most polymorphic and immunogenic blood group system in humans. This system has great importance in transfusion medicine because of its role in developing HDFN, HTR, and autoimmune hemolytic anemia. [1-5]. Of the 61 antigens present in the Rh blood group system, five antigens (CDEce) have clinical significance [6].

The lack of expression of the D antigen causes the Rh-negative phenotype. This phenotype is caused by three genetic mechanisms, including the complete deletion of the RHD gene, RHD pseudogene, and RHD-CE-D hybrid gene [7, 8].

In white Europeans and Americans, the D-negative phenotype is caused by the complete deletion of the RHD gene. In Africans, the D-negative phenotype is due to the formation of RHD-CE-D hybrid gene RHD pseudogene: a premature termination codon and with the presence of an extra 37 base pair duplication. The Del phenotype, which is the result of mutations in the RHD gene exons, is found in 10% to 30% of Asians[7, 9, 10].

There are two nearly identical Rhesus boxes in the upstream and downstream of the RHD gene. When

crossing-over occurs between these two alleles, the RHD gene is lost, and a hybrid Rhesus box is generated. The direct identification of this fragment is an indicator of the complete deletion of the RHD gene. The presence of this hybrid Rhesus box is used to confirm the removal of the RHD gene. The clinical applications of hybrid Rhesus box are the determination RhD typing in patients with repeated blood transfusions, patients with autoantibodies, and RHD zygosity. The use of this hybrid marker is applicable in many of the mentioned clinical cases in our country, provided that the Rh-negative phenotype occurs in the rhesus box due to gene deletion [7, 8].

Genetic alterations and mutations cause quantitative or qualitative changes in the D antigen expression, resulting in a variety of D alleles. These RHD alleles are classified as weak D, partial D, and Del [11]. Mutations in the weak D variants lead to decreased RHD gene expression without altering the protein structure. Partial D, however, results in alterations of the epitopes in the D antigen structure. The weak D type 1, 2, 3 no produce allo anti-D, which can be identified only through the genotyping method. Therefore, determining the type of weak D phenotype, clinically relevant D alleles can be transfused more precisely Rh-matched blood into patients and since there is no standard serological method for detecting and interpreting different D variants, they use molecular methods to identify this variant [8, 12-14].

This study aimed to identify Rh-negative phenotype mechanisms and common mutations in weak D phenotype using molecular analysis in blood donors.

MATERIALS AND METHODS

Blood Samples

This study was conducted in the Sistan and Baluchestan province, Southeast Iran. This descriptive cross-sectional study was performed on 200 Rh-negative blood samples and 26 weak D phenotype samples from donors at Sistan and Baluchestan blood transfusion centers from February to April 2019. Blood samples were collected in (EDTA) anticoagulant. Due to the rarity of the weak D phenotype, blood donors with serologically confirmed phenotypes were invited to participate in the study. Inclusion criteria are all samples with Rh-negative phenotype, samples with weak D phenotype that must have two criteria of reactionless than +2 or reaction only in the anti-human globulin [AHG] stage by hemagglutination method in determining D antigen and Samples without the above conditions are not included in the study. The project was carried out in accordance with the recommended standards of the Humanities Ethics Committee of the High Institute for Research and Education in Transfusion Medicine and written informed consent was obtained from all participants. (IR.TMI.REC code.1397.032).

Serologic Rh typing

The RhD antigen status was determined by automated method (Qwalys, Diagast automated pretransfusion blood testing system, France) with a monoclonal immunoglobulin reagent anti-D Lorne (Monoclonal IgM/IgG RUM-1, MS-26, Bio Clone, Ortho, UK). The samples with no or less than +2 agglutination reaction in IS phase, was re-evaluated manually by tube method with blend Anti D (human IgM/IgG, clone TH-28, MS-26, Immundiagnostik, Germany) and further examined by IAT test (AHG, Immundiagnostik, GmbH, Germany).

For phenotyping of C, c, E, e antigens by tube hemagglutination assay, commercially-available monoclonal antibodies (Immundiagnostik, GmbH, Germany) were used.

DNA Extraction

DNA was extracted using a genomic DNA extraction kit (Yekta-Tajhiz Azma, Tehran, Iran kit), according to the manufacturer's instructions. The quality and quantity of extracted DNA were evaluated using electrophoresis and optical absorbance readings (Thermo Scientific NanoDrop, USA) respectively.

Hybrid Rhesus box analysis

The hybrid Rhesus box was amplified using the specific primers, as previously described by Perco et al [15]. PCR was carried out in a total volume of 25 µL in 35 cycles using a thermal cycler (PEQSTAR 2X 95-

08002, PEQLAB Biotechnologie GmbH, Germany) under the following conditions:
Initial denaturation at 95 ºC for 10 minutes, secondary denaturation at 92 ºC for 20 seconds, annealing at 64 ºC for 30 seconds, extension at 68 ºC for 3 minutes, and a final extension at 72 ºC for 5 minutes.
The PCR products were electrophoresed on agarose gel 0.8%.

PCR-RFLP to detect the RHD zygosity
The PCR-restriction fragment length polymorphism (RFLP) technique was used to identify the presence or deletion of the RHD gene in both RH gene alleles. The 3030 bp genomic fragment was amplified from the hybrid Rhesus box (RHD gene deletion) and a genomic fragment with the same length was also amplified from the downstream Rhesus box, i.e. the presence of the RHD gene using the specific primers referred to by Wagner et al. [7]. If the genomic fragment was found to belong to the hybrid Rhesus box, it would have three cleavage sites for Pst-1 restriction enzyme (Jena Bioscience, GmbH, Germany), and if it belonged to the downstream Rhesus box, it would have two cleavage sites for Pst-1. PCR condition was similar to the PCR-SSP, except for the number of cycles and the annealing temperature that was 30 and 68°C, respectively. The PCR products were incubated with Pst-1 at 37 °C for 1 hour and were placed at 80 °C for 20 minutes so that the enzyme would completely cleave the intended product. The cleaved products were then separated by agarose gel 2%.

RHD exons 5, 7 and 10 analysis by Real-time PCR
The Rh-negative phenotype was confirmed via a Real-time PCR method using specific primers [16]. RT-PCR was performed in a volume of 25 µl in 30 cycles using a thermal cycler (Rotor-Gene, RG3000, Corbett, Australia) and RealQ Plus 2x Master Mix Green (Ampliqon, Copenhagen, Denmark) under the following conditions:
Initial denaturation at 94 ºC for 2 minutes, secondary denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, and extension at 72 ºC for 30 seconds. The concentration of the primers in the reaction was 0.4 µm.

RHD nucleotide sequencing
Direct DNA sequencing was used to identify the RHD gene variants. The RHD gene exons were amplified by the primers described by Doescher et al. [17]. PCR was performed in a 25-µl volume under the following conditions:
95 ºC for 2 minutes, 35 cycles at 95 ºC for 30 seconds, 62 ºC for 30 seconds (exons 1, 4, 5, 6, 8 and 10), 40 ºC (exon 2), 64 ºC (exon 3) and 63 ºC (exon 7), 72 ºC for 30 seconds, and final extension at 72 ºC for 10 minutes.
The PCR product was sent to Macrogen Co (Seoul, Korea) for sequencing. The sequencing results were analyzed using Chromas-2 software, and the allele was identified as the D variant if mutations were observed and located.

RESULTS
Serologic RhD typing
All samples with weak D phenotype had weak positive results in phases IS and 37 ºC with two monoclonal antibody reagents. The samples then had a stronger reaction of +1 to +3 in the AHG phase. The serological results of donor samples with Rh-negative and weak D phenotype are described in Table 1.
Analysis of the hybrid Rhesus box
All the 200 Rh-negative samples were positive in terms of the existence of hybrid Rhesus box fragment. All the 26 D variant samples were heterozygous, as one RH gene allele showed a hybrid Rhesus box fragment.

Zygosity analysis of hybrid Rhesus box by PCR-RFLP
A 3030-bp-long genomic DNA fragment was amplified by PCR and specific primers and then cleaved by Pst-I. The number and size of the cleaved fragments on agarose gel 2% showed that 198 samples (99%) were homozygous in terms of the hybrid Rhesus box genomic fragment, while two samples (1%) were heterozygous in both RH alleles,

with one RH gene allele having the hybrid Rhesus box fragment and the other allele having the downstream Rhesus box fragment. The Human growth hormone (HGH) gene was used to control the PCR process, the homozygous Rh-positive sample used as the positive control, and the homozygous Rh-negative sample as the negative control and heterozygous sample.

RHD exons' analysis

An examination of the samples for the presence of exons 5, 7 and10 of the RHD gene showed that 198 (99%) of the donors were negative for exons 5, 7 and 10 of the RHD gene while two samples (1%) were positive for these exons.

In the latter two samples, all three exons (5, 7 and 10) were amplified. For further molecular analysis, the RHD gene exons were sequenced. The results of RHD sequencing revealed one sample, RHD*01N.73 c.443C>G. 3p. Thr148Arg, and in the other sample, RHD*01N.18 c.807T>G. 6p. Tyr269Ter (Table 2).

RHD Sequencing

Of the 26 samples with weak D phenotype, 16 partial DLO, 4 partial DBT1, 2 partial DV type 2, 1 weak D type 1, 1 was weak D type 4.2.3, 1weak D type 105 and 1 RHD(S103P) were determined (Table 3).

Table 1: Serologic characteristics of Rh D typing by the Manual Tube method

RHD allele	IS	37 ºC	AHG
Gen Deletion	0	0	0
RHD*01N.18	0	0	0
RHD*01N.73	0	0	0
Weak D type 1	1+	1+	2+
Weak D type 4.2.3	1+	1+	3+
Weak D type 105	1+	1+	1+
Partial DLO	0	1+	1+
Partial DBT1	0	0	1+
Partial DV type 2	0	1+	1+
RHD(S103P)	0	0	2+

IS: Immediate spin, AHG: Anti-Human Globulin

Table 2: The frequency of the RHD gene alleles in the RHD-negative samples

RHD Gene Alleles	Number	Phenotype	Hybrid Rhesus Box	RHD Gene Deletion	RHD Exons 5, 7, 10
Gene deletion	198	D-	Positive	Homozygote	Negative
RHD*01N.73	1	D-	Positive	Heterozygote	Positive
RHD*01N.18	1	D-	Positive	Heterozygote	Positive

Table 3: D variant alleles and their associated phenotypes.

Variant D	Nucleotide Change	Exon(s)	Phenotypes	Number of Samples
Partial DLO	C. 851 C>T	6	D+C+c+E–e+ D+C+c–E–e+	16
Weak D type 1	C. 809 T>G	6	D+C+c+E–e+	1
Weak D type 4.2.3	C. 602 C>G C. 667 T>G C. 744 C>T C. 1025 C>G	4,5,7	D+C+c+E–e+	1
Weak D type 105	C. 200 C>G	2	D+C+c–E–e+	1
Partial D (S103P)	C. 307 T>G	2	D+C+c+E–e+	1
Partial DBT1	c.667T>G c.697G>C c.712 G>A c.733G>C c.744C>T c.787G>A c.800A>T c.916G>A c.932A>G c.941G>T c.968C>A c.974G>T c.979A>G c.985 G>C c.986 G>A c.989 A>C c.992 A>T c.1025T>C c.1048G>C c.1053C>T c.1057G>T c.1059A>G c.1060G>A c.1061C>A	D-CE(5-7)D*RHD	D+C+c+E–e+	4
Partial DV type 2	c.667T>G c.697G>C c.712G>A c.733G>C c.744C>T c.787G>A c.800A>T	D-CE(5)D*RHD	D+C+c–E–e+ D+C–c+E–e+	2

DISCUSSION

The presence of this hybrid Rhesus box is used for confirming RHD gene deletion and zygosity RHD determination. The clinical applications of hybrid Rhesus box are the determination RhD typing in patients with repeated blood transfusions, patients with autoantibodies, zygosity determination in Rh-positive fathers, and RHD determination in fetuses with Rh-negative mothers. The molecular investigation of the RHD gene seems to be necessary for terms of the existence of a hybrid Rhesus box gene and the identification of frequent weak D mutations in different ethnicities [14, 15].

Khosroshahi et al. in 2018 reported that 100% of the Rh-negative donors in Tehran Blood Transfusion Center were positive for the genomic fragment of the hybrid Rhesus box gene by PCR-SSP. They showed that 98% of the donors had a hybrid Rhesus box genomic fragment in both RH gene alleles. Two percent of the donors had a non-deletion RHD gene allele by PCR-RFLP [18]. Which is consistent with the present study; therefore, the genetic mechanism of the Rh-negative phenotype in Sistan and Baluchestan ethnic groups in Iran is also due to forming a hybrid Rhesus box.

Results from other Studies in East Asia suggested that the most frequent mechanism causing the RhD negative phenotype is the Del allele. These results are in contrast with our findings. However, is similar to European and American studies [19].

Limited studies have been carried out to identify the RHD*01N.18 and RHD*01N.73 phenotypes. The RHD*01N.73 allele in exon 3, 443C>G, leading to Thr148Arg in the Rh protein. And RHD* 01N.18 allele had a mutation in exon 6, 807T>G, showing, leading to a Tyr269Ter termination codon.

RHD * 01N.73 allele was introduced by Haer - Wigman L in the Netherlands in 2012, and our study is the second to report it. In addition, this allele contains the Cce antigen, which has not been reported so far [20, 21].

Both alleles have been classified as non-functional RHD phenotypes by the International Society of Blood Transfusion [ISBT] [10, 22].

These alleles are non-functional and cannot produce anti-D in the Rh-negative blood recipients. In the present study, the pack cell of a donor with the Rh-negative phenotype and the RHD*01N.18 genotype was transfused into a thalassemia patient with the Rh-negative phenotype but had not produced anti-D in the patient. The results of the present study also showed that the RHD*01N.18 allele has a Ce antigen, and Taeo et al. also reported the same findings in a 2019 study in Korea [21].

Since serologic tests cannot identify a variety of weak D phenotypes, these people are sometimes considered as Rh negative. Moreover, if a donor has the D antigen, with low expression, the Rh-negative recipient produces the D antibody. Studies conducted in European and American populations show that more than 80% of blood recipients have weak D types 1, 2, and 3. When these recipients receive Rh-positive red blood cells, they are not at risk for producing D-antibodies and can be considered as Rh-positive [5, 23].

Consequently, by genotyping the weak D phenotype, less Rh negative blood consumption will be required against and there will be no need for RhIg injections in weak D phenotype mothers with Rh-positive neonates or fetuses either. Therefore, considering the importance of this issue in blood transfusion medicine, it is necessary to determine the frequency of all D variants.

Numerous studies in European and American countries have shown that the frequency of weak D types 1, 2, and 3 is between 67 and 90 percent. The findings of these studies are very different from those of the present study [23-25]. This study also showed that the weak D type 1 sample had the Cce antigen, which Laget et al. Reported in 2019 [25].

In the present study, partial D type DLO was detected in 61% of our weak D samples. This allele was reported in 2004 by Ansart-Pirenne et al. in France, with a frequency of less than 1% in the white population. This allele would be capable to producing anti D antibodies. Serologically, the present study was consistent with the previous study [26].

The prevalence of DAR1.3 * RHD variant (Weak D type 4.2.3) in the white population (European and American) is less than 2%, common in the African people and not reported in the Asian population, so our study population is similar to the white people (European and American)[27]. This phenotype also

contained Cce antigens, which was consistent with a 2013 Lejon Crottet S study that reported this Partial D phenotype DBT has been reported in white European and American people (some being Italian) as well as in Morocco, Japan, and Thailand [28].

People with the DBT and DV phenotypes become more easily alloimmunized against D antigen than people with other partial D phenotypes [3, 30]. It is difficult to identify the partial D phenotype DBT through serology with IgG and IgM monoclonal antibodies [31].

Ansart-Pirenne H et al. In 2004 reported the prevalence of partial D phenotype DBT and partial D type V phenotype in 4% and 1.5% of the white population, which is close to our study [26]. On the other hand, this type of phenotype had Cce antigens, similar to the above study. Also, the results of our study were serologically consistent with the results of studies conducted in this field to identify this type of phenotype [31].

In 2014, Arnoni CP et al. Reported a partial D type V of 0.27%, consistent with our study. This allele also contained ce antigens that were consistent with our study [29].

The RHD(S103P) allele has been reported in the Netherlands and Belgium. G antigen has been identified as Ser103 on RHD and RHCE proteins. This allele lacks the G antigen due to this position. We detected this allele. In the study of Van Sandt VS et al., This allele contained ccEe antigens, while it contained Ccee antigens in our study [24].

In 2015, Ogasawara K. introduced a new type of weak D phenotype in Japan called type 105. The present study is the second to have reported this phenotype. [32].

CONCLUSION

Our study showed that the main mechanism of RHD gene deletion in Sistan and Baluchestan ethnicities is the Rhesus box hybrid. Therefore, the hybrid Rhesus box marker can be used for paternal RHD zygosity, determining fetal RhD, and solving RhD group typing discrepancies.

The present findings suggest that molecular tests have to be formulated in immunohematology reference laboratories to identify the phenotype of phenotype[27]. This phenotype is capable to stimulate anti D antibodies [6].

serologically-weak D samples so that clinically relevant D alleles can be transfused into patients in need of blood transfusion.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest relevant to this manuscript.

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