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Investigation of Paternal RhD Zygosity by Two Molecular Methods among Blood Donors in Kurdistan Province, Iran

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ABSTRACT

Background: RhD antigen system is the leading cause of hemolytic disease of the fetus and newborn (HDFN). Paternal molecular RhD zygosity test is valuable to decide on the use of anti-D immunoglobulin prophylaxis in Rh D-negative pregnant women. We aimed to investigate the paternal RhD zygosity by two molecular methods among blood donors in Kurdistan province, the west of Iran. We also compared these two methods in determining RhD zygosity.

Methods: 100 RhD positive blood samples were collected from male blood donors with RhD negative spouses who were referred to Kurdistan Blood Transfusion Center. The phenotype of all samples was tested for Rh D, C, c, E and e antigens by standard hemagglutination methods. Then, RhD zygosity of all samples was evaluated in terms of Rhesus box marker by SSP-PCR and PCR-RFLP methods.

Results: Among 100 RhD positive samples, 37% were heterozygote and 63% were homozygote for RhD gene. Both SSP-PCR and PCR-RFLP methods were able to detect zygosity with similar accuracy. Moreover, Rh phenotyping revealed that DCCee (38%) and Dccee (2%) were the most and the least frequent phenotypes in our sample, respectively.

Conclusion: RhD zygosity determination in men who have an RhD negative partner by molecular methods such as PCR-SSP and PCR-RFLP could be the first step in preventing HDFN and avoiding unnecessary administration of Rh immunoglobulin in Iran.

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Introduction

The Rhesus D antigen (ISBT004.001; RH1) as one of the clinically important blood group systems is the main causative antigen for hemolytic disease of the fetus and newborn (HDFN).[1] Currently, Rh immunoglobulin is administrated at 28th week of gestation and 72 hours postpartum to avoid alloimmunization against D antigen in Rh (D) negative women with Rh (D) positive partners and also in Rh (D) negative women with history of abortion.[2] Although; anti D immunoglobulin can reduce the risk of HDFN in RhD negative pregnant women, this strategy can result in shortage of anti D immunoglobulin and imposes high costs to the health system.[2, 3] On the other hand, anti D immunoglobulin is isolated from pooled human plasma which could be assumed as a potential source of infectious agents. It is demonstrated that RhD heterozygote fathers have a 50% lower chance of having RhD positive children compared with RhD homozygotes.[4, 5] Accordingly, zygosity determination in fathers could be an initial step in the management of alloimmunization to avoid HDFN.[2, 6, 7]

The most frequent mechanism responsible for Rh negativity in European population is RhD gene deletion which occurs as a consequence of crossing over between two rhesus box sequences which results in a single hybrid rhesus box gene.[4] The presence of this hybrid rhesus box is

the basis of RhD zygosity determination and confirms the deletion of the RhD gene. The two available methods for determination of RhD zygosity include sequence-specific primer (SSP)-PCR and PCR-restriction fragment length polymorphism (RFLP) which are performed to determine the presence of hybrid rhesus box sequence.[1, 2, 4, 6, 8]

In the present study, we used SSP-PCR and PCR-RFLP methods to reach an estimation of zygosity state of D positive fathers with D negative partners in order to manage the consumption of anti D immunoglobulin. We also compared these two molecular methods in determining of RhD gene zygosity.

Materials and Methods

100 EDTA blood samples was collected from male volunteer blood donors who were RhD positive and had RhD negative partners, referring to "Kurdistan Blood Transfusion Center". Ethical forms and questionnaires that were certified by the "Iranian blood transfusion organization (IBTO)" were used to collect demographic data. The data collection about the race was based on self-identification.

Serologic Typing

The RhD antigen status of the samples was tested by automated methods (Qwalys, Diagast automated pre-transfusion blood testing system, France) with a monoclonal immunoglobulin (IgM) reagent (anti-D IgM, clone RUM10, Immundiagnostika, Germany). The RhCcEe phenotype of all donors was determined using routine Anti-C, Anti-E, Anti–c and Anti-e monoclonal antibodies (Immundiagnostika, GmbH) according to the manufacturers 'instructions.

DNA Extraction

DNA was extracted using a commercially available kit (Yekta tajhiz azma, Iran) according to the manufacturer's instructions. The concentration and purity of isolated DNA were assessed by the Nanodrop (Thermo, Germany). DNA samples were preserved at -25°C for further experiments.

SSP-PCR

Hybrid rhesus box sequence (2778 bp) is comprised of three fragments: upstream rhesus box (775 bp), identity region (1467 bp) and downstream rhesus box (536 bp). To determine Rhesus box nucleotide sequences, SSP-PCR was performed using U1s/rnb31 primers described by Perco and colleagues.[9] The U1s primer was specific for hybrid and upstream rhesus box and rnb31 was specific for hybrid and downstream rhesus box (table 1). PCR was performed as denaturation at 95° C for 10 min, and then 35 cycles of 92° C for 20 s, 64° C for 30 s and 68°C for 3 min. Reaction was completed finally at 72°C for 5 min. PCR products were visualized on 0.8% agarose gel.

PCR-RFLP

To confirm the presence of hybrid rhesus boxes, PCR-RFLP method was performed as previously described by Wagner & Flegel (1). Primers rez7 and rnb31 were used for amplification of the downstream and hybrid rhesus boxes (table 1). Amplification was performed as follows: denaturation at 95° C for 2 min, and then 30 cycles of 95° C for 30 s, 61.6° C for 30 s and 72° C for 5 mins. Reaction was completed finally at 72° C for 5 mins. PCR amplicon was digested using PST1 enzyme (Jena Bioscience, Germany) for 60 minutes at 37° C and then 15 minutes at 55° C. Cleavage products that represent hybrid rhesus box or downstream fragments were separated using a 0.8% agarose gel.

Results

The most prevalent phenotypes among RhD positive volunteer men included: DCCee (n=38, 38%), followed by DCcee (n=29, 29%), DCcEe (n=18, 18%), DccEe (n=9, 9%), DccEE (n=4, 4%), and two who were Dccee (2%). As is shown in table 2, R1R1 was the most probable genotype among our samples followed by R0R0 and R0r.

Table 1: Oligonucleotide primers used for the polymerase chain reaction (9, 12)

Primer	Primer Nucleotide sequence (5′–3′)
u1-s	TGA GCC TAT AAA ATC CAA AGC AAG TTA G
Rnb31	CCT TTT TTT GTT TGT TTT TGG CGG TGC
Rez7	CCT GTC CCC ATG ATT CAG TTA CC

Table 2: The RhD zygosity, phenotypes and most probable genotypes according to genotyping and serological assessment (n=100)

Phenotypes	Number	Most probable phenotypes	Number	RhD zygosity
D+C+E+c+e+	18	R^1R^2	15	D/D
		_	3	D/d
D+C-E+c+e+	9	R^2r	5	D/D
		R^2r'	4	D/d
D+C+E-c+e+	29	R^1r	11	D/D
		R^1R^0	18	D/d
D+C-E-c+e+	2	R^0r	1	D/D
		R^0R^0	1	D/d
D+C+E-c-e+	38	R^1R^1	33	D/D
		_	5	D/d
D+C-E+c+e-	4	R^2R^2	4	D/D

SSP-PCR: Based on SSP-PCR analysis, hybrid rhesus box was amplified in only 37 samples indicating heterozygosity of RhD gene in 37% of donors. This fragment was not amplified in the remaining 63 samples (63%), indicating homozygosity of RhD gene in our sample (figure 1).

PCR-RFLP: Results of PCR-RFLP confirmed the zygosity status of all donors detected by SSP-PCR. In this method, a 3029 bp hybrid rhesus box fragment was amplified in all donors. In heterozygote donors (37 samples), pstl digestion of hybrid rhesus box sequence led to detection of genomic fragments with a length of 1888, 744, 564 and 379 bp. However, PCR-RFLP revealed loss of one pstl digestion site due to the presence of downstream Rhesus box haplotypes in 63 homozygotes. So, in these samples the 546 bp fragment was not observed. (figure 2).

Discussion

RhD gene encodes D antigen which is considered as the most important antigen in the Rh blood group system and is the leading cause of HDFN.[1, 4] RhD gene is surrounded by two sequences called rhesus box upstream and downstream. These sequences are 9000 base pairs in length and are 98.6% identical; this homology is due to the presence of a 1463 base pair known as "identity region". Regarding to high homology of these two regions, crossing over occurs during replication, so that one of the alleles completely loses identity region and another allele generates the hybrid rhesus box gene.[1, 8, 10, 11] In the clinical point of view, determination of the RhD zygosity of the father could help to estimate the risk of HDFN, since the chance of having a D positive child for a homozygote (D/D) father and a D negative mother is 50% higher than in a heterozygote (D/d) father.[2] In populations where Rh negative phenotype is caused by deletion in RhD gene, the RHD zygosity can be determined through the presence of a hybrid Rhesus box gene.[1, 8]

In the present study, RhD zygosity of D positive Kurdish male donors was evaluated using PCR-RFLP and SSP-PCR methods. In our study, 37% of the

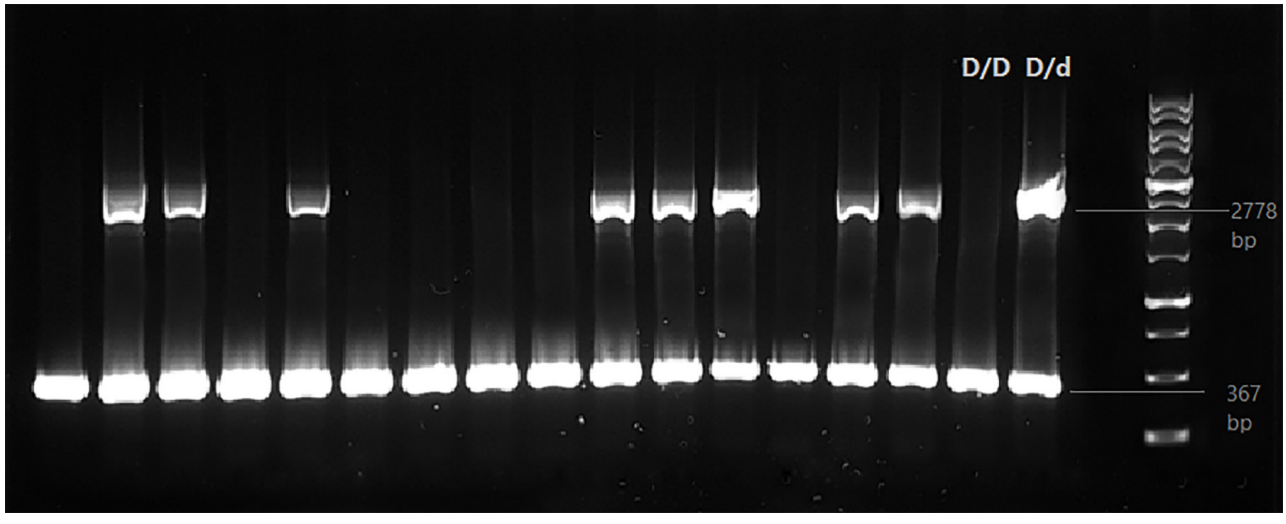

Figure 1: Gel electrophoresis showing banding pattern of a heterozygous D+ (D/d) and homozygous D+(D/D) detected by SSP-PCR method. All lanes with and without 2778 bp band represented D/d and D/D genotypes, respectively. 376 bp band represents the internal control.

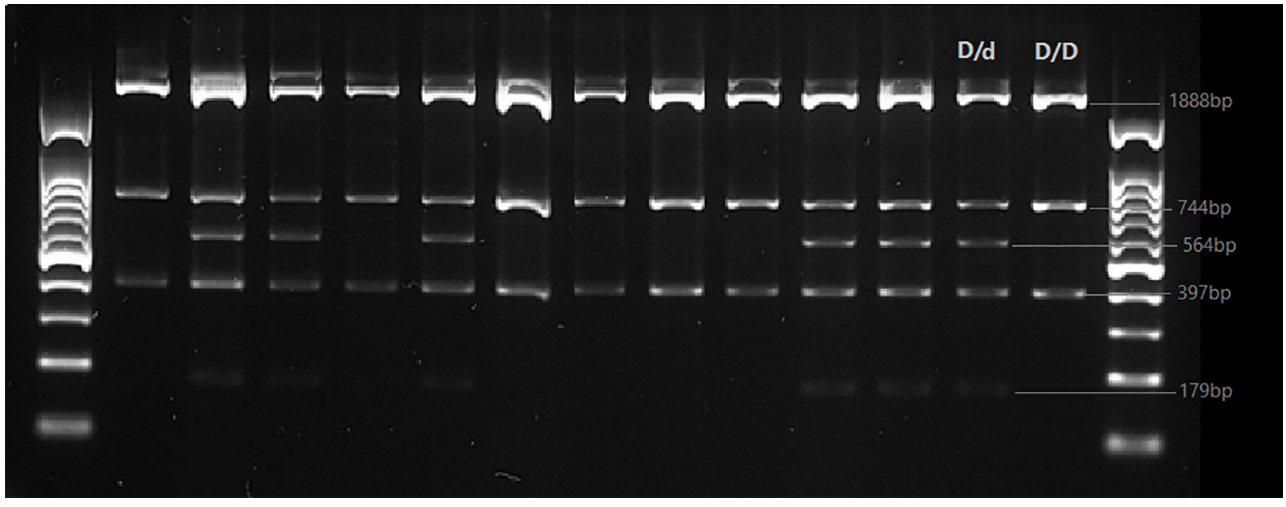

Figure 2: Gel electrophoresis showing banding pattern of a heterozygous D+ (D/d), and homozygous D+(D/D) detected by PCR–RFLP method. The PCR amplicons (primer rez7 and rnb31) were digested with Pstl. In D-negative haplotypes, there were 3 Pstl sites in the amplicon resulting in fragments of 1888 bp, 564 bp, 397 bp, and 179 bp. The downstream Rhesus box of D-positive haplotypes lacks 1 Pstl site, resulting in fragments of 1888 bp, 744 bp, and 397 bp. D^{+}/D^{-} heterozygotes showed both fragments of 744 bp and 564 bp and D+/D+ homozygotes showed three fragments of 1888 bp, 744 bp and 397 bp. Primer rnb31 does not amplify the upstream Rhesus box of D-positive haplotypes.

subjects were heterozygote and 63% were homozygote for RhD gene. The obtained results by the two different methods confirmed each other. The phenotyping data revealed that $D^+C^+c^-E^-e^+$ was the most frequent immunophenotype. Moreover, SSP-PCR and PCR-RFLP showed 33 homozygote individuals among this phenotype. Regarding to the frequency of Rh system, R^1R^1 could be assumed as the most proper genotype for this phenotype. Five cases were also determined heterozygote for this haplotype, which R^1r is predicted as the most frequent genotype for this group. Another 5 cases were heterozygote for D+C+c+E+e+. Considering the chance of 2 different genotypes in this condition and regarding to low prevalence of both genotypes, prediction of genotypes for this group was not feasible (table 2).

Several similar studies in other populations have also been conducted. Perco and colleagues demonstrated a hybrid Rhesus box gene in all weak D and D negative blood donors in Germany by a newly developed SSP-PCR and RFLP methods.[9] Aggarwal and colleagues found that among 104 partners of Indian D negative women, only 26% were homozygote (D/D), while 74% were heterozygotes (D/d) by SSP-PCR and PCR-RFLP methods.[12] Kacem and co-workers revealed 54% heterozygosity (D/d) and 46% homozygosity (D/D) by SSP-PCR among 466 Tunisian blood donors. The most and the least frequent phenotypes in their study were DCcee and DccEE, respectively.[13] In a study conducted by Peirli and co-workers performed by Quantitative fluorescence polymerase chain reaction (QF-PCR) method, the most frequent phenotypes in Europe and African Americans were DCcee and Dccee, while the less frequent were DccEE and DCCee.[5]

Recently, a study from Iran on 200 Rh negative blood donors from Tehran Blood Transfusion Center, detected hybrid Rhesus RhD box in all samples. PCR-RFLP confirmed that 198 (99%) were homozygous for RhD gene deletion. They concluded that the frequency of RhD gene deletion was high among Iranian populations, so hybrid Rhesus box can be used as an efficient marker to detect RhD gene deletion.

Conclusion

Determination of RhD zygosity in men who had D negative partners by molecular methods such as SSP-PCR and PCR-RFLP could be the first step in preventing HDFN and avoiding unnecessary Rh immunoglobulin administration.

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Informed consent: "Informed consent was obtained from all individual participants included in the study."

Ethical Approval: Ethical forms and questionnaires which were certified by Iranian blood transfusion organization were used to collect demographic data.

Conflict of Interest: None declared

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