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# Rhesus Box as the Primary Mechanism of RHD Gene Deletion in RhD-Negative Blood Donors from Eastern Iran

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**ABSTRACT**

**Background:** The Rh blood group system is highly significant in transfusion medicine because of the strong immunogenicity of the D antigen. The RhD-negative phenotype arises through various molecular mechanisms in different populations, most commonly complete deletion of the *RHD* gene caused by unequal recombination between upstream and downstream Rhesus box sequences. Although this mechanism has been well documented in some populations, limited data are available from Iran, particularly its eastern regions. This study aimed to determine the molecular basis of the RhD-negative phenotype among blood donors in eastern Iran.

**Materials and Methods:** In this cross-sectional study, a total of 16,190 blood donors referred to blood transfusion centers in South Khorasan Province, eastern Iran, over a one-year period were screened serologically for RhD status. Among them, 2,198 individuals were identified as RhD-negative, and 100 serologically confirmed RhD-negative donors were randomly selected for molecular evaluation. RhD typing was performed using standard serologic methods and verified by indirect antiglobulin testing. Molecular investigations included PCR–sequence-specific priming (PCR-SSP) targeting *RHD* exons 5, 7, and 10, real-time PCR for confirmation, and PCR–restriction fragment length polymorphism (PCR-RFLP) to detect the hybrid Rhesus box and determine *RHD* zygosity.

**Results:** Among 16,190 blood donors screened during the study period, 2,198 (13.57%) were identified as RhD-negative. From this group, 100 samples were randomly selected for molecular analysis. Both PCR-SSP and real-time PCR confirmed the absence of *RHD* exons 5, 7, and 10 in all samples, indicating complete deletion of the *RHD* gene. PCR-RFLP analysis further confirmed that all donors were homozygous for the hybrid Rhesus box, with full concordance observed between exon-specific assays and hybrid Rhesus box genotyping.

**Conclusion:** These findings indicate that the RhD-negative phenotype in eastern Iran is primarily due to homozygous *RHD* gene deletion mediated by the hybrid Rhesus box. Hybrid Rhesus box analysis may therefore serve as a reliable molecular marker for accurate RhD typing, which could improve transfusion safety and perinatal management in this population.

**Keywords:** RhD-negative; RHD gene deletion; Hybrid Rhesus box; PCR-SSP; PCR-RFLP

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## INTRODUCTION

The Rh blood group system is among the most clinically significant blood group systems, second only to the ABO system. This distinction is largely attributable to the high immunogenicity and polymorphism of the D antigen[1,2]. In transfusion medicine, the presence or absence of the RhD antigen is of critical importance in the prevention of both hemolytic disease of the fetus and newborn (HDFN) [3]. RhD-negative individuals are susceptible to alloimmunization when exposed to RhD-positive red blood cells. This exposure can result in hemolytic transfusion reactions or neonatal anemia [4].

The Rh system is genetically intricate, comprising two closely linked genes, RHD and RHCE, situated on chromosome 1. An analysis of the genes reveals the presence of 10 exons in both, suggesting that they encode highly homologous proteins, exhibiting approximately 94% nucleotide sequence similarity. The RHD gene is responsible for encoding the RhD protein, which is known to carry the immunogenic epitopes of the D antigen. In contrast, the RHCE gene is responsible for encoding the C/c and E/e antigens, as well as several minor variants [5-7]. The frequency of RhD-negative individuals varies globally, ranging from approximately 15–17% in Caucasians, 5–8% in Africans, less than 1% in East Asians, and around 8–10% in the Iranian population[8, 9].

At the molecular level, three primary mechanisms have been identified as the underlying causes of the RhD-negative phenotype: (i) The first component of the gene is the complete deletion of the RHD gene via recombination of the upstream and downstream Rhesus Box sequences; (ii) The second component is the presence of inactive or pseudogenized RHD alleles (RHDΨ); (iii) The third component is the presence of RHD-RHCE hybrid genes. The identification of the hybrid Rhesus Box is clinically significant, as it allows accurate determination of RhD zygosity, resolution of serological discrepancies in blood donors, and improved management of multi-transfused patients. Furthermore, molecular genotyping can guide the targeted administration of anti-D immunoglobulin in RhD-negative pregnant women, thereby minimizing unnecessary exposure and reducing the risk of HDFN [1, 10, 11].

The relative prevalence of these mechanisms varies among different populations. In Caucasian populations, complete RHD gene deletion is responsible for the vast majority of RhD-negative cases. In African populations, approximately 66% of the RhD negativity is attributable to the RHD pseudogene, while 15% is attributable to the RHD-RHCE hybrid allele[12]. A subsequent study revealed that 14% of the Brazilian population possessed the pseudogene mechanism, while 3% exhibited the RHD-CE-D hybrid mechanism[13]. In Iran, studies on various populations have demonstrated that the dominant mechanism of RhD negativity is the complete deletion of the RHD gene mediated by the hybrid Rhesus Box, similar to Caucasian populations. The occurrence of alternative mechanisms, such as RHD pseudogenes or hybrid RHD-CE-D genes, is comparatively infrequent [14, 15]. This finding indicates the presence of a largely uniform genetic basis for RhD negativity within the Iranian population, a finding with significant ramifications for molecular typing, transfusion safety, and the management of alloimmunization.

Despite extensive global research on RhD molecular mechanisms, molecular data from Eastern Iran remain limited. This study examines whether the hybrid Rhesus Box is the main cause of RhD negativity among blood donors in Eastern Iran, given the high national prevalence of RHD gene deletion and possible regional variation. Using PCR-SSP and PCR-RFLP to confirm RHD deletion, we aim to clarify Iran's overall RH genetic landscape.

## MATERIALS AND METHODS

### Study Design and Sample Collection

In this descriptive cross-sectional study, a total of 16,190 blood donors who referred to the blood transfusion centers of South Khorasan Province during a one-year period were evaluated. Among them, 2,198 (13.57%) donors were identified as RhD-negative, and 100 RhD-negative samples were randomly selected for further molecular investigation. For this purpose, two separate blood samples were collected from each selected donor into EDTA-containing tubes; one sample was used for routine serological testing and the other for molecular analysis.

**Serological Phenotyping**

The RhD antigen status was determined using an automated pretransfusion testing system (Qwalys, Diagast automated pretransfusion blood testing system, France) with monoclonal anti-D IgM/IgG reagents (clone RUM-1, BioClone, UK). Samples showing weak or no reaction were re-evaluated manually using the tube method and confirmed by indirect antiglobulin test (IAT).

**DNA Extraction**

Genomic DNA was extracted from whole blood using the Salting-out method. DNA quality and quantity were assessed using agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific, USA). The 260/280 absorbance ratio was used to evaluate purity, with ratios between 1.8–2.0 considered acceptable.

**RHD Exon Analysis by PCR-SSP**

To detect the presence of RHD gene exons, PCR-SSP was performed targeting exons 5, 7, and 10. Reactions were carried out in a 25 µL volume using sequence-specific primers listed in Table 1. PCR amplification was performed by using thermal cycler (PEQSTAR 2X 95-08002 PEQLAB Biotechnologie GmbH, Germany) for 35 cycles under the following conditions: initial denaturation at 94 °C for 2 min, denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72°C for 30 s, and a final extension at 72 °C for 3 min. The PCR products were electrophoresed on a 2% agarose gel. This method allowed specific detection of the presence or absence of RHD exons in each donor sample.

**RHD Exons Analysis by Real-Time PCR**

To confirm the Rh-negative phenotype, the presence of RHD exons 5, 7, and 10 was analyzed by real-time PCR. Reactions were carried out in 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. A known RHD-positive sample was included as a control, and melting curve analysis was performed to ensure specificity of the amplification.

**PCR-RFLP for Hybrid Rhesus box analysis**

The PCR–restriction fragment length polymorphism (RFLP) method was employed to determine the presence or deletion of the RHD gene in both RH alleles. A 3030 bp genomic fragment was amplified from the hybrid Rhesus box representing the RHD gene deletion, and an identical-length fragment was also amplified from the downstream Rhesus box corresponding to the presence of the RHD gene, using primers described by Wagner et al.[17] (Table 2). If the fragment originated from the hybrid Rhesus box, it contained three PstI restriction sites (Jena Bioscience, GmbH, Germany), whereas fragments from the downstream Rhesus box had two PstI sites. PCR was carried out in a total volume of 25 µL in 35 cycles using a thermal cycler 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 digested with PstI at 37 °C for 1 hour, followed by heat inactivation at 80 °C for 20 minutes. Finally, the digested fragments were separated using 2% agarose gel electrophoresis.

**Table 1:** Primers' sequence of exons 5,7, and 10 of the RHD gene

| Exon RHD gene | F/R | Sequence | Size |
|---|---|---|---|
| Exon 5 | Forward | 5′-CGCCCTCTTCTTGTGGATG-3′ | 82bp |
| | Reverse | 5′-GAACACGGCATTCTTCCTTTC-3′ | |
| Exon 7 | Forward | 5′-CTCCATCATGGGCTACAA-3′ | 90bp |
| | Reverse | 5′-CCGGCTCCGACGGTATC-3′ | |
| Exon 10 | Forward | 5′-CCTCTCACTGTTGCCTGCATT-3′ | 74bp |
| | Reverse | 5′-AGTGCCTGCGCGAACATT-3′ | |

* Reproduced with permission from (16).

**Table 2:** Sequence and specificity of primers comprising segments of hybrid Rhesus box

| Gene | Sequence | Size |
|---|---|---|
| *Rez7* | 5′ - CCTGTCCCCATGATTCAGTTACC -3′ | 3030bp |
| *Rnb31* | 5 ′- CCT TTT TTT GTT TGT TTT TGG CGG TGC -3′ | |

## RESULTS

In this study, a total of 16,190 blood donors referred to the blood transfusion centers of South Khorasan Province over a one-year period. Among them, 2,198 donors (13.57%) had the RhD-negative phenotype. From these individuals, 100 samples were randomly selected and collected for further analysis. The demographic characteristics of the study population, including age and sex distribution, are summarized in Table 3.

**Table 3:** Demographic characteristics of Rh-negative donors

| *Parameter* | | *Number* | *%* |
|---|---|---|---|
| ***Age*** | <20 | 25 | 25% |
| | 20-50 | 58 | 58% |
| | >50 | 17 | 17% |
| ***Gender*** | Man | 41 | 41% |
| | Woman | 59 | 59% |

### Serologic RhD Typing and RHD Exons' Analysis

An indirect antiglobulin test was performed as part of serologic evaluation, and all donor samples showed negative reactions in this test. Then, all RhD-negative samples were evaluated for the presence of RHD exons 5, 7, and 10 using the PCR-SSP technique. The results demonstrated that none of the RhD-negative samples (100%) showed amplification of exons 5, 7, or 10, indicating the complete absence of these exons (Figure 1). These findings were further confirmed using real-time PCR. Consistent with the PCR-SSP results, no amplification of exons 5, 7, or 10 was observed in any of the samples, confirming the complete deletion of the RHD gene in all studied donors.

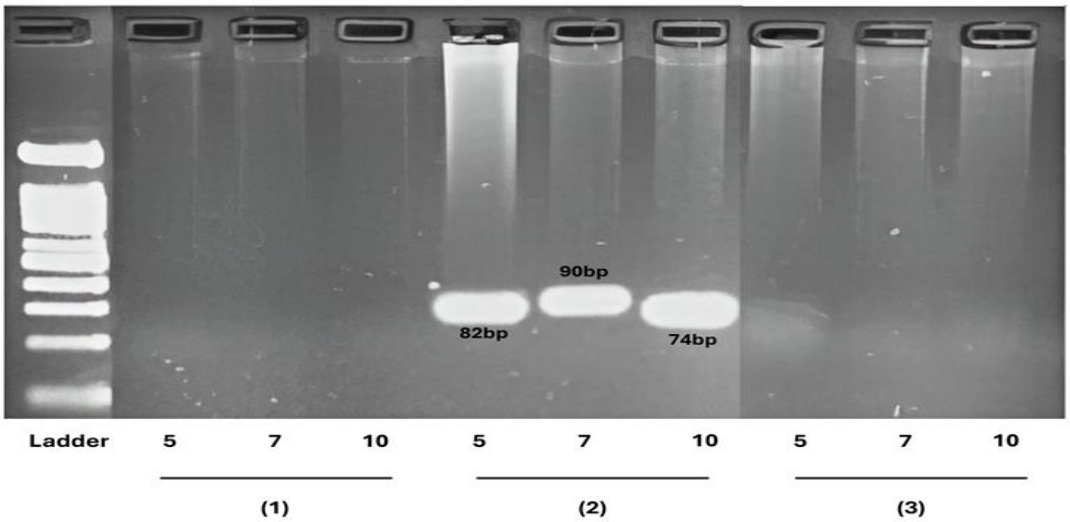


Figure 1. Electrophoresis of PCR-SSP products targeting the hybrid Rhesus box and downstream Rhesus box. Genomic DNA from RhD-negative donors was amplified, and the expected band sizes for exon 5, exon 7, and exon 10 were 82 bp, 90 bp, and 74 bp, respectively. A 30-bp DNA ladder was used as a molecular size marker. Lane 1 represents an RhD-negative sample lacking amplification of exons 5, 7, and 10; lane 2 contains the RhD-positive sample serving as the positive control; and lane 3 contains the no-template reaction used as the negative control.

**Hybrid Rhesus Box Analysis with PCR-RFLP**

The genomic fragment with a length of approximately 3030 bases was amplified by PCR and specific primers and then cut by the enzyme Pst-Ⅰ. The number and size of the cut fragments on a 2% agarose gel showed that all 100 samples from the donor population (100%) were homozygous for the Rhesus Box hybrid genomic fragment in both alleles of the RH gene. Human growth hormone was also used to control the PCR process, and the RH+ sample was used as a positive control and the RH- sample was used as a negative control (Figure 2).

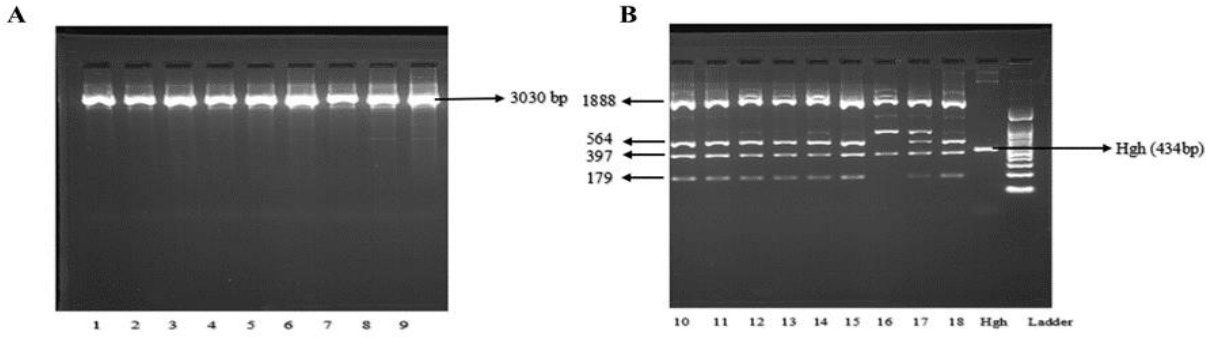


Figure 2. PCR-RFLP analysis of the Rhesus box for determination of RHD zygosity. (A) Lanes 1–9 show the undigested PCR product (3030 bp) amplified from genomic DNA samples prior to enzyme digestion. (B) Lanes 10–15 represent digested PCR products from homozygous RhD-negative samples, demonstrating the expected fragments of 1888, 564, 397, and 179 bp that confirm the presence of two hybrid Rhesus box alleles. Lane 16 shows the digested product from a homozygous RhD-positive sample, displaying fragments of 1888, 744, and 397 bp consistent with two downstream Rhesus box alleles. Lane 17 corresponds to a heterozygous RhD-positive sample. Lane 18 contains the homozygous Rh-negative positive control. Lane 19 shows the growth hormone internal control band, and lane 20 contains the 100-bp DNA ladder used as a molecular size marker.

**DISCUSSION**

The RhD antigen, which is encoded by the RHD gene, occupies a pivotal role in the fields of transfusion medicine and perinatal care. Its absence can result in alloimmunization and HDFN[7,18]. The RHD gene is flanked by two highly homologous sequences known as the upstream and downstream Rhesus boxes, which exhibit 98–99% identity over a span of approximately 9 kb. Unequal crossing over between these boxes can result in the formation of a hybrid Rhesus box and complete deletion of the RHD gene. This is recognized as the most frequent mechanism causing the RhD-negative phenotype in Caucasian populations [19]. Also hybrid Rhesus box , is a reliable molecular marker for identifying the RHD gene allele and zygosity status (homozygous or heterozygous)[14]. Hence, the molecular underpinnings of the RhD-negative phenotype have been the subject of extensive research across diverse populations, highlighting notable ethnic and geographic variations.

The results of this study showed that among a total of 16,190 blood donors in South Khorasan Province over a one-year period, 2,198 individuals (13.57%) had the RhD-negative phenotype. This prevalence is slightly higher than the average reported in Iran (approximately 10%), but it still falls within the range reported for Iranian populations. In comparison with global data, this prevalence is roughly similar to that reported in European populations (15–17%), whereas it is about 8% among individuals of African descent and less than 0.1% in Asian populations [20, 21]. These differences are likely related to genetic, ethnic, and historical characteristics of different populations and highlight the importance of regional studies on the distribution of Rh phenotypes.

Among the RhD-negative donors, 100 samples were randomly selected for molecular analysis. The results showed that no amplification of RHD gene exons 5, 7, and 10 was detected in any of the samples, and all samples exhibited the hybrid Rhesus Box fragment in a homozygous state in both alleles. The concordance of PCR-RFLP and exon analysis illustrates the reliability of employing the hybrid Rhesus box as a genetic marker for RHD gene deletion. The findings of the present study are consistent with those of prior studies conducted in Iran. Sadeghi-Bojd et al. (2021) reported that among 200 Rh-negative donors from Sistan and Baluchestan, 198 (99%) were homozygous for the hybrid Rhesus box, while only two samples carried a non-deletion RHD allele (14). Notably, Sistan and Baluchestan is geographically adjacent to South Khorasan, where the current study was conducted, suggesting that the close proximity of these two provinces may reflect similar genetic backgrounds and account for the comparable prevalence of hybrid Rhesus box–mediated RHD deletion observed in both populations. In a parallel investigation, Khosroshahi et al. (2018) determined that 198 out of 200 Rh-negative donors in Tehran were homozygous for the hybrid Rhesus box. Two heterozygous individuals were observed, carrying a weak D type 11 or RHD-CE(2-9)-D2 hybrid allele [15]. These findings indicate that complete deletion of the RHD gene is the main mechanism underlying the RhD-negative phenotype in Iranian populations. Therefore, considering the relatively high prevalence of RhD negativity, proper management of compatible blood resources, accurate donor screening to prevent alloimmunization, and appropriate strategies for the prevention of hemolytic disease of the fetus and newborn in RhD-negative pregnant women remain important considerations in transfusion medicine [22].

Comparisons with other populations further highlights the ethnic variability in RhD-negative mechanisms. European and American populations demonstrate a high frequency of complete RHD gene deletions via hybrid Rhesus box formation, which is consistent with the findings observed in Iran[23]. In contrast, African populations exhibit a more diverse molecular basis, including the RHDΨ allele and point mutations that lead to inactive RHD. These factors contribute to the RhD-negative phenotype in only a subset of individuals[24]. In East Asian populations, where the prevalence of RhD negativity is exceptionally low (less than 0.1%), the predominance is characterized by the presence of non-functional or variant RHD alleles, as opposed to the occurrence of complete gene deletions[25]. Among Arab populations, studies from Saudi Arabia (Alalshaikh et al., 2024) and Oman (Lawati et al., 2023) indicate that while the hybrid Rhesus box is

present in the majority of RhD-negative donors, a small proportion carry non-deletion alleles or partial hybrid genes [26, 27.] In Tunisia, a study by Sassi et al. (2014) demonstrated that of 400 Rh-negative donors, 390 exhibited complete RHD deletions, while 10 carried non-deletion alleles. These included 4 RHDΨ pseudoalleles, 5 RHD-CE-D hybrid alleles, and 1 weak D type 4 allele [2].

Molecular RhD typing has important clinical relevance, as accurate identification of RHD variants helps prevent alloimmunization in transfusion recipients and reduces the risk of hemolytic disease of the fetus and newborn in perinatal care [28]. These data further indicate that although the deletion mechanism is dominant in many populations, regional and ethnic differences exist that must be considered in transfusion strategies and clinical decision-making.

The identification of the hybrid Rhesus box and the determination of RHD zygosity provide a reliable approach to detecting true RhD-negative donors, particularly in populations where conventional serological tests may fail to distinguish weak or variant D alleles. The implementation of molecular genotyping is imperative for the effective management of blood transfusions, particularly in patients who are dependent on transfusions. This approach is crucial for ensuring the safety of blood products and for maximizing the efficacy of prophylactic anti-D immunoglobulin in perinatal care settings [7, 17].

Also, in countries with ethnically and racially diverse populations, accurate knowledge of the genetic structure of the RHD gene is of particular importance for the design of blood banks and the management of transfusion strategies based on localized molecular data. In Iran, given the high frequency of homozygous RHD deletions among RhD-negative donors, hybrid Rhesus box analysis can serve as an efficient and accurate genetic marker for routine donor screening. Our findings, while confirming the predominance of hybrid Rhesus box–mediated RHD deletion in Iran, provide the first documented evidence from Eastern Iran and offer region-specific data with practical implications for blood bank policy and local genetic counseling. Nevertheless, this study focused exclusively on detecting complete RHD deletion based on hybrid Rhesus box analysis; therefore, alternative non-deletion mechanisms of RhD negativity such as the RHDΨ pseudogene, point mutations, or RHD-CE-D hybrid alleles were not evaluated. This represents a limitation of the study and highlights the need for future work employing broader molecular genotyping approaches.

## CONCLUSION

The RhD-negative phenotype in blood donors from eastern Iran is predominantly caused by homozygous RHD gene deletion mediated by the hybrid Rhesus box. High concordance between PCR-SSP, real-time exon analysis (exons 5, 7, and 10), and PCR-RFLP zygosity testing confirms the hybrid Rhesus box as a reliable molecular marker for RHD deletion. These results align with findings in Iranian and European populations but differ from those in African and East Asian groups, underscoring the importance of population-specific Rhesus box–based genotyping for accurate RhD typing and transfusion safety.

### Abbreviations

| | |
|---|---|
| **HDFN** | Hemolytic disease of the fetus and newborn |
| **RHDΨ** | Pseudogenized RHD alleles |
| **IAT** | Indirect antiglobulin test |
| **RFLP** | Restriction fragment length polymorphism |

## CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

### Ethics approval and consent to participate

This study was approved and supported by Birjand university of medical sciences (Ethical code: IR.BUMS.REC.1404.112). All procedures were conducted in accordance with the ethical standards of the institutional and national research committees and with the Declaration of Helsinki. Written informed consent was obtained from all participants prior to inclusion in the study.

### Consent for publication

Not applicable.

**Data availability**

All data generated or analyzed during this study are included in this published article. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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