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Molecular Characterization of RhD Variants in the Turkish Population: A Multicenter Study for Population-Specific Diagnostic Strategy Development

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Abstract

Background and Aims: RhD variants pose significant transfusion challenges due to alloimmunization risks. While molecular characterization exists for many populations, comprehensive Turkish data remains limited despite the country's unique genetic heritage. This study aimed to determine the molecular profile of RhD variants in Turkey and establish foundations for population-specific diagnostic strategies.

Materials and Methods: This multicenter prospective study, conducted between July 2022 and January 2025 and supported by the Turkish Society of Hematology (Project No: 2022-14), included 35 samples comprising 21 RhD variants, 9 RhD negative, and 5 RhD positive control cases collected from four university hospital transfusion centers. Genomic DNA was isolated from EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany). All 10 exons of the *RHD* gene were selectively amplified using gene-specific primers and subjected to bidirectional Sanger sequencing for molecular characterization. Serological evaluation was performed using column agglutination (gel centrifugation) techniques with standardized monoclonal anti-D reagents to confirm phenotypic D status and assess serological discrepancies.

Results:A total of 35 samples were analyzed (17 males, 48.57%; 18 females, 51.43%). DNA concentrations ranged from 28-435 ng/µL (mean: 157.9±94.3 ng/µL). All five Rh positive control samples included in the study were successfully amplified by PCR and demonstrated normal sequencing results. In contrast, PCR amplification failed in all nine Rh negative control samples. Among the 21 samples with variant Rh phenotypes included in the study, 15 samples showed partial PCR amplification failure (ranging from one to three exons). For these cases, further analyses were recommended to investigate potential gene-level deletions, duplications, or hybrid alleles, in order to establish genotype correlation. RhD variants were identified in 13 samples with variant Rh phenotypes: two homozygous for *RHD*09.01.02 (RHD*DAR1.02)*, seven heterozygous for *RHD*01W.1 (RHD*weak D type 1)*, one heterozygous for *RHD*01W.5 (RHD*weak D type 5)*, one heterozygous for *RHD*07.01 (RHD*329C)*, one heterozygous for *RHD*165T (DBO-1)*, and one homozygous for *RHD*05.05 (RHD*697A).* Preliminary analysis revealed distinct rates from European variant patterns' rates.

Conclusion: This study represents the first comprehensive molecular characterization of RhD variants in Turkey, delineating population-specific allelic patterns' rates that differ substantially from those reported in European and Asian cohorts. The high prevalence of technically challenging variants, including potential *RHD-CE* hybrid alleles, coupled with rare polymorphisms underrepresented in global databases, underscores the critical need for Turkey-specific molecular diagnostic strategies. Furthermore, our data demonstrate that while serological tests remain the cornerstone of routine RhD typing, their limitations in detecting weak D and partial D variants necessitate the integration of molecular approaches. A total of 35 cases were analyzed in our study. Nine of these were serologically RhD-negative, five were RhD-positive controls, and the remaining 21 cases were variants initially assessed as RhD-negative by routine blood banking serological methods. Molecular analysis revealed that these 21 cases carried different RhD variants (3 partial D, 8 weak D, and 2 rare variants). These results highlight the critical complementary role of molecular genotyping in preventing unnecessary anti-D prophylaxis and ensuring the accurate identification of clinically significant variants. These results provide a robust scientific rationale for the implementation of national RhD genotyping programs and the development of population-tailored diagnostic kits, which would significantly enhance transfusion safety and mitigate healthcare costs associated with RhD mistyping and alloimmunization risk.

Introduction and Aim

The Rh blood group system, the most complex after ABO, comprises 56 antigens encoded by the highly homologous *RHD* and *RHCE* genes on chromosome 1p36.11 [1,2]. RhD antigen demonstrates exceptional immunogenicity, with approximately 70% of D-negative individuals developing anti-D antibodies following D-positive erythrocyte exposure, potentially causing severe hemolytic transfusion reactions and hemolytic disease of the fetus and newborn (HDFN) [3,4].

The RH locus molecular complexity, characterized by 97% *RHD-RHCE* sequence homology, predisposes this region to frequent gene conversion and recombination events, resulting in the generation of more than 700 documented *RHD* allelic variants. [5]. These variants, weak D (quantitative reduction) and partial D (qualitative alteration), present significant diagnostic challenges.

Weak D variants express approximately 60–4,000 D antigen sites per erythrocyte compared to 10,000–30,000 sites on normal D-positive cells, whereas DEL variants, characterized by extremely low antigen density (<30 sites per cell), often escape serological detection and are typically identified only through adsorption-elution studies or molecular testing [6,7].

Population-specific RhD variant distribution demonstrates remarkable ethnic variation. European populations predominantly carry weak D types 1, 2, and 3 (>90% of weak D phenotypes) [8]. African populations exhibit high partial D variant frequencies, particularly DAR, DAU and DIIIa alleles [9]. Asian populations uniquely harbor DEL variants, particularly *RHD* (1227G>A), at frequencies reaching 30% among D-negative individuals [10]. This ethnic heterogeneity necessitates population-specific diagnostic approaches.

Turkey's unique demographic composition, shaped by historical migration patterns and regional consanguinity rates reaching 23.2%, creates a distinctive genetic landscape [11]. Recent genomic studies revealed 28% of exome variants and 49% of genome variants in the Turkish population occur at very rare frequencies (allele frequency <0.005), distinguishing it from global reference populations [12]. This genetic uniqueness likely extends to blood group polymorphisms, yet comprehensive RhD variant characterization remains absent.

Current serological methods demonstrate variable sensitivity for D antigen detection. European molecular RHD screening reports clinically significant variants in 0.1-0.47% of serologically D-negative donors [13,14,15]. The Croatian experience demonstrates regional variation within Europe, with 0.35% of D-negative donors carrying RhD variants, predominantly *RHD-CE* (2-9) D hybrids rather than common weak D types [16].

The absence of Turkish RhD variant data creates critical clinical challenges: potential alloimmunization from mistyped blood products, unnecessary anti-D immunoglobulin prophylaxis, inability to develop national guidelines, and missed opportunities for regional leadership in molecular transfusion medicine. This multicenter study aimed to establish the molecular profile of RhD variants in Turkey through comprehensive *RHD* gene sequencing, providing scientific foundation for rapid diagnostic kit development.

Materials and Methods

Study Design and Ethical Considerations

This prospective multicenter study (July 2022-January 2025) was coordinated by Afyonkarahisar Health Sciences University Department of Molecular Diagnostics. The study received Turkish Society of Hematology Research Projects Support Program funding (Project No: 2022-14, Date: 22.07.2022, Budget:50,000 TL). Institutional ethics committee approvals were obtained from all participating centers. Written informed consent was acquired from all participants following Declaration of Helsinki principles.

Participating Centers and Sample Collection

This multicenter study included four university hospital transfusion centers: Afyonkarahisar Health Sciences University Hospital (coordinating center), Dokuz Eylül University Hospital, Marmara University Hospital, and Eskişehir Osmangazi University Hospital. Although a total of 40 samples (20 RhD variants, 10 Rh-negative, and 10 Rh-positive controls) were initially targeted, DNA quality and amplification adequacy were not achieved in some samples during the molecular analysis process, and duplicate patient samples were excluded from the study. Therefore, the analysis was performed on a total of 35 unique samples, including 21 RhD variants, 9 Rh-negative, and 5 Rh-positive control samples.

Inclusion criteria encompassed serological discrepancies in D typing (reaction strength ≤+3), discordant results obtained with different anti-D reagents, and a positive indirect antiglobulin test in the presence of negative direct agglutination findings. Exclusion criteria included a documented history of transfusion within the preceding three months, a confirmed diagnosis of autoimmune hemolytic anemia, and inadequate sample volume for subsequent molecular analyses. Peripheral blood samples (2 mL, EDTA-anticoagulated) were obtained from eligible patients, coded, and transported under cold-chain conditions (2-6 °C) to the blood transfusion center laboratory in less than 48 hours.

Serological Testing

Initial RhD typing was performed in accordance with standard transfusion laboratory protocols utilizing automated blood group analyzers. Confirmatory testing was conducted using gel column agglutination technology (DiaMed ID-Cards, Bio-Rad, Switzerland) incorporating both DVI-negative and DVI-positive microtubes preloaded with IgM/IgG monoclonal anti-D reagents (clones D415, 1E401/172-2). Weak D expression was assessed through the indirect antiglobulin test (IAT). Agglutination strength was semi-quantitatively graded from negative (-) to +4 according to standard hemagglutination scoring criteria.

DNA Extraction and Quality Assessment

Genomic DNA was isolated from 200 µL of EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) in strict accordance with the manufacturer's protocol. DNA concentration and purity were determined using NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific, USA). Quality control thresholds were defined as A260/A280 ratios between 1.7 and 2.0 and A260/A230 ratios exceeding 2.0, indicative of protein- and reagent-free nucleic acid preparations. DNA samples meeting these criteria were standardized to a working concentration of 20–80 ng/µL for downstream molecular assays.

Primer Design and PCR Amplification

RHD gene-specific primers were designed using Primer3 software, based on the reference sequence (GenBank accession NG_007494.1), with stringent in silico validation to ensure specificity against RHCE homologous regions. Polymerase chain reaction (PCR) was performed in a 15 µL reaction volume containing 2X PCR Master Mix, HotStart Taq DNA Polymerase, an optimization buffer, gene-specific primers, and genomic DNA template. Primer sequences covering all 10 exons of the RHD gene are provided in Table 1.

Thermal cycling conditions consisted of an initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 96°C for 30 seconds, annealing at 60°C for 60 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. Amplification products were confirmed by 2% agarose gel electrophoresis under ethidium bromide staining and visualized using a UV transilluminator.

Table 1. *RhD* gene-specific primer sequences and PCR amplification parameters

Exon	Forward Primer (5'→3')	Reverse Primer (5'→3')	Amplicon Size (bp)	Annealing Temperature (Tm, °C)
1	TAGAGAGGCCAGCAGACACCA	TGCCCCTGGAGACACCACA	298	60
2	CCTTATTCCTATGCCCAGCAGGC	ACAGGAGTCCCGAATAATTTGAATC	342	60
3	CTATTCGCACAGAAGATAGTGCC	GGGCTCAAACATCCTGCAAC	315	60
4	CCTCTCACTGAAGACTGTCACT	TTGAATCTGCATACCCCAGGC	287	60
5	CCTCCTCTTTACACAGAGTTCCC	TCTACCTAGACTGCACTGAGAG	324	60
6	CAGTATGGAGCTGTGGACTC	GACACTGCTGGAAGGCTTTG	296	60
7	CTTATTTTACAAACATCCCCCACTC	GCTCTGTTGTTTGGGGACTAC	312	60
8	CTTCTCTGGAGCTGACTACT	AGAGTGGGGCAACTAGACAC	289	60
9	GGCTCAGAGATGAAGACTGTCCA	ACAGTGCAGACAATATATCTCAGT	356	60
10	GGCTGTTTTAGCAGTTGCAGCC	GCTTACTGGATGACACCATCATA	378	60

DNA Sequencing and Analysis

PCR amplicons were purified using the CleanDTR Sequencing Purification Kit (CleanNA, Netherlands) in accordance with the manufacturer's instructions to remove unincorporated primers and nucleotides. Cycle sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing reactions were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems) to generate bidirectional sequence reads.

Raw chromatograms were inspected using Chromas v2.6.6 software, with manual curation to confirm base-calling accuracy and resolve ambiguous peaks. High-quality sequences were aligned against the RHD reference sequence (GenBank accession NG_007494.1) using the ClustalW algorithm implemented in MEGA X software, enabling precise identification of nucleotide substitutions.

Statistical Analysis

All statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). The data in this study were analyzed using descriptive statistics. Continuous variables, including DNA concentration and purity ratios (A260/A280 and A260/A230), were assessed and expressed as mean ± standard deviation (SD). DNA concentrations ranged from

28–435 ng/μL, with a mean value of 157.9 ± 94.3 ng/μL. Categorical variables, such as sex distribution (male: 17 [48.57%], female: 18 [51.43%]), RhD variant classification, and sequencing success (complete vs. partial), were presented as absolute frequencies and percentages.

Results

Study Population Demographics

A total of 35 samples fulfilled the inclusion criteria and were subjected to analysis. Among these, 21 (60.0%) were identified as Rh variants, 9 (25.7%) as Rh negative, and 5 (14.3%) were included as Rh positive controls.

The study cohort consisted of 17 males (48.57%) and 18 females (51.43%). No repeated or duplicate patient samples were included in the analysis.

DNA Quality and Sequencing Success

All extracted DNA samples demonstrated acceptable quality parameters (A260/A280: 1.7–1.9), with DNA concentrations ranging from 28 to 435 ng/μL (mean: 157.9 ± 94.3 ng/μL).

Complete bidirectional sequencing was successfully achieved in all Rh-positive controls (100%). Among Rh-negative controls, the expected RHD gene deletion pattern was confirmed, characterized by variable exon-specific amplification failures consistent with the Rh-negative phenotype.

Table 2. Sequencing success rates by sample type and exon

Sample Type	n	Complete Sequencing	Partial Sequencing	Failed Exons
Rh positive	5	5 (100%)	0	None
Rh negative	9	0	9 (100%)	Variable*
RhD variant	21	6 (28.6%)	15 (71.4%)	Mainly 1,4 or 5

*Rh-negative samples showed expected absence of RHD-specific amplification with variable patterns

RhD Variant Characterization

Among the RhD variant samples, complete sequencing was achieved in 6 patients (28.6%).

Exon-specific failures were observed in the remaining cases (see table 4):

11 patients (52.4%) exhibited amplification failure limited to exon 1,

2 patients (9.5%) showed failures in exons 4 and 5,

1 patient (4.8%) demonstrated combined failure of exons 1, 4, and 5, and

1 patient (4.8%) exhibited failure in exons 1 and 9.

In this study, a variant allele was identified in 13 samples with RhD variant phenotypes (see table 3).

Table 3: Distribution of *RHD* Gene Variants Among Samples with Variant Rh Phenotypes

RHD Genotype	Zygosity	Sample Size
*RHD*09.01.02 (RHD*DAR1.02)*	Homozygous	2 (15.38%)
*RHD*01W.1 (RHD*weak D type 1)*	Heterozygous	7 (53.85%)
*RHD*01W.5 (RHD*weak D type 5)*	Heterozygous	1 (7.69%)
*RHD*07.01 (RHD*329C)*	Heterozygous	1 (7.69%)
*RHD*165T (DBO-1)*	Heterozygous	1 (7.69%)
*RHD*05.05 (RHD*697A)*	Homozygous	1 (7.69%)
Total		13 (100%)

Table 4: PCR Success and RhD Variant Distribution in Samples

Sample No	Phenotype	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9	Exon 10
S1	Positive	+	+	+	+	+	+	+	+	+	+
S2	Positive	+	+	+	+	+	+	+	+	+	+
S3	Positive	+	+	+	+	+	+	+	+	+	+
S4	Positive	+	+	+	+	+	+	+	+	+	+
S5	Positive	+	+	+	+	+	+	+	+	+	+
S6	Negative	-	*RHCE*	*RHCE*	-	-	*RHCE*	-	*RHCE*	*RHCE*	-
S7	Negative	+	*RHCE*	*RHCE*	+	+	*RHCE*	+	*RHCE*	*RHCE*	*RHCE*
S8	Negative	+	*RHCE*	*RHCE*	+	+	*RHCE*	+	*RHCE*	*RHCE*	*RHCE*
S9	Negative	+	*RHCE*	*RHCE*	+	+	*RHCE*	+	*RHCE*	*RHCE*	*RHCE*
S10	Negative	+	*RHCE*	*RHCE*	-	-	*RHCE*	+	*RHCE*	*RHCE*	*RHCE*
S11	Negative	-	*RHCE*	*RHCE*	-	-	*RHCE*	-	*RHCE*	*RHCE*	-
S12	Negative	-	*RHCE*	*RHCE*	-	-	*RHCE*	+	*RHCE*	*RHCE*	-
S13	Negative	-	*RHCE*	*RHCE*	-	-	*RHCE*	-	*RHCE*	*RHCE*	*RHCE*
S14	Negative	-	*RHCE*	*RHCE*	-	-	*RHCE*	+	*RHCE*	*RHCE*	*RHCE*
S15	Variant	+	+	+	c.602 C>G	c.667 T>G c.744 C>T	+	c.957G>A c.1025T>C	+	+	+
S16	Variant	+	+	c.446 C>A	+	+	+	+	+	+	+
S17	Variant	-	+	+	+	+	c.809T>G	+	+	+	+
S18	Variant	+	+	+	-	-	+	+	+	+	+
S19	Variant	+	+	+	-	-	+	+	+	+	+
S20	Variant	+	+	+	+	c.697 G>A	+	+	+	+	+
S21	Variant	-	+	+	+	+	+	+	+	+	+
S22	Variant	-	c. 329C	+	+	+	+	+	+	-	+
S23	Variant	-	+	-	+	+	c.809T>G	+	+	+	+
S24	Variant	-	+	+	+	+	c.809T>G	+	+	+	+
S25	Variant	-	+	+	+	+	c.809T>G	+	+	+	+
S26	Variant	-	+	+	+	+	c.809T>G	+	+	+	+
S27	Variant	-	+	+	+	+	+	+	+	+	+
S28	Variant	+	c.165 C>T	+	+	+	+	+	+	+	+
S29	Variant	-	+	+	+	+	+	+	+	+	+
S30	Variant	-	+	+	+	+	c.809T>G	+	+	+	+
S31	Variant	-	+	+	+	+	+	+	+	+	+
S32	Variant	+	+	+	c.602C>G	c.667T>G c.744C>T	+	c.957G>A c.1025 T>C	+	+	+

S33	Variant	-	+	+	+	+	+	+	+	+	+
S34	Variant	-	+	+	-	-	+	+	+	+	+
S35	Variant	+	+	+	+	+	c.809T>G	+	+	+	+

RHCE: Considering the high sequence homology between *RHD* and *RHCE*, this interpretation was made based on the detection of *RHCE*-specific SNP's. In certain exons, distinguishing between *RHD* and *RHCE* may not be feasible (Positive: Rh positive, Negative: Rh negative, Variant: RhD Variant)

Discussion

This study constitutes the first comprehensive molecular characterization of RhD variants in Turkey, delineating a distinct genetic profile that diverges from those reported in European and other populations. These results carry important implications for transfusion medicine, underscoring the necessity of population-tailored molecular diagnostic strategies to accurately resolve serological discrepancies and mitigate alloimmunization risk.

The frequent amplification failures observed in particular exons such as 1, 4, and 5 in our study underscore the technical challenges inherent in *RHD* genotyping. The high (97%) sequence homology between *RHD* and *RHCE*, especially in these regions, likely contributes to non-specific amplification and dropouts, particularly in the context of gene conversion events [17]. These findings highlight the necessity for optimized primer design and consideration of alternative amplification or sequencing strategies to improve coverage of these exons.

Among the 13 samples with variant Rh phenotypes, a diverse spectrum of *RHD* alleles was identified, reflecting the molecular heterogeneity underlying RhD antigen expression. The most prevalent allele was *RHD*01W.1* (weak D type 1), observed in seven heterozygous individuals (53.85%), consistent with its frequent occurrence in European populations [18]. The detection of *RHD*09.01.02* (DAR1.02) in two homozygous samples is notable, as this allele, often associated with partial D phenotypes, is more commonly reported in individuals of African ancestry but has also been described in admixed populations [20,21]. The identification of rare alleles such as *RHD*01W.5 (*weak D type 5*)*, *RHD*07.01 (RHD*329C)*, *RHD*165T (DBO-1)*, and *RHD*05.05 (RHD*697A)*, each in a single sample, underscores the allelic diversity within the Turkish population and the potential presence of underrecognized or population-specific variants. The presence of both weak and partial D alleles in this cohort emphasizes the importance of high-resolution genotyping in transfusion settings, as serologic methods alone may fail to differentiate between clinically significant subtypes, increasing the risk of alloimmunization. These findings support the need for comprehensive molecular screening strategies tailored to the local population. This has profound implications for diagnostic kit design, as commercial platforms optimized for European populations may inadequately detect Turkish variants.

The identification of rare SNPs with limited representation in established databases suggests population-specific genetic features rather than novel variants. Turkey's historical role as a migration corridor, coupled with regional endogamy practices, may have contributed to the presence and persistence of these rare RhD variants within the population [19].

A comparison of RhD variant frequencies and predominant alleles across European populations is summarized in Table 5. This table presents data on RhD variant frequencies among serologically D-negative donors from selected European studies. The most common alleles demonstrate marked inter-population variability, emphasizing the importance of population-specific molecular reference data.

Table 5. Comparison of Rh D variant frequencies in different populations

Population	D-negative Screened	RHD-positive n (%)	Clinically Significant (%)	Most Common Variants	Reference
Croatia	6,523	23 (0.35%)	12 (0.18%)	RHD-CE(2-9)D (47.8%), weak partial 11	[17]
Germany	46,133	96 (0.21%)	47 (0.10%)	Weak D types 1, 2, 3	[14]
Austria	23,330	94 (0.40%)	74 (0.32%)	Variable	[15]
Switzerland	25,370	120 (0.47%)	37 (0.15%)	Variable	[16]

Our findings carry immediate clinical relevance. Among the 21 RhD variant samples analyzed, complete sequencing was achieved in 6 cases (28.6%), while exon-specific amplification failures were observed in 15 cases (71.4%), predominantly involving exons 1, 4, and 5. Notably, 4 cases (19.0%) demonstrated amplification patterns suggestive of potential RHD-CE hybrid alleles, underscoring the technical challenges inherent in genotyping within regions of high RHD-RHCE homology. Importantly, partial D alleles identified in this cohort, including RHD09.01.02 (DAR1.02) and RHD07.01 (RHD*329C), highlight the risk of alloimmunization if such individuals are misclassified as RhD-positive. These results reinforce the necessity of integrating molecular testing to accurately distinguish weak D (types 1 and 5) from clinically significant partial D variants, thereby guiding appropriate transfusion management and minimizing alloimmunization risk.

Development of Turkey-specific diagnostic tools requires; establishing reference variant database through expanded screening, developing multiplex PCR assays targeting prevalent Turkish variants, collaborating with Turkish biotechnology companies, implementing pilot programs, and creating national guidelines through Turkish Society of Hematology leadership.

Economic feasibility appears favorable. Current imported molecular typing kits cost 50-100 EUR/test; local production could reduce costs to 10-20 EUR. With approximately 2.5 million annual blood donations in Turkey, selective molecular typing of serologically ambiguous cases (estimated 1-2%) would require 25,000-50,000 tests annually, creating sustainable market for local manufacturers.

Novel Findings

Preliminary sequence analysis revealed:

1. Population-specific SNPs not commonly reported in European populations
2. Evidence of both quantitative (weak D) and qualitative (partial D) variants

Serological-Molecular Correlation

The integration of serological findings with molecular genotyping in this study underscores the complementary value of both approaches in resolving RhD typing discrepancies. Among the 21 samples with RhD variant phenotypes identified serologically, molecular characterization confirmed the presence of both quantitative (weak D) and qualitative (partial D) variants, demonstrating the heterogeneity underlying serologic weak or discrepant D phenotypes (Table 6).

Table 6. Discrepancies between serologic and molecular RHD typing results

Sample Code	**Serological Result**	**Genetic Findings**	**Genotype Classification**	**Cause of Discrepancy**	**Correct Result (Clinical Management)**
S15	RhD-negative	***RHD*09.01.02 (RHD*DAR1.02)*** c.602C>G; c.667T>G; c.744C>T; c.957G>A; c.1025T>C	Partial D	Epitope loss / structural change	Patient: RhD negative; Donor: RhD positive
S16	RhD-negative	***RHD*01W.5 (RHD*weak D type 5)*** c.446C>A	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S17	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S18	RhD-negative	Exon 4-5 amplification failure	Indeterminate (suspected hybrid)	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S19	RhD-negative	Exon 4-5 amplification failure	Indeterminate (suspected hybrid)	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S20	RhD-negative	***RHD*05.05 (RHD*697A)*** c.697G>A	Rare RHD variant	Possible qualitative epitope alteration	Patient: RhD negative; Donor: RhD positive
S21	RhD-negative	Failure of amplification of exon 1	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S22	RhD-negative	***RHD*07.01 (RHD*329C)***	Partial D	Epitope loss / structural change	Patient: RhD negative; Donor: RhD positive
S23	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S24	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S25	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S26	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S27	RhD-negative	Failure of amplification of exon 1	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S28	RhD-negative	***RHD*165T (DBO-1)*** c.165C>T	Rare RHD variant	Possible qualitative epitope alteration	Patient: RhD negative; Donor: RhD positive
S29	RhD-negative	Failure of amplification of exon 1	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S30	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive
S31	RhD-negative	Failure of amplification of exon 1	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended

S32	RhD-negative	***RHD*09.01.02 (RHD*DAR1.02)*** c.602C>G; c.667T>G; c.744C>T; c.957G>A; c.1025T>C	Partial D	Epitope loss / structural change	Patient: RhD negative; Donor: RhD positive
S33	RhD-negative	Failure of amplification of exon 1	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S34	RhD-negative	Failure of amplification of exons 1,4,5	Indeterminate	Possible RHD-CE hybrid / exon deletion	Further testing recommended
S35	RhD-negative	***RHD*01W.1 (RHD*weak D type 1)*** c.809T>G	Weak D	Reduced antigen density	Patient: RhD negative; Donor: RhD positive

Serologically weak D phenotypes (reaction strength ≤+3) correlated strongly with weak D type 1 (RHD*01W.1) and weak D type 5 (RHD*01W.5) alleles, both associated with reduced antigen density (60-4,000 sites per RBC). Conversely, samples displaying atypical or inconsistent serological reactivity were associated with partial D alleles such as RHD*09.01.02 (DAR1.02) and RHD*07.01 (RHD*329C), which lack specific D epitopes and confer alloimmunization risk.

Exon-specific amplification failures, particularly in exons 1, 4, and 5, were noted in several serologically weak or discrepant samples. These failures reflect the technical challenges posed by RHD-RHCE homology and potential hybrid alleles, complicating molecular resolution in ambiguous cases.

While serological tests remain the primary method for routine identification of the RhD antigen, they have significant limitations, particularly in the accurate classification of weak D and partial D variants. This is not due to serological inaccuracy, but rather to inadequate method sensitivity in cases where antigen density is low (weak D) or epitope loss occurs (partial D). Differences in epitope recognition among the anti-D reagents used can lead to some variants being incorrectly reported as RhD negative or indeterminate. Furthermore, distinguishing between partial D types is often not possible with serology. Therefore, serology provides incomplete information due to its technical limitations. However, molecular typing plays a critical complementary role by ensuring the accurate identification of serologically equivocal or discordant cases. Clinically, this approach avoids unnecessary anti-D immunoprophylaxis and improves transfusion safety by accurately identifying variants at risk for alloimmunization. Our study demonstrates the regular identification of distinct features in some cases serologically classified as RhD-negative, highlighting the critical importance of integrating these two methods into patient and donor regimens. A total of 35 cases were analyzed: 9 were serologically RhD-negative, 5 were RhD-positive controls, and the remaining 21 were routinely classified as RhD-negative in blood banking but exhibited different RhD markers upon analysis. Specifically, of these 21 cases, 3 were partial D, 8 were weak D, and 2 were rare variants. These results, combined with the detection of weak D and partial D types in addition to rare regimens, strongly support the limitations of serological methods and the additional diagnostic value of treatment analyses.

Clinical Implications

The findings of this study have direct and clinically relevant implications for transfusion medicine practice. Accurate differentiation between weak D variants (types 1 and 5) and partial D alleles (e.g., DAR1.02 and RHD*329C) is critical to ensure appropriate transfusion management and alloimmunization prevention.

Patients with partial D alleles, which lack specific D epitopes, remain at risk of anti-D alloimmunization and therefore should be managed as RhD-negative for transfusion and anti-D prophylaxis purposes. In contrast, individuals harboring weak D type 1 or type 5-confirmed by molecular analysis-can be safely classified as RhD-positive, thereby reducing unnecessary administration of anti-D immunoglobulin and avoiding overtreatment.

Furthermore, the identification of exon-specific amplification failures and suspected RHD-CE hybrid alleles in this cohort highlights the technical limitations of serology alone and underscores the importance of incorporating molecular testing in cases of serological discrepancy. Integrating molecular genotyping into routine transfusion workflows would provide greater diagnostic precision, prevent alloimmunization events arising from undetected partial D alleles, and support evidence-based national transfusion guidelines tailored to the Turkish population.

Study Limitations

Several limitations warrant acknowledgment. First, the relatively small sample size limits the precision of allele frequency estimates, and the geographic distribution predominantly represents western Turkey, potentially reducing generalizability. Second, technical constraints prevented full characterization of all detected variants. Importantly, the Sanger sequencing approach used in this study cannot reliably detect large deletions or duplications, nor can it identify hybrid alleles or complex *RHD* rearrangements, which are increasingly recognized in serologically challenging cases.

Future research priorities include expanding sample collection to at least 1,000 donors to improve statistical power, implementing long-read sequencing technologies to address current limitations in detecting hybrid alleles and complex rearrangements, conducting cost-effectiveness analyses of molecular typing strategies, and establishing a biobank for the preservation and further study of rare *RHD* gene variants.

Conclusion

This multicenter study provides foundational evidence that the Turkish population harbors a unique spectrum of Rh D variants distinct from established European and Asian patterns. The convergence of clinical need, technical feasibility, and economic viability creates an opportune moment for advancing Turkish transfusion medicine into the molecular era. These findings establish the scientific rationale for national molecular blood typing implementation, ultimately improving transfusion safety for millions of Turkish citizens.

Abbreviations

HDFN: hemolytic transfusion reactions and hemolytic disease of the fetus and newborn

PCR: Plymerase Chain Reaction

SNP:Single Nukleotide Polymorphism

Declarations

Ethics Approval and Funding

The study received Turkish Society of Hematology Research Projects Support Program funding. This study was approved by the Afyonkarahisar Health Sciences University Non-Interventional Research Ethics Committee (Project No: 2022-14, Date: 22.07.2022, Budget:50,000 TL). Institutional ethics committee approvals were obtained from all participating centers. Written informed consent was acquired from all participants following Declaration of Helsinki principles.

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Authorship Contributions

Concept: İ.E., N.A.; Design: İ.E., D.A.Ö., N.A.; Data Collection/Processing: S.A., Ö.Ö., C.K., Ö.T., F.Y., F.D.; Analysis/Interpretation: İ.E., D.A.Ö., G.K., H.O.; Literature Search: İ.E., S.H.Y., M.Ö.E.; Writing: İ.E., D.A.Ö.

Competing of Interest

The authors report there are no competing interest to declare This study was not influenced by any commercial or financial relationships that could be construed as a potential conflict of interest.

Availability of data and materials

The datasets generated and analysed during the current study are stored as Excel files and are not publicly available due to institutional data protection policies. However, the data are available from the corresponding author upon reasonable request.

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