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ORIGINAL ARTICLE

# Rare Occurrence of *RHD* Null Alleles With Del Expression Among Serologically D-Negative Blood Donors

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## Abstract

**Background/Aim:** An investigation into the diversity of serologically D-negative blood donors in the Republic of Srpska entity of Bosnia and Herzegovina forms the basis of this research. The primary purpose of the study was the examination of *RHD* variants over a period of five years.

**Methods:** A comprehensive depiction of the *RHD* distribution in D-negative blood donors is achieved through a combination of serological observations and DNA testing (PCR-SSP with fluorometric signal detection), involving 74,149 blood donors. The adsorption/elution method was used to confirm the Del phenotype.

**Results**: A small fraction (0.31 %) of the serologically D-negative blood donors was found to contain eight different *RHD* alleles. The Del phenotype of the *RHD*01N.03* and *RHD*01EL.44* alleles was highlighted, challenging the common perception that these alleles are associated exclusively with a D-negative expression.

**Conclusion:** The importance of molecular methods in analysing and understanding Del variants, which typically elude conventional serological assays, is underscored by the findings. A group of donors seemingly having the *RHD*01* allele but who lacked D antigen expression was encountered, hinting at the potential presence of still unidentified, possibly geographically restricted, *RHD* variants or alterations in other genes responsible for the expression of Rh proteins in the erythrocyte membrane.

**Key words:** Blood group antigens; Polymerase chain reaction; Blood donors; Phenotype; Genetic variation.

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## Introduction

Recombination between the two Rh genes are not uncommon and result in hybrid alleles.[1-3] Several other genetic events also influence the expression of Rh antigens (weak, partial, Del or null phenotype).[1, 2] In this paper, as suggested previously, Del was used for the phenotype and DEL for the allele designation.[4]

In Del individuals, D antigen expression is quantitatively very low.[4] They are usually typed as D-negative in routine serological testing and can only be detected by the adsorption/elution technique.[4-6] Only with routine molecular screening for Del variants the carriers could be successfully detected and not mistyped as D-negative.[7, 8] Anti-D alloimmunisation with Del-mistyped red blood cells (RBC) has been reported, although rarely.[3, 4, 6, 9-13]

The frequency of Del has been well documented for large populations.[6] It is significant in Asian populations, where the frequency of Del variants among serologically D-negative donors is 1:5–1:8 in comparison with 1:350–1:2,000 for the populations of European origin.[14] The *RHD*01EL.01* allele is the most prevalent DEL allele in Asian populations, as exemplified by Chinese[15, 16] and Korean.[17, 18] Pregnant women with this allele do not receive RhIg and patients could be transfused with D-positive RBCs.[6] The most common alleles with Del expression in populations of European origin are *RHD*11* and *RHD*01EL.08*.[4, 19-21]

Adsorption/elution testing can assist in Del detection, particularly when molecular methods are not available.[13] The RhCE phenotypes could be significant for Del detection because of their strong association with C+/E+ phenotypes in many populations.[5,14,18,21-25] With the advancement of molecular techniques, the detection of Del variants is becoming increasingly feasible, underscoring the importance of integrating both methodologies into standard blood typing procedures, as illustrated in Asia, where Del transfusion may lead to alloimmunisation.[12, 26]

The primary focus of this study involved the examination of *RHD* variants within the population of D-negative blood donors over a period of five years from the Republic of Srpska entity of Bosnia and Herzegovina. Through the assessment of Rh phenotype associations, the presence of RhD epitopes and the analysis of different *RHD* alleles, objective was to strengthen and enhance the safety protocols in blood transfusion procedures.

# Methods

## Participants

A prospective study was conducted at the Institute for Transfusion Medicine of the Republic of Srpska in Banja Luka, Bosnia and Herzegovina, with 74,149 unrelated healthy regular blood donors. The samples have been collected for five years (2017–2021). They represented the majority of the blood donors in the study period of the entity the Republic of Srpska from Bosnia and Herzegovina.

Initial routine serological testing was performed for all samples. If the result was D-negative in direct agglutination, for participants with C+/E+ indirect antiglobulin test (IAT) was performed. If the results of both tests were negative, samples were screened for the presence of *RHD* exons 3, 5 and 10. Every sample reactive at the screening assay was further tested by specific PCR-SSP (PCR-sequence-specific primers) assays.

The study was approved by the Ethical Board of the Clinical Centre of Banja Luka. The research was conducted ethically, with all study procedures being performed in accordance with the requirements of the World Medical Association's Declaration of Helsinki. Written informed consent was obtained from each participant for study participation and data publication.

## Serological testing

For D antigen determination, two direct agglutination methods were used:

- tube method using anti-D monoclonal IgM/IgG, anti-D blend (TH-28/MS-36, anti-D blend 175 2-415 1E4, *CE Immunodiagnostika*, Germany);
- gel method and ID-Cards „DiaClon ABO/Rh for Donors" (monoclonal anti-D:ESD-1M, 175-2, *BioRad*, USA).

Verification of weak D by IAT: ID-DiaClon Anti-D (reagent containing monoclonal IgG anti-D for confirmation of D weak by IAT), Coombs Anti-IgG (ID-Cards containing anti-human globulin rabbit anti-IgG for verification of D weak by IAT), LISS/Coombs (ID-Cards of 6 microtubes containing anti-IgG and anti-C3d), all manufactured by *BioRad*.

The RhCE antigens were also detected by two methods:

- tube method using human monoclonal anti-C test reagent (clones: P3X25513GB+MS24), anti-E (clone 906), anti-c (clone 951) and anti-E (clones: P3GD512+MS63), all by *Diagast*, France;
- DiaMed-MP Test C, c, E, e, K (*BioRad*) consisting of microplates with dried antibodies: anti-C cell line MS-24; anti-c MS-33; anti-E MS-260; anti-e MS-63 and anti-K MS-56.

## DNA testing

DNA was extracted from 200 µL of whole blood sample by using the Ready DNA Isolation Spin kit (*Inno-train Diagnostik*, Germany). The donor-

derived DNA samples were tested for the presence of *RHD* sequences by three RBC-FluoGene assays (RBC-FluoGene CDE, RBC-FluoGene D weak/variant, RBC-FluoGene CDE eXtend), which were based on PCR-SSP, with the fluorometric results evaluation involving the fluorescence reading of TaqMan probes by the FluoVista instrument according to the manufacturer's instructions (*Inno-train Diagnostik*).

## Adsorption/elution test

In serologically D-negative, C+/E+ participants where *RHD* sequence was confirmed by DNA testing, D antigen was later re-examined in the Blood Transfusion Institute of Serbia (BTIS), Belgrade, Serbia, by adsorption of human polyclonal anti-D antibodies (produced "in house" from anti-D test sera of human origin by the Department for Production of Diagnostic Test Reagents of BTIS). Afterwards, RBCs were washed five times and subsequently eluted (DiaCidel, *Bio-Rad*) for antibody identification. Anti-D from the eluate was detected by the gel method using NaCl and Liss/Coombs ID-cards by ID-DiaCell IP-IIP-IIP and ID-DiaCell I-II-III screening test red cells (both *Bio-Rad*). Antibody specificity was determined using ID-DiaPanel P and ID-DiaPanel (both *Bio-Rad*).

## Statistical methods

Absolute frequencies of antigens and alleles were collected by direct counting. The Pearson's chi-squared test was used to determine significance, set at $p < 0.05$ as statistically significant, in frequency comparisons. Proportion 95 % confidence interval (CI) was calculated using the Wilson score interval. IBM SPSS software version 18.0 was used.

# Results

Initially, 74,149 blood donors were routinely serologically tested for D antigen. There were 12,827 serologically D-negative donor samples (17.30 %), both in direct agglutination as well as in IAT. Among these, 481 were D-negative, C+ or E+ (0.65 % of all samples, or 3.75 % of D-negative samples). The distribution of the main RhCE antigens among these 481 donors was as follows: C 35.86 % (345/962), c 64.14 % (617/962), E 15.28 % (147/962) and e 84.72 % (815/962). The frequencies of serologically determined Rh phenotypes in D-negative blood donors are given in Table 1. Homozygous *RHCE* genotype was determined in three D-negative blood donors with C+/E+ (0.62 %): *RHCE*02/*02* in two and *RHCE*03/03* in one.

In the serologically D-negative blood donor population with C+/E+, there were mostly *RHD*-negative donors (441/481, 91.68 %), whose final result of *RHD* deletion (homozygous *RHD*01N.01*) was defined by the negative result in the *RHD* screening assay. In each of the remaining samples (40/481, 8.32 %), some *RHD* sequence was detected; therefore, there were 40 *RHD*-positive samples. Summary frequencies for *RHD* variants in this study are presented in Table 2.

*Table 1: The frequencies of Rh phenotypes in serologically D-negative blood donors*

| Serologically determined Rh phenotypes | Donors (N) | Frequency in D-negative C+/E+ blood donors (N = 481) | Frequency in all D-negative blood donors (N = 12,827) |
|---|---|---|---|
| ddccee | 12,346 | NA | 96.250 % |
| ddCcee | 333 | 69.231 % | 2.596 % |
| ddCCee | 2 | 0.416 % | 0.016 % |
| ddCcEe | 8 | 1.663 % | 0.062 % |
| ddccEe | 137 | 28.482 % | 1.068 % |
| ddccEE | 1 | 0.208 % | 0.008 % |
| Other D-negative phenotypes | 0 | 0.000 % | 0.000 % |
| Total | 12,827 | 100.000 % | 100.000 % |

*NA: not applicable;*

*Table 2: Summary frequencies of RHD variants in blood donors*

| Blood donors' subgroup | Total blood donors | Frequency of blood donors with *RHD* variants | 95 % confidence interval[a] |
|---|---|---|---|
| All blood donors | 74,149 | 0.054 % or 1:1,853 | 0.040 % – 0.073 % |
| D-negative | 12,827 | 0.312 % or 1:321 | 0.229 % – 0.424 % |
| D-negative, C+/E+ blood donors | 481 including:<br>• 441 RHD-negative<br>• 40 *RHD-positive* | 8.316 % or 1:12 | 6.166 % – 11.130 % |

*a: Proportion confidence interval was calculated using the Wilson score interval;*

*Table 3: Distribution of detected RHD alleles (N = 40) with the associated Rh phenotype*

| Rh phenotype | Result designation | *RHD*11* | Seemingly *RHD*01* | *RHD*01N.03* | *RHD*01W.1* | *RHD*01W.2* | *RHD*01W.3* | *RHD*01W.14* | *RHD*05.05* | *RHD*01EL.44* | No conclusive result, *RHD*-positive | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ccee | A | 11 | 15 | 3 | 1 | 1 | - | 1 | 1 | 1 | 2 | **36** |
| | B | 27.500 % | 37.500 % | 7.500 % | 2.500 % | 2.500 % | | 2.500 % | 2.500 % | 2.500 % | 5.000 % | **90.000 %** |
| | C | 2.287 % | 3.119 % | 0.624 % | 0.208 % | 0.208 % | | 0.208 % | 0.208 % | 0.208 % | 0.416 % | **7.484 %** |
| | D | 0.086 % | 0.117 % | 0.023 % | 0.008 % | 0.008 % | | 0.008 % | 0.008 % | 0.008 % | 0.016 % | **0.281 %** |
| ccEe | A | - | 1 | - | - | - | - | - | - | - | 1 | **2** |
| | B | | 2.500 % | | | | | | | | 2.500 % | **5.000 %** |
| | C | | 0.208 % | | | | | | | | 0.208 % | **0.416 %** |
| | D | | 0.008 % | | | | | | | | 0.008 % | **0.016 %** |
| Ccee | A | - | - | - | - | - | 1 | - | - | - | - | **1** |
| | B | | | | | | 2.500 % | | | | | **2.500 %** |
| | C | | | | | | 0.208 % | | | | | **0.208 %** |
| | D | | | | | | 0.008 % | | | | | **0.008 %** |
| CcEe | A | 1 | - | - | - | - | - | - | - | - | - | **1** |
| | B | 2.500 % | | | | | | | | | | **2.500 %** |
| | C | 0.208 % | | | | | | | | | | **0.208 %** |
| | D | 0.008 % | | | | | | | | | | **0.008 %** |
| Total | A | 12 | 16 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 3 | **40** |
| | B | 30.000 % | 40.000 % | 7.500 % | 2.500 % | 2.500 % | 2.500 % | 2.500 % | 2.500 % | 2.500 % | 7.500 % | **100.000 %** |
| | C | 2.495 % | 3.326 % | 0.624 % | 0.208 % | 0.208 % | 0.208 % | 0.208 % | 0.208 % | 0.208 % | 0.624 % | **8.316 %** |
| | D | 0.094 % | 0.125 % | 0.023 % | 0.008 % | 0.008 % | 0.008 % | 0.008 % | 0.008 % | 0.008 % | 0.023 % | **0.312 %** |

*A – absolute number in each cell designates the number of samples;*
*B – the first percentage is the frequency in all RHD positive donors (N = 40);*
*C – the second percentage is the frequency in serologically D-negative C+/E+ donors (N = 481);*
*D – the third percentage is the frequency in all serologically D-negative donors (N = 12827);*
*The minus sign denotes no alleles within the Rh phenotype.*

Eight different *RHD* alleles were found in this study. There were 19 samples that need further clarification. Out of these, genotyping of three samples did not reveal any known *RHD* allele and they remained unresolved (without a conclusive result, labelled as "*RHD*-positive"). The remaining 16 samples were designated as seemingly *RHD*01*, indicating just the presence of all *RHD* exons, as all the exons' respective reference SNVs (single nucleotide variant) were detected in these samples. Table 3 shows the distribution of the *RHD* genotyping results linked to the associated Rh phenotype.

The donors with hybrid *RHD-RHCE* alleles (*RD*01N.03* and *RHD*01EL.44*) were afterwards serologically tested by adsorption/elution. All four donors gave positive result, thus indicating

the Del expression of both respective alleles. The same adsorption/elution technique showed positive result in the donor with *RHD*05.05* and selected donors with *RHD*11*.

## Discussion

In this study, the diversity of serologically D-negative blood donors in the Institute for Transfusion Medicine of the Republic of Srpska that collects blood donor samples from the whole entity of the Republic of Srpska in Bosnia and Herzegovina was described, so this study may be considered population-wide research. After initial study in 2019,[21] this research focused on the Rh diversity of the serologically D-negative blood donor population in the period of 5 years.

Three of eight different *RHD* alleles were previously determined in our country (*RHD*11*, *RHD*01W.1* and *RHD*01W.3*)[21] and the five remaining were encountered locally for the first time. As established for other European populations, *RHD*11* is the most frequent allele with the Del phenotype also in the studied population.[4, 19-21] The *RHD*01W.2* and *RHD*01W.14* alleles were not encountered within their most common haplotype (cDE),[27] as both donors had the Ccee phenotype. All but one *RHD*11* donors had the Ccee phenotype (the remaining one had CcEe), which validated the established observation of *RHD*11* Del expression linkage to *CDe* haplotype.[27] All eight alleles were previously described in neighbouring Croatia.[22, 28-30] All but *RHD*05.05* and *RHD*01EL.44* alleles were detected in neighbouring Serbia.[21]

Interestingly, while in authors' initial study, with a smaller sample size,[21] the frequency of *RHD* alleles in D-negative C+/E+ donors was relatively high (9.8 %), the comparison with results of this study (8.3 %) yields a nonsignificant difference, p = 0.645. The difference in comparison between the results in this study and the same ratio from Serbia[21] is not significant (p = 0.688), but it is significant with the percentage values from the two Croatian donor studies (first study[30]: 3.59 %, p < 0.001; second study[22]: 1.74 %, p = 0.013).

The three rare *RHD* alleles in the focus of this study were *RHD*05.05*, *RHD*01N.03* and *RHD*01EL.44* (*RHD*DEL44*). The allele *RHD*05.05* was originally described in Asian populations, but also in Austria and Greece.[31, 32] It has been reported in Dalmatia, the coastal region by the Adriatic Sea in Croatia;[29] therefore, its occurrence was not completely inconceivable. This allele codes for the DHK (DYO) antigen, first described in Asia.[33, 34] The phenotype varies from D-negative through weak D to D-positive.[35] Since there was an anti-D reactivity detected in the eluate from the donor's RBCs, the donor's status was changed to D-positive.

This is the first publication with any *RHD* null allele other than *RHD*01N.01* detected in Bosnia and Herzegovina. The *RHD*01N.03* allele is infrequently detected among serologically D-negative individuals. In the Han Chinese population, it was the most common after the homozygous *RHD* deletion.[36] In a Croatian study, it was present in almost half of all *RHD*-positive D-negative blood donors.[30] Three samples in this study were negative in IAT, but anti-D was identified after adsorption/elution technique. The haplotype association of *RHD*01N.03* is predicted[37] to be with the *RHCE*02 (*Ce)* allele. The designation *RHD*01N.03* was chosen in this paper, as it was the result indicated by the FluoGene software. For clarification purposes, since all three donors with this allele have the Ccee phenotype, it is impossible to distinguish the *RHD*01N.03* (*RHD*D-CE(2-9)-D*) allele from the *RHD*01N.04* (*RHD*D-CE(3-9)-D*) allele with the used molecular test system due to the exon 2 sequence equivalence of *RHD* and *RHCE*02* (*RHCE*Ce*).

The *RHD*DEL44* allele has been sporadically registered globally and to the authors' knowledge, this research represents the first description of any DEL allele in Bosnia and Herzegovina. In Europe, the reports came from Austria,[19] Switzerland,[38, 39] Portugal[40] and the most recent one from Croatia.[22] Outside Europe, the allele presence was documented in China,[41-43] Australia,[7] India,[44] Argentina,[45] Thailand[46] and Oceania.[47] It is a hybrid allele, with the gene structure *RHD*D-CE(4-9)-D*.[1, 27, 35, 47] Its expression varies as either Del or D-negative. It is mostly reported as D-negative,[3, 4, 27, 35, 43] but ISBT categorises it within the DEL alleles (hence the official name) and not the null allele group.[1] It has just been reported that the allele of the same gene structure can even express a C antigen.[47] Recently, there was a description of the *RHD*DEL44* allele and its expression within the blood donors in northwestern Croatia.[22] It seems likely that there could exist at least a central or southeastern European basin of this allele with detectable antigens on the RBCs accounting for the Del expression. Both the results of the Croatian study[22] and this study

suggest the need to verify the expression of this allele, thereby preventing its reporting as a null allele by default solely due to its hybrid structure. *RHD*DEL44* in presented donor was most probably associated in haplotype[37] with the *RHCE*02* (**Ce*) allele.

For the four donors with the rare hybrid alleles (*RHD*01N.03* and *RHD*01EL.44*) their Del expression was indicated. Accordingly, their statuses have been changed to D-positive, so their blood products can be properly administered in the future. Both alleles are commonly interpreted as having a D-negative expression[30, 36] which is further inconsistent considering their official nomenclature.[1, 3] The *RHD*01N.03* allele has a structure of *RHD*D-CE(2-9)-D*, which suggests it should typically behave as a null allele.[1] The *RHeference* database[35] has numerous references to the D-negative phenotype and only one to the Del phenotype like presented in this study, from Thailand.[48] Although *RHD*01EL.44* is catalogued as the DEL allele by ISBT, its Del phenotype was observed only originally in China[41, 42] and Croatia.[22] The phenotype of this allele could not be verified for the carrier in Oceania[47], as it was a case of heterozygous *RHD* genotype of *RHD*DEL44* with *RHD*01* in *trans* position.

Satisfactory conclusive results were not obtained for three donor samples. It is confirmed they definitely do have *RHD* sequences. However, the final result was not clear due to discrepancies and their evaluation is still pending. The most problematic but possibly exciting results involve the group of 16 donors (3.33 %), all *RHD*-positive, seemingly *RHD*01*. All reference SNVs detecting each of the 10 *RHD* exons were present in all 16 samples and consistently, no other SNV from any other *RHD* allele than *RHD*01* was detected. The authors share genuine reservations that these really are reference sequences of the *RHD* gene and that there are intact standard D antigens on RBCs of these donors. Serological reactivities in both assays were negative. It can be confirmed that data of these 16 blood donors were subsequently examined and evidence of any blood relationship among them was not found. Consequently, it could be the case of a rare *RHD* variant, probably a geographically specific one. The high frequency of these samples in serologically D-negative C+/E+ donors (3.33 %) corroborates that hypothesis. PCR assays based on SNV detection disclose very little of the entire *RHD* sequence.[49] Therefore, there could be many sequence variants that remained undetected by the PCR setup in this study. There could also be some variation in the genes required for expression of Rh proteins in the RBC membrane (like *RHAG*).[50] Regrettably, in our region, there are no sequencing opportunities and it is also not financially feasible for our institutions to have them all sequenced remotely. The authors are keen to contribute these samples to any future research involving whole-gene *RHD* sequencing (both exons and introns of the *RHD* gene) that would be willing to explore and finally identify the underlying molecular causes of the absence of D antigen expression in these seemingly *RHD*01* donors.

This research demonstrates the importance of verifying the expression of large hybrid Rh alleles. Although alloimmunisation occurrences are extremely rare, blood bank facilities should take all precautions to make sure that D-negative units truly are D-negative, especially for the blood donor population. Molecular Rh examination already marks an important stage, but the results displayed in this research shed new light on the canon for two different large hybrid alleles. A supporting view is articulated by Srivastava et al[47] where, in addition, the importance of hybrid allele's breakpoint regions to the expression and stability of mRNA transcripts is discussed, as well as the need to distinguish different expression patterns of hybrid alleles by blood transfusion services.

## Conclusion

The *RHD* diversity in 74,149 serologically D-negative blood donors was demonstrated by the *RHD* frequency of 0.31 % in this study, which also challenges the common perception for specific alleles, like *RHD*01N.03* and *RHD*01EL.44*, that they do not express D antigen. This underlines the critical role of molecular techniques in identifying Del variants, which are often undetectable by standard serological tests. The presence of donors with seemingly *RHD*01* allele variants points towards the possibility of undiscovered *RHD* polymorphisms or other genetic factors affecting Rh protein expression. These insights reinforce the need for molecular screening in blood donation to ensure safety. Furthermore, this highlights the study's contribution to the broader understanding of *RHD* allele variations and their implications for blood transfusion compatibility.

## Ethics

The study was approved by the Ethical Board of the University Clinical Centre of Banja Luka, Banja Luka, the Republic of Srpska, approval No 01-9-382.2/15, dated 29 September 2015.

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## Conflicts of interest

ML is employed as a scientific advisor by a local distributor of *Inno-Train Diagnostik*, which was not involved in this research in any way, particularly scientifically, financially, or in writing the paper. All other authors declare that there is no conflict of interest.

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## Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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