ORIGINAL ARTICLE

Molecular Typing of RhD-Negative Blood Donors With C and/or E Antigen

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ABSTRACT

Background: Most people are either RhD positive or RhD negative, but there is also a number of persons with D antigen variants. The aim of this study was to prove, by using molecular diagnostic tests, whether the RHD gene and D antigen on the red cell membrane of the blood donors serologically typed as RhD-negative with RhD phenotype Ccddee and ccddEe, are so weak that they cannot be proven by serology techniques or the available anti-D test serums.

Methods: Samples used are those of regular voluntary donors who were serotyped as RhD-negative, C and/or E positive. Samples were collected from voluntary donors at the Institute for Transfusion Medicine of the Republic of Srpska during the period from April 2016 to December 2018.

Results: Among the serologically proven RhD-negative donors, 346 had C and/or E in their phenotype and those were subjected to molecular screening test.

Conclusion: The first results of molecular typing match those published in literature, i.e. the RHD gene is present in some serologically RhD-negative forms, which was proven by molecular testing.

Key words: Rh system, D antigen, weak D, partial D, antigens, blood donors, phenotype.

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INTRODUCTION

Red cell blood types are hereditary polymorphisms located on proteins, glycoproteins and glycolipids of erythrocytes. Thus far, the Working Party on Red Cell Immunogenetics and Blood Group Terminology of the International Society of Blood Transfusion (ISBT) has registered more than 340 antigens.[1-2] The molecular basis has been defined for most antigens known so far, and they have been classified as one of the 36 blood group systems. The Rh system is one of the most complex blood group systems and, in addition to the ABO system, it is the most important one for clinical practice. Besides presence and absence of the D antigen, other common Rh phenotypes imply allelic C and c, and E and e antigens.

The RH locus implies two genes, RHD and RHCE, whose rear ends (tail-to-tail orientation) are facing the end of the short arm of chromosome 1 (p34-36). Another gene, SMP1, is interspersed between the two RH genes and in close proximity to the 3' end of the RHCE gene.[3]

Critical components of the structure of RhD and RhCE proteins are the amino acids residing in the extracellular vestibule of the Rh protein and making part of the transmembrane polypeptide that forms the eponymous channel. Amino acid substitution in the protein vestibule alters the molecular structure of the antigen sufficiently to make those persons susceptible to the formation of anti-D antibody, regardless of the fact that they are RhD-positive.[4-5]

The two genes (RHD and RHCE), which are in close proximity of each other on chromosome 1, encode Rh proteins on the red cell membrane;

one protein carries D antigen, and the other carries various combinations of the remaining CE antigens (ce, Ce, cE or CE).[6-8] Each gene consists of 10 exons which are 97% identical and the result of gene duplication.[9] The RhD and RhCE proteins differ by 32-35 of the total of 416 amino acid residues, cross the red cell membrane twelve times, and form six extracellular loops. Both Rh polypeptide ends (NH2 and COOH) are located in the intracellular region.

The RHD and RHCE genes have almost identical genome organization and each consists of 10 exons. Exons 1 through 7 each encode 50 to 60 amino acids, while exons 8 through 10 encode the remaining 58 amino acids.[7,8,10] These genes share 93.8% homology in the structure of introns and encoding exons. The main difference is a deletion of about 600 base pairs (bp) in intron 4 of the RHD gene as compared to the RHCE.

The terms 'Rh-positive' and 'Rh-negative' refer to the presence or absence of the D antigen respectively. The most common D-negative haplotype in all populations is caused by the deletion of the whole RHD gene, with the concomitant presence of the hybrid Rhesus box. However, there are also other D-negative haplotypes.[11-12] A weak D antigen proven by serology techniques or a serologically weak D phenotype is defined as reactivity of red blood cells with an anti-D reagent giving no or weak ≤2+ reactivity in direct agglutination, while reacting moderately or strongly with antihuman-globulin reagent. Discordant results in serological typing of the weak D antigen forms are frequent in routine practice, and this can be overcome by RHD genotyping, which provides a more precise analysis.[13] Red cells with a weak D antigen presence are almost always C+ or E+.

The main aim was to prove, using molecular diagnostic tests, whether the RHD gene and D antigen on the red cell membrane of the blood donors serologically typed as RhD-negative with the RhD phenotype Ccddee and ccddEe, are so weak that they cannot be proven by serology techniques or the available anti-D test serums. The additional aim was to establish, by means of a retrospective study, whether some of the RhD-negative recipients with the Rh phenotype ccddee, who received Ccddee and ccddEe phenotype blood, have created anti-D antibody after the application of blood with the above phenotypes.

METHODS

Samples used were those of voluntary donors of the Institute for Transfusion Medicine of the Republic of Srpska (ITM RS) from April 2016 to December 2018, typed as RhD-negative with C and/or E antigen in the Rh phenotype, using serology techniques, test tube method, microplate method and gel method. An automated system (Techno Twin Station, Diamed Switzerland) was used.

Each subject was taken two blood samples in EDTA anticoagulant: one for serological RhD status testing, Rh phenotyping, antibody screening, and the second for molecular RhD typing and Rh phenotyping.

To determine ABO system blood groups and RhD antigen, the methods of gel cards and microplates manufactured by BioRad, USA, on the automated Techno Twin Station were used in this study. The reverse group was processed on the gel cards A, B, DVI-, ctl/A1, B using the following reagents: ID Card DiaClon ABO/D, monoclonal anti-A (cell line A5), anti-B (cell line G1/2), anti-D (cell line LHM 59/20 (LDM3)+175-2). The microplate method used the microplates DiaClon-MP test A, B, DVI+, ctl and the following test reagents: DiaClon-MP Anti-A (cell line BIRMA-1), DiaClon-MP Anti-B (cell line LM306/686 (LB-2)), DiaClon-MP Anti-AB (cell line ES131 (ES-15), ES-4) and DiaClon-MP Anti-DVI+/IgM/IgG (cell line MS-26), all products manufactured by BioRad, USA. The microplates for Rh and Kell phenotyping on Techno Twin Station: Anti-C cell line MS-24; Anti-c cell line MS-33; Anti-E cell line MS-260; Anti-e cell line MS-63, Anti-K cell line MS-56; negative control, buffer without antibodies (BioRad, USA).

All samples identified as RhD-negative were further tested by monoclonal anti-D IgG serum (cell line ESD1) to determine weaker D antigen forms by gel method, using the gel manufactured by BioRad, USA.

The samples typed as D negative, C and/or E positive, were weak D tested using three protocols: DiaClonMP Anti-DVI-/IgM (cell line TH-28) and DiaClon-MP Anti-DVI+/IgM/IgG (cell line MS-201/MS-26), microtiter plate (BioRad, USA); anti-D (cell line LHM 59/20 (LDM3)+175-2); gel card (BioRad, USA); anti-D IgG serum (cell line ESD1).

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The samples typed as RhD-negative with C and/or E antigen in the Rh phenotype were further referred to molecular testing by PCR-SSP polymerase chain reaction. The analysis is based on a specifically modified TaqMan® probe system detected in the FluoVista apparatus manufactured by Inno-Train Diagnostik, Germany.

Statistical data processing was performed by using the SPSS 22 statistical package for Windows. The primarily obtained data were analysed by descriptive statistical methods. The used descriptive statistical methods included: measures of central tendency, measures of variability and structure indicators expressed as percentage.

RESULTS

Blood was collected from 95,525 donors. Among them, 79,563 (83.29%) were RhD-positive, 15,710 (16.44%) were RhD-negative and 252 (0.26%) were RhDw. (Table 1)

Table 1: Overview of RhD status in voluntary blood donors

RhD status	RhD-positive	RhD-negative	RhDweak
	79,563	15,710	252
	(83.29%)	(16.44%)	(0.26%)
Total	**95,525 (100%)**		

There were 346 RhD-negative blood donors whose phenotype included C and/or E antigen. Of them, 102 (29.47%) were female and 244 (70.52%) were male. Their blood groups were: A in 137 (39.59%), O in 116 (33.52%), B in 63 (18.20%) and AB in 30 (8.67%). (Table 2)

Table 2: OABO system blood groups representation in subjects

Blood group	Representation
A	137 (39.59%),
O	116 (33.52%)
B	63 (18.20%)
AB	30 (8.67%)
Total	**346 (100%)**

Rh phenotype distribution was as follows: a) Ccddee in 232 subjects (67.05%); b) ccddEe in 105 subjects (30.34%); c) CcddEe in 3 subjects (0.86%); d) CCddee in 5 subjects (1.44%); e) ccddEE in 1 subject (0.28%). (Table 3)

Table 3: Rh phenotype distribution by blood group

	A	O	B	AB	Total
Ccddee	83 (35.77%)	86 (37.06%)	44 (18.96%)	19 (8.18%)	232
ccddEe	47 (44.76%)	29 (27.61%)	19 (18.09%)	10 (9.52%)	(100.0%)
CcddEe	2 (66.66%)	0 (0.0%)	0 (0.0%)	1 (33.33%)	105
CCddee	4 (80.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)	(100.0%)
ccddEE	1 (100.0%)	0 (0.0%)	0 (0.0%))	0(0.0%)	3 (100.0%)
Total	137(37.0%)	116(33.52%)	63(18.20%)	30(8.67%)	346(100.0%)

The RhD-negative blood donors with C+ and/or E+ antigen in their phenotype were subjected to molecular typing, primarily using the molecular screening kit, and then D weak and CDE kits, the PCR-SSP method, with fluorometer reading on the FluoVista, to confirm the findings obtained by serological methods.

Molecular screening was positive in 31 (8.95%) blood donors and negative in 315 (91.04%). By using the CDE and D weak kits on the 31 subjects with positive molecular screening, D weak type 11 was proven in 8 donors (25.8%), D weak type 1 in 3 donors (9.67%), D weak type 3 in 3 donors (9.67%) and RhD status was unknown in 17 (54.8%) donors. (Table 4)

Table 4: Distribution of molecularly typed RhD antigen variants by Rh phenotype in subjects found positive after molecular screening

	type 11	type 1	type 3	unknown	Total
CcDwee	8	3 (25.0%)	1 (8.33%)	0	12 (100.0%)
CCDwee	(66.66%)	0	2 (100.0%)	0	2 (100.0%)
Ccddee	0	0	0	16 (100.0%)	16 (100.0%)
ccddEe	0	0	0	1 (100.0%)	1 (100.0%)
Total	8 (25.8%)	3 (9.67%)	3 (9.67%)	17 (54.8%)	31(100.0%)

DISCUSSION

These are the first test results of molecular typing of RhD-negative blood donors with C and/or E antigen in the phenotype in the Republic of Srpska. The molecular testing was enabled after serological testing performed by using RhD status testing algorithm at the ITM RS.

According to the data from the information system of the ITM Banja Luka, the total number of blood donors during the period from April 2016 to December 2018 was 95,525. Among the tested

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blood donors, 15,710 (16.44%) were RhD-negative, and C+/E+ antigen was present in the phenotype of 346 RhD-negative donors.

In the group of 346 serologically RhD-negative blood donors, C+/E+, type A blood group was present in 137 (37.0%), type O in 116 (33.52%), type B in 63 (18.2%) and type AB in 30 (8.67%). These results are in a correlation with the published data about the ABO system blood groups distribution in healthy population in the territory of the former Yugoslavia. Data from 1989 show that in former Yugoslavia, the ABO system antigen frequency in the population of voluntary blood donors in compulsory military service was: A (42%), O (35%), B (16%), AB (7%). According to the data available to the Blood Transfusion Institute of Serbia for 2008, the frequency of ABO blood groups in voluntary blood donor population of Serbia for both sexes, in a sample of 52,732 donors was as follows: A (41.8%), O (34.9%), B (16.2%), AB (7.1%).[14]

The term phenotype means results of red cell testing with specific antiserums. Rh phenotyping uses five main antiserums: anti-D, anti-C, anti-c, anti-E and anti-e. In the group of 346 serologically RhD-negative blood donors, molecular methods gave the following Rh phenotype testing results: a) Ccddee was proven in 232 (67.05%); b) ccddEe in 105 (30.34%); c) CcddEe in 3 (0.86%); d) CCddee in 5 (1.44%); e) ccddEE in 1 (0.28%);

Based on a comparison between the test results obtained in this study and those published in literature, it can be concluded that Rh phenotype distribution in the tested blood donor population in the Republic of Srpska is in line with the Rh phenotype distribution in other Caucasian populations.[6] The frequency of various Rh phenotypes in RhD-negative persons ranges as follows: ccddee phenotype is present in 15% of Caucasian persons, Ccddeee and ccddEe in about 1%, Ccddee and ccddEE in about 0.01%, while other RhD phenotypes of D-negative persons are much less present, according to the data published in the works of Daniels and Jovanović Srzentić.[9,14]

Following contemporary strategy in D antigen testing in donors and pregnant women, the Institute for Transfusion Medicine in Banja Luka introduced molecular RHD and RHCE gene testing in donors, pregnant women and patients in early 2017, by using the FluoGene method. It implies PCR-SSP, including fluorometer reading on the FluoVista.

Our testing involved 346 blood donors who were typed, using serology techniques, as RhD-negative with the Rh phenotype D-neg. (-), C and/or E-pos. (+), to be subjected to molecular typing to establish potential presence of the RHD and RHCE gene. The molecular type testing was preceded by molecular screening for all serologically RhD-negative blood donors. This screening enables detecting the RHD gene exons, specifically exons 3, 5 and 10 in a single reaction, and the test is suitable for molecular confirmation of serologically negative samples. The molecular screening produced 31 (8.95%) positive samples in the tested group.

Positive reactions obtained by the screening test were further investigated using the RHD and CDE kits. The test using the PCR-SSP method with fluorometer reading of fluorescence on the FluoVista in our laboratory proved that among the 14 blood donors serologically typed as RhD-negative, 8 actually had weak RhD type 11 (25.8%), 3 donors had weak RhD type 1 (9.67%) and 1 had weak RhD type 3 (3.22%) with the Rh phenotype Ccddee, while 2 CCddee phenotype donors were proven to have weak D type 3 (6.45%). In 17 donors (54.83%) who had a positive screening, the available D weak and CDE kits could not determine the RhD status. The obtained results indicate the necessity of introducing routine molecular testing for RHD gene presence in donors in whom the RhD antigen was not detected by serologic methods, which would in turn prevent the use of D-positive blood in RhD-negative recipients, as well as their immunisation by the RhD antigen.

Data about the distribution of D antigen variants are of strategic importance because the persons with weak forms of the RhD antigen types 3 and 1, as blood recipients, are considered RhD-positive and may receive RhD-positive blood, thus preserving the RhD-negative blood stocks, and pregnant women with these RhD variants do not need to receive the RhD immunoprophylaxis; the patients and pregnant women with other D antigen variant types are considered RhD-negative. Besides, the weak form of D antigen type 11 was proven in our subjects, which is characterised by a small number of antigen sites and cannot be proven by standard serological methods. These forms can cause creation of anti D-antibodies in negative blood recipients.[15]

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CONCLUSIONS

In 31 (8.95%) of the total 346 tested RhD-negative blood donors with the Rh phenotypes Ccddee, ccddEe, Ccddee, ccddEE, molecular testing established presence of the RHD gene and some of the types of weak D antigen variants.

Data about the distribution of D antigen variants are of strategic importance because the persons with weak forms of the RhD antigen types 3 and 1, as blood recipients, are considered RhD-positive and may receive RhD-positive blood, thus preserving the RhD-negative blood stocks, and pregnant women with these RhD variants need not receive RhD immunoprophylaxis; the patients and pregnant women with other D antigen variant types are treated as RhD-negative.

Introducing routine molecular testing of donors and pregnant women with serologically weak form of the D antigen and those RhD-negative with C and/or E in the phenotype, would lead to savings in funds allocated for serological typing of blood groups, because it is done only once, not at each blood group testing.

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CONFLICT OF INTEREST

None.

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