

Molecular – genetic variance of RH blood group system within human population of Bosnia and Herzegovina

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ABSTRACT

There are two major theories for inheritance of Rh blood group system: Fisher – Race theory and Wiener theory. Aim of this study was identifying frequency of RHDCE alleles in Bosnian – Herzegovinian population and introduction of this method in screening for Rh phenotype in B&H since this type of analysis was not used for blood typing in B&H before.

Rh blood group was typed by Polymerase Chain Reaction, using the protocols and primers previously established by other authors, then carrying out electrophoresis in 2-3% agarose gel.

Percentage of Rh positive individuals in our sample is 84.48%, while the percentage of Rh negative individuals is 15.52%. Inter-rater agreement statistic showed perfect agreement (K=1) between the results of Rh blood system detection based on serological and molecular-genetics methods.

In conclusion, molecular – genetic methods are suitable for prenatal genotyping and specific cases while standard serological method is suitable for high-throughput of samples.

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KEY WORDS: RHD gene, RHCE gene, frequency, Rh blood group system, allele specific PCR method

INTRODUCTION

The Rh blood group system (commonly known as Rh factor) is considered to be the most important blood group system in the world. This blood group system is involved in several life-threatening diseases like hemolytic disease of the newborns and autoimmune hemolytic anemia [1]. Rh antigens have major clinical impact in transfusiology, since they can produce hemolytic transfusion reactions. Molecular testing has shown that the Rh-locus of human genome, which is located on the short arm of chromosome 1 (1p36.2-p34), consists of two closely related and structurally homologous genes, RHD and RHCE. RHD and RHCE genes consist of 10 exons and they stretch along 75kb of DNA sequence [2]. So far, the only observed difference between these two genes is a partial deletion of RHD gene in the fourth exon of this gene [3]. In cases of deletion of RHD gene in individual genotypes, there is an Rh (D-) phenotype on erythrocytes, which is different if there is no deletion on RHD gene, since in that case there is an Rh (D+) phenotype. In previously described

studies [4, 5] it is reported that the difference between the Rh E and e alleles encoding these polypeptides is in only one nucleotide at position 676 of RHCE gene [6]. Comparing the Rh C and c alleles there are six nucleotides differences, four of which result in amino acid substitutions. These substitutions are 48 G>C, 178 C>A, 203 A>G and 307 C>T [3, 7]. There are two major theories that try to explain inheritance of Rh blood group system: Fisher – Race theory (gene complex theory) and Wiener theory (multiple – allele theory). Fisher and Race used letter symbols, which make CDE nomenclature, for antigen and gene marking. In this theory, there are 6 antigens in Rh blood group (C, D, E, c, d, e) inherited through 3 gene alleles, located on 2 gene loci. Gene alleles (C/c, E/e, D/d) are labeled with same letters as antigens. Every gene complex or haplotype is inherited from each parent. With simple serological blood tests, it is possible to determine hetero- or homozygosity of Rh blood group, regarding antigens C, c, E and e. Serological test for antigen D will show only if erythrocytes contains antigen D or not. On the other hand, molecular-genetic typing of RHD gene will identify exact genetic variance regarding heterozygosity or homozygosity. Prenatal testing for fetal RHD status was the first application of RHD DNA genotyping, and this method is now in use in many different laboratories. In Bosnia and Herzegovina, there is mostly serological method of determining RhD phenotype in use, so we tried to introduce RHD and RHCE genotyping with use of PCR-based method.

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Submitted: 7 August 2012 / Accepted: 12 November 2012

MATERIALS AND METHODS

Subjects

Total of 1 ml of peripheral blood from 290 healthy volunteers of different age and sex were collected. A number of these subjects (36) were submitted to the routine blood group determination, using the serology method for comparison with DNA genotyping. All subjects were introduced to aim of this study and signed letter of acknowledgment. Ethical aspects of use of healthy volunteers were considered and approved by Science Council of Institute for Genetic Engineering and Biotechnology, Sarajevo. DNA was isolated from 1 ml of whole blood, with the standard salting – out method [8]. After isolation, DNA concentration was determined, and DNA was diluted to the concentration of 500 ng/μl.

Procedures

To perform RHD specific PCR, we used previously described primer sets [3], forward primer A9 5'- AC-GATACCCAGTTTGTCT – 3' and reverse primer A6 5'-TGACCCTGAGATGGCTGT-3', which generate 2 amplicons, one control fragment of 1200 bp and Rh positive phenotype specific fragment of 600 bp. PCR reaction was carried out in 25 μl of total volume, with 12,5 μl RED-Taq® ReadyMix™ PCR reaction mix (Sigma, Saint Louis, Missouri, USA), 400 pM of each primer (Biotex, Berlin, Germany) and 1 μg/μl of DNA in each reaction. PCR procedure consisted of 35 cycles of 95 °C for 1 minute, 55 °C for 1.5 minutes and 72 °C for 2.5 minutes, with initial denaturation at 95 °C for 5 minutes and final extension at 72 °C for 9 minutes. PCR products were separated on 1% agarose gel and visualized with ethidium bromide. For fragments sizing we used 1kb DNA Ladder (Fermentas, Maryland, USA). For RHCE genotyping we use also previously described primer sets, for RH E/e genotyping we used two allele specific PCR-reactions with following primers: set A (for E genotyping) 5' – CCAAGTGTCAACTCTC – 3' and 5' – TGACCCTGAGATGGCTGT – 3'; and set C (for e genotyping) : 5' – ACAGACTACCACATGAAC – 3' and 5' – GCTTTGGCAGGCACCAGGCCAC – 3' [4]. These primer sets generated different size amplicons – 108 bp for set A and 141 bp for set C. PCR reaction was carried out in 25 μl of total volume, with 12.5 μl REDTaq® ReadyMix™ PCR reaction mix (Sigma, Saint Louis, Missouri, USA) with addition of 0.5 mM MgCl₂, 200 pM of each primer (Biotex, Berlin, Germany) and 1 μg/μl of DNA in each reaction. PCR procedure consisted of 30 cycles of 94 °C for 1 minute, 54 °C for 1.5 minutes and 72 °C for 1.5 minutes, with initial denaturation at 94 °C for 3 minutes and final extension at 72 °C for 5 minutes. PCR products were separated on 2.7 % agarose gel and visualized with ethidium bromide. For fragments sizing

we used 50bp DNA Ladder (Fermentas, Maryland, USA). For RH C/c genotyping multiplex PCR primer sets were used: Rh C set – 5' – CAGGGCCACCACCATTTGAA – 3' and 5' – GAACATGCCACTTCACTCCAG – 3', Rh c set – 5' – TCGGCCAAGATCTGACCG – 3' and 5' – TGAT-GACCACCTTCCCAGG – 3' [9]. These sets generated PCR products of 320 bp for C allele and 177 bp for c allele. Multiplex PCR reaction was carried out in 25μl of total volume, with PCR Master Mix (2X) (Fermentas, Maryland, USA), 300 pM of Rh C primer sets and 100 pM of Rh c primer sets, with 1 μg/μl of DNA in each reaction. Touch – down PCR procedure consisted of 30 cycles overall – initial denaturation at 94 °C for 2 minutes, then 10 cycles of 94 °C for 10 seconds and 65 °C for 1 minute, 20 cycles at 94 °C for 30 seconds, 61 °C for 1 minute and 72 °C for 30 seconds and final extension at 72 °C for 5 minutes. PCR products were separated on 2 % agarose gel and visualized with ethidium bromide. For fragments sizing we used 50bp DNA Ladder (Fermentas, Maryland, USA).

Statistical analysis

Haplotype frequencies and gene diversity [10] was estimated for all observed Rh loci, since allele and genotype frequencies, PIC (Polymorphism Information Content) [11] and exact p-value test for Hardy-Weinberg equilibrium departure test [12] were calculated for C and E loci separately. In order to achieve these estimations POW-ERMARKEER software v.3.25 [13] was implemented. Allele frequencies were calculated with FreqSim software [14]. Inter-rater agreement statistic (K, Kappa) with 95% confidence interval was calculated to estimate accordance between results based on serological and molecular-genetics methods. Total of 36 samples among 290 was included in this comparison. For this analysis MedCalc ver. 12.3 (MedCalc software, Mariakerke, Belgium) has been used.

RESULTS

Total number of subjects in this study was 290, of both sex and different age. Individuals were from different regions in Bosnia and Herzegovina. We amplified two amplicons from each subject using PCR method, one of 1200 bp and other from 600 bp. 1200 bp amplicon was reaction control fragment (exon 4 of RHD gene), while the 600 bp fragment was specific RHD +/- fragment. In case of amplification of this fragment, we concluded that individual was RhD positive, and in case of non-amplification of this fragment, individual was RhD negative (Figure 1.). For the RHC genotyping we used multiplex PCR reactions which generated two amplicons of 320 base pairs for C allele and 177 base pairs for c allele (shown in Figure 2). RHE genotyping was performed in two separated PCR

TABLE 1. Allele and haplotype frequencies in B-H population

| Allele frequency | | | | | | | |
|---------------------|--------|--------|--------|--------|--------|--------|--------|
| | D | d | E | e | C | c | |
| | 0.6061 | 0.3939 | 0.1293 | 0.8707 | 0.412 | 0.588 | |
| Haplotype frequency | | | | | | | |
| cde | cDe | CDe | cDE | Cde | cdE | CdE | CDE |
| 0.1034 | 0.0827 | 0.5448 | 0.1000 | 0.0379 | 0.0068 | 0.0034 | 0.1206 |

TABLE 2. RHDCE genotype frequencies in B-H population

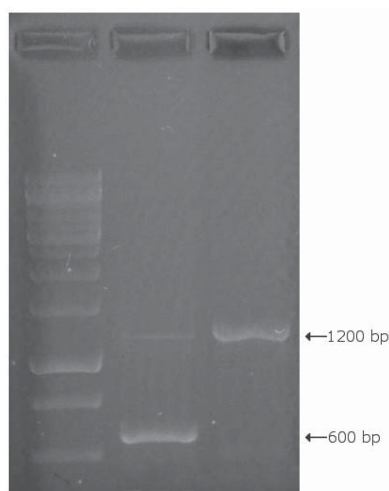
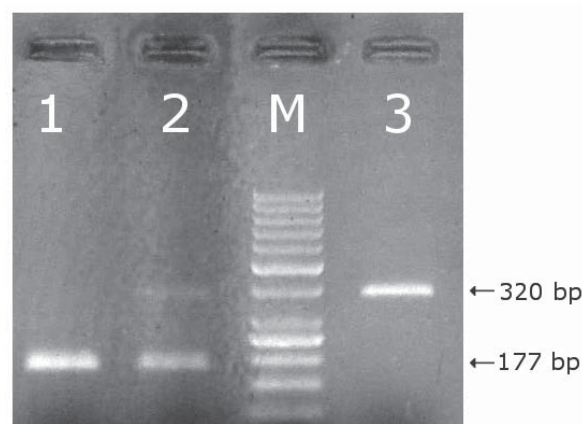
| Genotype | Rh positive | | Rh negative | | |
|----------|-----------------|---------------|-------------|-----------------|---------------|
| | Observed number | Frequency - % | Genotype | Observed number | Frequency - % |
| DCCEE | 0 | 0.00% | dCCEE | 0 | 0.00% |
| DccEE | 3 | 1.03% | dCcEE | 0 | 0.00% |
| DccEe | 5 | 1.72% | dccEE | 0 | 0.00% |
| DCCee | 0 | 0.00% | dCCee | 0 | 0.00% |
| DCCeE | 37 | 12.76% | dCCeE | 0 | 0.00% |
| DCcEe | 32 | 11.03% | dCcEe | 1 | 0.34% |
| DccEe | 24 | 8.28% | dccEe | 2 | 0.69% |
| DCcee | 121 | 41.72% | dCcee | 11 | 3.79% |
| Dccee | 24 | 8.28% | dccee | 30 | 10.34% |

reactions for each allele. Size of the PCR product for allele E was 108 bp, and for allele e was 141 bp. With the RHE genotyping, agarose gel was loaded in following order: PCR mix with primer set for E allele and in next lane PCR mix with primer set for e allele from the same sample. Percentage of Rh positive individuals in our sample is 84.48%, while the percentage of Rh negative individuals is 15.52%. Regarding homozygote/heterozygote for RHCE, we observed following results: dominant homozygote CC – 12.41%, heterozygote Cc – 57.59%, recessive homozygote cc – 30.00%; dominant homozygote EE – 2.76%, heterozygote Ee – 20.34% and recessive homozygote ee – 76.90%. Allele frequencies

for D, d, C, c, E and e allele and haplotype frequencies – percentages are shown in Table 1. Estimated gene diversity values are between 0.262 for D, 0.334 for C and 0.479 for E loci. Significant deviation from Hardy-Weinberg equilibrium was observed for C and E loci ($p < 0.01$). The highest polymorphism information content was detected for E locus (0.364). We also calculated frequency of every possible genotype (18 genotypes) and the number of samples corresponding to those genotypes. Genotype frequency are shown in Table 2. Inter-rater agreement statistic showed perfect agreement ($K=1$) between the results of Rh blood system detection based on serological and molecular - genetics methods.

DISCUSSION

In blood group systems diagnostics (ABO and Rh system) there is rising demand for molecular – genetic methods (genotyping) of these blood group systems, apart from serological method which is routinely used in transfusion laboratories in Bosnia and Herzegovina, as well as around the world. The question that rises from employment of different methods is: is it really necessary to use molecular – genetic methods for RH blood group typing? One of the advantages of PCR allele specific method is that this method does not require blood sample for determina-

**FIGURE 1.** PCR reaction result. In position 1 is 1 kb DNA ladder. RhD positive individual is in lane 1, and RhD negative individual is shown in lane 2.**FIGURE 2.** RhC genotyping: Lane 1 recessive homozygote (cc), lane 2 heterozygote (Cc), M – 50bp DNA ladder, lane 3 dominant homozygote (CC).

tion of blood group haplotype. For example, this is of extreme importance in prenatal diagnostics for determination of fetal D antigen status, where fetal blood does not need to be collected, because PCR can be performed on DNA isolated from amniotic fluid. Also, there is a new trend in prenatal diagnostics for screening of fetal Rh phenotype from mother's blood. Allele specific PCR also has major impact in transfusion medicine because of inability of determining D weak and D partial antigens with routine serological method. This method is used in case of Rh negative blood transfusion to Rh phenotype negative person with D weak or D partial antigen. In these cases there is present immunization of receptor and late transfusion reaction in individuals with formed anti-D antigen in blood serum. On the other hand, serological typing is faster and cost – effective. For serological typing person only needs drop of blood and commercially available serums. Reaction time is measured in minutes (as opposed to days for molecular – genetic typing), but, as we already suggested, serological typing is not as sensitive as molecular - genetic typing and only material for serological typing is blood (as opposed to molecular – genetic typing where is possible to use every type of cells in humans, including fetal cells). In conclusion, molecular – genetic methods are more expensive and more complicated than standard serological method, but they are also very precise and reliant compared to serological methods of blood group diagnostics. For high-throughput samples it is enough to use serological methods, but in case of special needs for typing (fetal typing, partial D and weak D typing, in case of other materials than blood) we suggest employment of molecular – genetic methods.

ACKNOWLEDGEMENTS

There are no acknowledgements in this paper

DECLARATION OF INTEREST

There is no conflict of interest in this paper.

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