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# Detection of fetal *RHD* pseudogene (*RHDΨ*) and hybrid RHD-CE-D$^s$ from RHD-negative pregnant women with a free DNA fetal kit

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**ABSTRACT.** Hemolytic disease of the newborn is a clinical condition in which maternal and paternal Rh blood group antigens are incompatible and the mother is negative for the antigen whereas the father is positive. Analysis of fetal cells recovered from maternal plasma can provide a highly sensitive prenatal diagnosis. The fetal *RHD* gene in plasma DNA is detected by real-time PCR amplification of two different segments of the *RHD* gene (exons 7 and 10). Each amplicon is revealed with specific probes. We examined 40 female blood samples to verify the specificity of RHD exons (7 and 10) amplified by real-time PCR. Thirty fetuses were predicted to be RHD-positive based on analysis of plasma DNA. Seven fetuses were predicted to be RHD-negative. One fetus was negative for RHD on exon 10, and positive for RHD on exon 7 (early gestation age); two fetuses were RHD-negative on exon 7, and RHD-

 

positive on exon 10 (RHD-CE-D$^s$ or *RHDΨ*), indicative of a maternal RHD allele. We conclude that it is necessary to analyze at least two exon regions in the *RHD* gene.

**Key words:** *RHD* genotyping; Cell-free DNA; Maternal plasma; Prenatal diagnosis

## INTRODUCTION

In the Rh (rhesus) blood group system, RHD is the most important and highly immunogenic antigen, and anti-D isoantibody is the major cause of hemolytic disease of the newborn (HDFN) and transfusion reactions (Chen et al., 2004). The RHD antigens encoded by the *RHD* gene on chromosome 1 determine the RHD blood group (Rh- or Rh+) of people, and in case of incompatibility between maternal and paternal RHD blood group, this may result in intrauterine fetal death, fetal anemia or neonatal icterus (Huang, 1998). If the fetus is D-negative, it is not at risk of HDFN. The determination of free fetal DNA and the *RHD* gene in maternal blood make it possible to detect the RHD status of a baby by a non-invasive method (Rouillac-Le et al., 2007). The Rh blood group system is a very polymorphic system. RHD and RhCE are located in the region of p36.13-p34.3 on chromosome 1, and they are 97% homologous to each other. Each of these genes consists of 10 exons, and they contain 69-kb DNA. The regions of exon 7 and exon 10 within the *RHD* gene are the areas of focus. In all positive results, the RHD blood group has also been found to be positive (Huang, 1998; Wagner and Flegel, 2000). In the white population, D-negative individuals are homozygous for a deletion of RHD. The D-negative phenotype shows the absence of the whole RHD protein from the red cell membrane. The genetic diversity of the Rh gene is evident, particularly in 82% black Africans. The most common RHD-negative phenotype is caused by the presence of the RHDΨ pseudogene (Bennett et al., 1993) carrying several mutations, and the other haplotype is RHD-CE-D$^s$. There are RHD variants in populations that show no expression or low expression, and this causes false-negative results. The RHDΨ pseudogene, which causes the RHD-negative phenotype in the black African population, is susceptible to very many mutations. It has variants that show low expression, which may in turn lead to false-negative and false-positive results (Rouillac-Le et al., 2004). RHD exon 7 PCR is positive in almost all *RHD* genes, but not in RHD-CE-D$^s$ and the RHDΨ pseudogene. The use of RHD exon 7 and RHD exon 10 prevents false-positive results in fetuses carrying only the RHDΨ pseudogene or RHD-CE-D$^s$ gene (Scheffer et al., 2011).

## MATERIAL AND METHODS

Five milliliters blood was drawn from each of 40 Rh (-) pregnant women showing Rh incompatibility with their husbands, who came to 11-23 weekly check-ups at Istanbul University, Faculty of Medicine, Department of Gynecology and Obstetrics, Kanuni Sultan Suleyman Hospital and Medicus Health Center. The day blood samples were obtained, centifuged for 15 min at 4100 *g*, and aliquots of 500 µL were then transferred to polypropylene cryogenic vials and stored at -80°C until the day of the isolation of the supernatant. DNA was extracted from 500 µL of the plasma sample containing 5 µL diluted maize DNA (1/100,

 

v/v), using a QIAmp DSP Virus kit (IVD CE), Qiagen Ref. 60704, according to manufacturer instructions. DNA was eluted using 30 µL of the elution buffer provided with the kit. For each series of extraction, three controls were added: RHD-negative and RHD-positive plasma controls provided with the kit and a blank control for which 500 µL water was used instead of plasma. A no-template control was included in each run as a negative and positive control for test. For each patient, PCR amplifications of exons 7 and 10 of the *RHD* gene were performed as well as of maize to check the absence of PCR inhibitor in the sample and the quality of the DNA extraction. Real-time PCR analysis was performed using a Stratagene Mx3005P. Amplification conditions were an initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 10 s, annealing and extension at 59°C for 45 s, and extension and fluorescence measurement at 72°C for 1 s.

## RESULTS

Between 2010 and 2011, RHD fetal genetic typing was carried out in 40 cases at the Department of Molecular Biology and Genetics. We found 30 (75%) cases with a prenatal and postnatal RHD-positive blood type; in 7 (17.5%) cases we detected a prenatal and postnatal RHD-negative status. In three of 40 cases, we observed discordant test results (7.5%), which are depicted in Table 1.

**Table 1.** Testing for the presence or absence of RHD.

| Case | Exon 7 | Exon 10 | Maize DNA | Newborn |
|---|---|---|---|---|
| 30 | Positive | Positive | Positive | Rh (+) |
| 7 | Negative | Negative | Positive | Rh (-) |
| 2 | Negative | Positive | Positive | Rh (-) |
| 1 | Positive | Negative | Positive | Rh (+) |

The results of the $C_T$ real-time PCR assay of exon 7 and exon 10 in plasma samples from 40 D-negative women are shown in Table 2. The fetal *RHD* genotyping $C_T$ values were in the range of 35-40 $C_T$. Thirty fetuses were predicted to be RHD-positive based on the results on plasma DNA, and seven fetuses were predicted to be RHD-negative. One fetus showed negative RHD exon 10 and positive exon 7 (early gestation age), and two fetuses showed negative exon 7 and positive exon 10. These results suggest the presence of hybrid RHD-CE-$D^s$ or *RHDΨ* allele expressing a maternal RHD.

**Table 2.** Cycle threshold values ($C_T$) for cell-free fetal DNA RHD exon 7 and exon 10.

| Case | Exon 7 $C_T$ | Exon 10 $C_T$ | Conclusion on fetal RhD status |
|---|---|---|---|
| 30 | 35.66 ± 1.55 | 35.9 ± 1.45 | RHD-positive |
| 7 | Negative | Negative | RHD-negative |
| 2 | Negative | $C_T \geq 35$ | RH-variant (RHDΨ pseudogene or RHD-CE-$D^s$ gene) |
| 1 | Positive | Negative | Exon 7 more sensitive than exon 10 |

## DISCUSSION

Several studies have examined the prenatal detection of fetal *RHD* genotyping from analysis of cell-free fetal DNA in maternal plasma (Bombard et al., 2011). Almost all labora-

tories carrying out fetal D typing on fetal DNA in maternal plasma employ quantitative real-time PCR technology with TaqMan chemistry (Daniels et al., 2007). Most protocols involve amplification of two or three exons to avoid false results with the more common variants of RHD (Daniels et al., 2009). The finding of this study shows the RHD genotyping real-time PCR technology for the detection of the RHD exon 10 and exon 7 from examination of cell-free fetal DNA in maternal blood.

Most of the studies examined samples from all three trimesters of pregnancy, but the majority were from the second and third trimesters (Bombard et al., 2011). The results of our study were from all trimesters. In our laboratory, 40 plasma samples from RHD-negative pregnant women showed that the fetal *RHD* gene could be detected by real-time PCR. We showed that non-invasive fetal blood group genotyping of rhesus D in alloimmunized women is accurate and applicable in a clinical diagnostic setting. We were able to report fully conclusive results in 40 tests performed. Thus, the sensitivity of assay was 100%.

In the Caucasian population, homozygous deletion of the *RHD* gene is the predominant cause of the D-negative phenotype. In contrast, 82% of D-negative black Africans do not have a homozygous deletion of RHD, but carry one or two RHD variant genes, the RHD pseudogene (Singleton et al., 2000) or the RHD-CE-D$^s$ hybrid gene (Faas et al., 1997; Rodrigues et al., 2002). In one of the largest validation studies published on non-invasive fetal *RHD* genotyping, Rouillac-Le et al. (2004) amplified RHD exon 7 and exon 10 in 893 maternal plasma samples. They had to exlude 26 D-negative women carrying an RHD pseudogene and five carrying the RHD-CE-D$^s$ gene, as they were unable to predict the fetal phenotype with the combination of these two targets. Chinen et al. (2010) tested 102 D-negative women in a Brazilian population and reported two false-positive results in women carrying an RHD pseudogene, using the exon 7 exon 10 approach. We found this hybrid gene in two cases. These samples were D-positive by exon 10 analysis but D-negative by exon 7 analysis (7.5%). Our data show the necessity of performing multiplex PCR for detecting more than one region of the *RHD* gene to avoid false-negative and false-positive results.

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