Original Article

Comparison of real-time polymerase chain reaction assay methods for detection of *RHD* gene in amniotic fluid

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Abstract

Hemolytic disease of the newborn is the clinical condition in which Rh blood group antigens in couples are incompatible with each other and mother is negative for the antigen, whereas father is positive. Although *RHD* antigen encoded by *RHD* gene that is localized on chromosome 1 determines person's Rh genotyping, this incompatibility can lead to delivery as anemia, jaundiced, or dead in mother's uterus. In recent years, improvements have occurred in the prenatal diagnosis of Rh incompatibility. Quantitative real-time polymerase chain reaction (Real-time PCR) has been improved and determining rapidly, reliably, and sensitively has been possible. In this study, the determination of *RHD* genotyping was investigated using fetal DNA obtained from amniotic fluid and SYBR Green I and TaqMan probe methods were compared, and reliability in prenatal diagnosis of these methods was determined. We studied 35 pregnant women in the second trimester of pregnancy. "SYBR Green I" and "TaqMan" probes results for *RHD* gene of genomic DNA extracted from total 35 different amniotic fluid samples acquired from 10 *RHD* (-) and 25 pregnant women randomly were analyzed. DNA extracted from amniotic fluid was analyzed for *RHD* gene with real-time PCR and the results were then compared with the *RHD* fetal genotype determined on *RHD* phenotype of the red blood cells of the infants at birth. The results of *RHD* TaqMan probes PCR analysis of amniotic fluid DNA were completely concordant with the fetal blood group analysis after birth. Real-time PCR using the TaqMan probes has proven to be more sensitive, accurate, and specific for *RHD* gene than SYBR Green I method.

Key words: Blood group antigens, fluorescent dyes, prenatal diagnosis

INTRODUCTİON

In the Rh 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 (HDN) and transfusion reactions.[1] Despite the widespread use of anti-D immunoglobulin prophylaxis (RhIg) to prevent the production of anti-D antibodies by *RHD* (-) mothers,[2] *RHD* alloimmunization still remains the major cause of severe hemolytic disease in fetuses and newborns.[3] In case mother is *RHD* (-) and father is heterozygous, HDN is, in 50% of cases, caused by maternal anti-D (IgG) antibody crossing the placenta and binding to fetal red blood cells, followed by their destruction, which results in anemia.[4,5]

During the past several years, prenatal determination of the fetal Rh blood type has been made by means of DNA testing on amniotic fluid cells.[6] Several polymerase chain reaction (PCR) techniques have been developed for the diagnosis of various genes. The recent advent of a real-time PCR technique has been proven to be useful in various applications, including pathogen detection, gene expression, regulation, and allelic discrimination. Real-time PCR utilizes the 5' nuclease activity of *Taq* DNA polymerase to cleave a nonextendible, fluorescence-labeled hybridization probe during the extension phase of PCR.

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The fluorescence of the intact probe is quenched by a second fluorescent dye, usually 6-carboxy-tetramethyl-rhodamine (TAMRA). The nuclease cleavage of the hybridization probe during the PCR releases the effect of quenching, resulting in an increase of fluorescence proportional to the amount of PCR product, and can be monitored by a sequence detector.[7]

SYBR Green I is a minor groove DNA-binding dye, its fluorescence is low; the fluorescence increases when it binds to double-stranded DNA. Among the detection chemistries available for real-time PCR, it is the least expensive, does not require the synthesis of a target-specific probe, and can be used with any pair of primers. Thus, it is particularly useful to develop a real-time quantitative assay when primers, which are known to generate a single product with high yield, are already available.[8]

The human *RHD* gene has been cloned,[9] and it is absent in *RHD* (-) subjects.[10] Real-time PCR,[11] which quantifies DNA, can be utilized to detect whether[3] the *RHD* antigen is present or not. Current study aims to compare the two different methods.

MATERİAL AND METHODS

The ethics committee of Istanbul University, Faculty of Medicine endorsed the study design (protocol number 1532).

Subject

We collected 1 ml amniotic fluid samples from 35 pregnant women. We obtained these samples at the department of Gynecology, Cerrahpasa and Istanbul Medical Faculties, Istanbul, Turkey. We used these samples to establish the accuracy of the *RHD* gene real-time PCR system.

DNA extraction

DNA was extracted from samples of amniotic fluid with High Pure PCR Template Preparation Kit (Roche Diagnostics, Manheim, Germany, Kat.No: 11796828001) according to the manufacturer's instructions. The DNA was eluted into 50 µl elution buffer (10 mM Tris HCl pH 7.4:1 mM EDTA), of which 5 µl was used as a template for the PCR reaction.

Real-time polymerase chain reaction using SYBR Green I

The *RHD* primers custom was synthesized by Tib MolBiol (Syntheselabor GmbH Eresburgstr. 22-23, D-12103 Berlin, Germany). The primers combinations were as follows: *RHD*-forward (5'-CCTCTCACTGTTGCCTGCATT-3') and *RHD*-reverse (5'-AGTGCCTGCGCGAACATT-3'). The real-time PCRs were set up according to the manufacturer's instructions (TaKaRa, SYBR Green I Real-Time PCR Kit, The Biotechnology Company ™ Japan) in a reaction volume of 50 µl. The PCR primers were used at a concentration of 900 nmol/l for *RHD* gene. Five microliters in each of the extracted genomic DNA were used for amplification.

Real-time PCR analysis was performed using a Stratagene Mx3005P. The thermal profile used for *RHD* gene analysis was as follows: after a 3-minute denaturation at 95°C, 40 cycles of PCR were carried out after 30 seconds of denaturation at 95°C and 60 seconds of annealing at 55°C, and then 30 seconds of extension at 72°C. Each sample was analyzed in duplicate. The SYBR Green I fluorescent signal was determined for each cycle at the end of the extension step.

Real-time polymerase chain reaction using the TaqMan probe

In this study, the real-time PCR amplification was carried out in a Stratagene Mx3005P. The *RHD* fluorogenic PCR system consists of the amplification primers *RHD*-forward (5'-CCTCTCACTGTTGCCTGCATT-3') and *RHD*-reverse (5'-AGTGCCTGCGCGAACATT-3') and a dual-labeled fluorescent probe, *RHD*-T (5'(FAM) TACGTGAGAAACGCTCATGACAGCAAAGTCT (TAMRA)3'); FAM [6-carboxyfluorescein] and TAMRA were the fluorescent reporter dye and quencher dye, respectively. The primers and probe were targeted toward the 3' untranslated region (exon10) of the *RHD* gene. The fluorescent probe contained a 3'-blocking phosphate group to prevent extension of the probe during the PCR. Sequence data for the *RHD* gene were obtained from the GeneBank data base (accession number, X63097).

The *RHD* fluorescent probe and primers custom were synthesized by Tib Mol Biol (Syntheselabor GmbH Eresburgstr. 22-23, D-12103 Berlin, Germany) and were used of concentrations of 25 nM and 300 nM, respectively. A total of 5 µl of the extracted amniotic fluid DNA was used for amplification.

RESULTS

Reaction results with SYBR Green I method

When total DNA extracted from amniotic fluid samples from 25 pregnant women, positive and negative controls, and reaction mixture not including DNA used as no template control (NTC) were analyzed with SYBR Green I method, all of results were found to be *RHD* (+) and NTC was not shown a amplification signal [Figure 1].

Reaction results with TaqMan Probe method

When total DNA extracted from amniotic fluid samples

from 25 pregnant women used in SYBR Green I method, positive and negative controls, and NTC were analyzed with TaqMan probe method, negative control, NTC, and a sample of belonging to a pregnant woman were *RHD* (-) [Figure 2].

When fetal DNA extracted from amniotic fluid samples from 10 *RHD* (-) pregnant women, positive and negative controls, and reaction mixture not including DNA used as NTC were analyzed with TaqMan probe and SYBR green method, two samples were found as *RHD* (-) and eight samples were found as *RHD* (+) in TaqMan probe method [Table 1].

All the results were confirmed with postnatal serological test and serological test results were same with results acquired with TaqMan probe method.

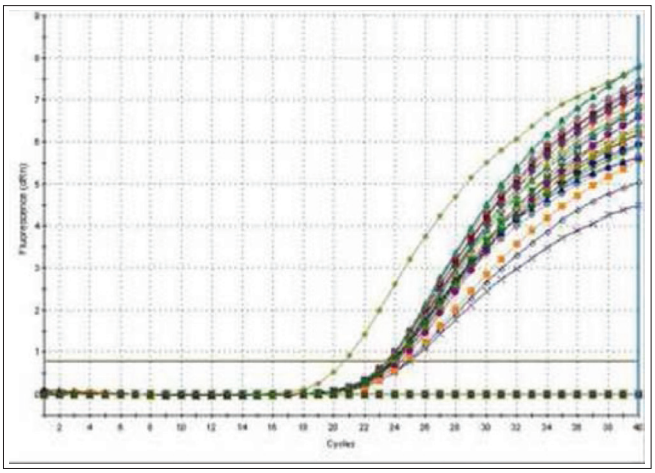

Figure 1: Amplification curve acquired result amplification of *RHD* gene with SYBR Green I method

DISCUSSION

This study aimed at the diagnostic reliability of prenatal *RHD* diagnosis from amniotic fluid with two different real-time PCR methods. This study showed that Real-time PCR with TaqMan probes is an accurate method for detection of fetal *RHD* genes. In 1996, Vanden Veyver *et al.* published their data about 114 amniotic fluid samples in which fetal *RHD* blood type was determined with PCR.[6] A caveat of this methodology is the requirement of development for appropriate strategies to counter incorrect diagnoses resulting from either false positives (contamination) or false negatives (no fetal DNA present in the sample).[12]

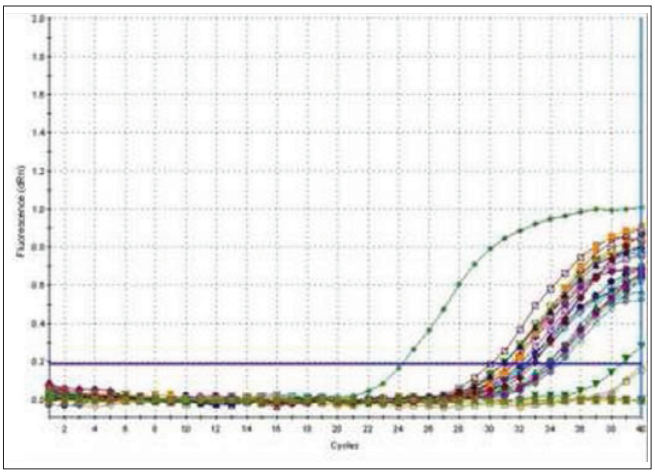

Figure 2: Amplification curve acquired result amplification of *RHD* gene with TaqMan probe method

The dsDNA-binding dye SYBR Green I and a TaqMan probe were tested in different real-time PCR systems (Stratagene Mx3005P) to determine the *RHD* gene in amniotic fluid.

The detection and quantification of gene rearrangement, amplification, translocation, or deletion were significant problems, both in research and in a clinical diagnostic setting.[13]

Real-time PCR analysis is sensitive, reliable, and rapid technique for determining *RHD* genotyping of genomic DNA extracted from amniotic fluid obtained from *RHD* (-) pregnant women.[3]

In this study, results obtained with TaqMan probe method were compared with that of SYBR Green I method and the former was found to be more reliable after demonstration. With TaqMan probe method, amplification was not found in negative control and NTC, and one sample was with no *RHD* DNA and

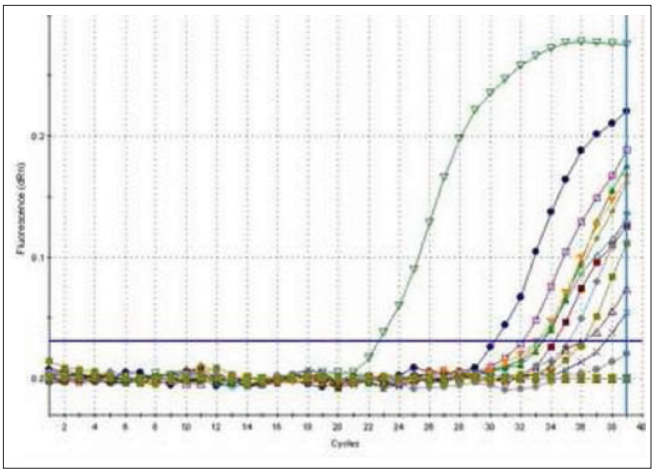

Figure 3: Amplification curve from *RhD* (-) pregnant women results amplification of *RhD* gene with TaqMan Probe Method

Table 1: Results of *RHD* genotyping of fetuses of *RHD*-negative women with the use of the *RHD* PCR assay

Samples	TaqMan Ct values	SYBR green values	Real-time PCR results	Newborn Rh phenotypes
NTC (No template control)	-	-	-	-
Positive control	32.42	29.15	Rh (+)	-
Negative control	-	-	Rh (-)	-
1	33.99	38.16	Rh (+)	Rh (+)
2	32.32	35.71	Rh (+)	Rh (+)
3	-	38.70	Rh (-)	Rh (-)
4	37.53	40.24	Rh (+)	Rh (+)
5	36.28	34.89	Rh (+)	Rh (+)
6	-	41.72	Rh (-)	Rh (-)
7	34.07	35.60	Rh (+)	Rh (+)
8	36.9	36.28	Rh (+)	Rh (+)
9	35.06	36.26	Rh (+)	Rh (+)
10	36.62	38.16	Rh (+)	Rh (+)

The *RHD* status was determined by serologic analysis of newborn blood samples

24 samples were *RHD* (+) DNA. These results have supported the avoidance of evoking false-positive results because of the sequence-specific complementary of TaqMan probe.[14]

When same samples were analyzed with SYBR Green I method, amplification was found in total of samples. When it was compared with serological results, false-positive result was determined in one sample. These results have supported the hindrance of accuracy because of primer dimers and nonspecific products, which were bound by the SYBR Green I dye.[12] Measurement of the ratio of specific PCR products against nonspecific ones was enabled by determining the melting peak integration.

As TaqMan probe method does not need melting curve analyses and is more sensitive than SYBR Green I, it is suggested for *RHD* genotyping.[15] When total DNA obtained from amniotic fluid of 10 pregnant women known as *RHD* (-), negative and positive controls, and NTC were analyzed with TaqMan probe method, amplification was found of eight amniotic fluid samples, while amplification was not found in two amniotic fluid samples [Figure 3]. These results were confirmed with serological tests. Real-time analyses are useful for *RHD* (-) pregnant women because of their sensitivity to *RHD* antigens. If the test indicates that the fetus is *RHD* (-), it can be reassured that the fetus is not at risk. On the other hand, if the test indicates that the fetus is *RHD* (+), treatment can be planned. The test gives a result in a short period. So, this is an advantage for a period of pregnancy times during the race.

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