Fetal *RHD* Genotyping by Analysis of Maternal Plasma in a Mixed Population

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Background: Maternal plasma analysis for the determination of the fetal *RHD* status is an exciting tool for the management of RhD-negative pregnant women, specially sensitized women. We assessed the accuracy of fetal *RHD* genotyping by analysis of maternal plasma in a multi-ethnic population. **Methods:** We analyzed plasma samples from 88 RhD-negative pregnant women between 11 and 39 weeks of gestation, median age of 28 years old to determine the fetal *RHD* genotype. This population was from Southeastern Brazil with high mixed ethnic background. Fourteen patients (16%) had anti-D alloantibody. We used Taqman primers and probes to detect by real-time PCR, exons 4, 5, and 10 of *RHD*. As internal controls we used primers/probes sets to *SRY* and *CCR5*. Peripheral or umbilical cord bloods from respective neonates were collected during

delivery and hemagglutination was performed. **Results:** Fifty-eight samples (66%) were genotyped as *RHD*+, 27 samples (31%) showed complete absence of *RHD* and 3 samples (3 %) presented a D variant (*RHD* ψ). All the results agreed with the neonatal typing, including the three fetuses with the *RHD* ψ , phenotyped as RhD-negative. Thus, the accuracy of the fetal *RHD* genotyping in this mixed population was 100%. The earliest pregnancy in which fetal *RHD* was detected was 11 weeks. **Conclusion:** Our findings indicate that the accuracy of *RHD* gene using three regions (exons 4, 5, and 10) can be sufficient for clinical application in a multi-ethnic population. This knowledge helped us on the development of a feasible protocol for fetal *RHD* genotyping on DNA from maternal plasma for our population. J. Clin. Lab. Anal. 25:100–104, 2011. © 2011 Wiley-Liss, Inc.

Key words: fetal *RHD* genotyping; multi-ethnic population; pregnant women; RhD-negative

INTRODUCTION

Hemolytic Disease of the Fetus and the Newborn (HDFN) still contributes to perinatal morbidity and mortality, in spite of the widespread immunoprophylaxis (1). Although the perinatal mortality rate continues to fall, mainly through advances in obstetric and neonatal care, the rates of new cases of RhD immunization in RhD-negative pregnant women have remained relatively constant (2). Currently, the main problem in the management of RhD-negative women is not once a decision for transfusion has been made, but instead it is how to avoid invasive investigations for as long as possible (3).

It is beneficial to know the fetal RhD phenotype of pregnant women with anti-D. If the fetus is RhD-negative, then it is not at risk from HDFN and no invasive procedures are required; if it is RhD-positive,

the appropriate management of a pregnancy at risk can be arranged (4).

However, traditional procedures for obtaining fetal samples (amniocentesis and cordocentesis) are expensive, invasive and present a risk to the fetus with fetal losses estimated at 1–15%. Furthermore, these procedures may aggravate the disease (4–6). The discovery of free fetal DNA in maternal plasma coupled with the use of molecular biology techniques has made noninvasive

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fetal *RHD* genotyping possible (5–9). Currently, maternal plasma analysis for the determination of the fetal *RHD* status has become an exciting new tool for the management of RhD-negative pregnant women, specially sensitized women. In addition, a simple, noninvasive fetal *RHD* typing would reduce wastage of anti-RhD Ig, making the test cost-effective (10).

Fetal *RHD* genotyping by conventional PCR can be easily performed in any basic molecular laboratory (1,11–14). However, it has been demonstrated that real-time PCR assays can provide more accurate results in a quick and nondemanding way (4–6,8,15–19).

There are, however, several challenges in fetal *RHD* genotyping. A major problem is the presence of *RHD* variants, in special, the presence of the *RHD* pseudogene (*RHD*Ψ) and the *RHD-CE-Ds* hybrid gene in Africans with an RhD-negative phenotype (20). Minon et al. (21) related, in an African population, that 44% of the population had the *RHD* pseudogene and 22% the hybrid gene *RHD-CE-D^s*. In Brazilians, Rodrigues et al. (22) reported the presence of the *RHD* pseudogene in 11% and the *RHD-CE-D^s* in 2% of the population phenotyped as RhD-negative. Although amplification of a single region of *RHD* can provide a correct phenotype prediction in the majority of the cases, most protocols involve amplification of two or three exons to avoid obtaining false results with the more common variants of *RHD*. It is important that false positives do not result from the presence of the inactive African genes *RHD*Ψ and *RHD-CE-D^s* (7,10,19).

Most of molecular protocols developed were based on sequences of individuals of Caucasian origin. It is known that there are different genetic causes for RhD-negative phenotype of an individual with an incidence that varies according to the ethnic origin. The Brazilian population is of heterogeneous ethnical origin and it is unevenly distributed within this country of continental dimensions. The intense process of miscegenation made the Brazilian population unique in its ethnic background without any clear distinction of race in the country (1,23).

For these reasons, we conducted a study to evaluate the clinical application of fetal *RHD* genotyping from maternal plasma using real-time PCR and primers and probes targeted toward *RHD* (exons 4, 5, and 10), by assessing its accuracy, in a multi-ethnic Brazilian population.

MATERIAL AND METHODS

Subjects

Ten milliliters of maternal peripheral blood were obtained from 88 RhD-negative pregnant women with singleton pregnancies and maternal age from 18 to 46 years (median 28 years) who agreed to participate in this study by signing an IRB approved informed consent during a routine prenatal visit. The gestational age at the

time of blood collection ranged from 11 to 39 weeks (median: 27 weeks). Two (2.27%) samples were from first trimester, 45 (51.13%) from second and 41 (46.60%) from third trimester of gestation. The pregnant women were from the Southeastern Brazil with high mixed ethnic background, and admixture between descendants of Europeans, Africans and Native Americans. Fourteen patients (16%) had anti-D in the serum.

Preparation of Samples

Blood samples were collected into EDTA-containing tubes and centrifuged at 4,000 rpm for 10 min, with less than 2 hr after collection. Maternal plasma was removed to a fresh tube without disturb buffy coat. Aliquots of plasma were then centrifuged at 13,000 rpm for a further 20 min. The supernatant was removed to a fresh tube and stored at –20°C until required. Maternal buffy coat was removed to a fresh tube to obtain maternal DNA, respectively. One plasma aliquot of each sample was thawed at room temperature and centrifuged again at 13,000 rpm for 10 min to isolate cryoprecipitate.

DNA Extraction

Fetal DNA was extracted from 800 μl of plasma supernatant using the QIAmp Blood DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Fetal DNA was eluted in 55 μl of nuclease-free water. DNA concentration was measured by spectrophotometry (Nanodrop, Wilmington, DE).

Real-time PCR Analysis

Real-time PCR analysis was performed using a sequence detector Rotor Gene 6.000 5-plex (Qiagen). TaqMan primers and probes used for fetal *RHD* genotyping were targeted toward exons 4, 5, and 10 of *RHD*. The primers for exons 4 and 5 were designed to amplify only the *RHD* gene and not *RHD*Ψ, whereas the primers for exon 10 were designed to amplify both genes. As internal controls we used primers/probes sets to *SRY* and *CCR5*. All primers and probes sequences used were previously described (24). The Real-time PCR was performed in accordance with a previously described protocol (24) and is briefly described below.

TaqMan amplification reactions were set up in a reaction volume of 15 μl using the TaqMan Universal PCR master Mix (Applied Biosystems, Foster City, CA). Primers and probes were optimized to determine the minimum prime and probe concentrations that give the minimum cycle threshold (*C_t*) values. The *RHD* exon 4 and *CCR5* primers were used at a final concentration of 300 nM, whereas *RHD* exons 5 and 10 and *SRY* were used at a final concentration of 150 nM. The *RHD* exons

4, 5, and 10 and *CCR5* probes were used at a final concentration of 50 nM, whereas *SRY* was used at a final concentration of 100 nM. The TaqMan PCR conditions used were 50°C for 2 min, 95°C for 10 min, then 50 cycles of 95°C for 15 sec, and 60°C for 1 min. Each sample was analyzed in three replicates for each *RHD* exon and for *SRY* and in two replicates for *CCR5*.

Amplification plots of each *RHD* exon, *SRY*, and *CCR5* for patient samples were checked. The fetus was predicted to be RhD-positive if at least 2/3 replicates for *RHD* exons 4, 5, and 10 were positive (C_t value <42), with an additional two positive replicates from any of the *RHD* exons. If, the difference between any C_t value for *RHD* exon 10 and *CCR5* was less than 2, this could indicate that the mother has an unexpressed *RHD* gene and the test result was considered unreliable and further investigation was carried out using the maternal genomic DNA. The fetus was predicted to be D-negative if no replicates, or only 1/9 replicates were positive. If the fetus was predicted to be D-negative, the *SRY* result was used to confirm the presence of fetal DNA if the fetus was male. In these cases, the presence of fetal DNA was confirmed when at least 2/3 replicates were positive. If *RHD* exon 10 replicates were positive, but exons 4 and 5 were negative, this could indicate that the fetus had a D-variant *RHD* gene (*RHD* Ψ or the hybrid *RHD-CE-D^s* gene) and we interpreted as RhD-negative.

Confirmation of the Fetal D Status and Sex

Peripheral or umbilical cord bloods from respective neonates were collected at birth and hemagglutination was performed. Information of the neonate's sex was also obtained at birth.

RESULTS

Fetal *RHD* Genotyping

We analyzed plasma samples of 88 pregnant women and correlated the results with the serological analysis of peripheral or umbilical cord bloods from respective neonates at birth.

RHD-positive fetuses were identified in 58 (65.9%) samples and *RHD*-negative in 27 (30.7%) samples. Three (3.4%) samples showed amplification for *RHD* Exon 10 and *CCR5* only (Table 1). These assays were repeated several times and showed the same results. These results could indicate the presence of *RHD* variants as *RHD* Ψ or the hybrid *RHD-CE-D^s* gene and therefore the fetuses were classified as RhD-negative. Of the 58 *RHD*-positive fetuses there was 1 in the first trimester, 29 in the second trimester and 28 in the third trimester. Of the 27 *RHD* negative fetuses there was 1 in the first trimester, 16 in the second trimester and 13 in the third trimester. Two *RHD* variants were detected in the second trimester and one in the third trimester (Table 1).

Among the 30 fetuses classified as RhD-negative (27 *RHD*-deletion and 3 *RHD*-variants) the *SRY* gene was identified in 11 (36.7%) confirming the presence of fetal DNA. In the 19 RhD-negative samples remaining, only the *CCR5* gene was detected confirming the successful of DNA extraction but not the presence of fetal DNA.

Fetal *SRY* Genotyping

All the 88 DNA samples from maternal plasma were tested for *SRY*. Interestingly, the *SRY* was positive in 44 samples and negative in 44 samples. Thirty-three fetuses predicted to be RhD-positive and 11 predicted to be RhD-negative were also predicted to be male (Table 1).

Accuracy of Fetal *RHD* and *SRY* Genotyping

An accuracy rate, sensitivity, specificity, positive and negative predictive values of 100% was achieved in the prediction of fetal D status and fetal sex, when results were compared with serological tests on infant's RBCs and sex information. Those three samples genotyped as *RHD* variant, and predicted to be RhD-negative, were confirmed to be RhD-negative by serology.

DISCUSSION

The discovery of fetal free DNA in maternal plasma has opened new possibilities for the determination of blood groups for prenatal noninvasive procedures (4,5).

TABLE 1. *RHD* and Sex Genotyping in 88 Pregnancies Using Maternal Plasma as a Source of Fetal DNA, Stratified by Gestational Age

Gestational age (weeks)	Number	<i>RHD</i> +		<i>RHD</i> –		Accuracy (%)
		Male	Female	Male	Female	
1–12	2	0	1	1	0	100
13–27	45	16	13	4	12 ^a	100
28–42	41	17	11	6	7 ^b	100
Total	88	33	25	11	19	100

a and b: Two samples and one sample, respectively, that showed amplification only for *RHD* Exon 10 and *CCR5* and were predicted to be RhD-negative.

Knowledge of *RHD* fetal genotype is very important for medical management of RhD-negative pregnant women mainly in a risk of occurrence of HDFN. It allows the identification of fetuses at risk for HDFN and also the identification of risk pregnancies, intensifying prenatal care for these groups. Many researchers have concentrated efforts in the investigation of a methodology for determining fetal *RHD* genotype using fetal DNA isolated from maternal blood. This prospective study demonstrated that the real-time PCR analysis of maternal plasma can be used for fetal *RHD* genotyping, even in a multiethnic population.

For high sensitivity and specificity (close to 100%), the methodology requires a number of criteria and controls. The use of probes methodology is necessary once the fetal DNA in maternal plasma is found in low concentrations and highly degraded. The real-time PCR technique is labor intensive and requires extensive standardization to obtain reliable results. Thus, the establishment of optimal conditions of reaction is of great importance.

Owing to high homology and complexity of the *RHD* gene, there is a general agreement that primers and probes must be carefully designed and should be analyzed at least two gene regions (25,26). To consider a positive *RHD* genotyping result, it is necessary that all *RHD* exons show amplifications. This minimizes the occurrence of false results and enables the identification of possible *RHD* variants. With the aim of improving accuracy of results, it is recommended that the amplification be performed at least in triplicate for each *RHD* region studied and that positive and negative controls be included. Owing to implementation of all these criteria in the protocol, this study obtained satisfactory results and a concordance rate of 100% when comparing results from genotyping and serology tests performed on the neonates RBCs at birth.

There is no consensus about which *RHD* regions should be used in fetal genotyping. *RHD* Ψ contains several single nucleotide polymorphisms in exons 4 and 5 and a negative result can be achieved by the use of primers or probes specific for the active sequence (7,20). Amplification of any *RHD* sequence within exons 4–7 will give a correct negative result with *RHD-CE-D^s*. Where possible, *RHD* genes producing variant RhD antigens should give a positive result and this can be achieved by including an amplification of exon 7 or 10 (27). Exons 4, 5, 6, 7, and 10 are the *RHD* regions most used. In this study, we decided to analyze exons 4, 5, and 10 and found three samples that showed amplification only for *RHD* Exon 10 and *CCR5*. This strategy allowed us to identify three fetuses with *RHD* variants, further investigated at birth and identified as *RHD* Ψ . These findings strongly suggest that in multiethnic populations

it is essential to include analysis of *RHD* Ψ in order to avoid false-positive results.

In this study we also had satisfactory results with the fetal sex with 100% concordance, although we had used the *SRY* as an internal control of the presence of fetal DNA. Eleven fetuses predicted to be RhD-negative were also predicted to be male. However, in 19 maternal blood samples analyzed, no *RHD* or *SRY* signal was detected. Although it was confirmed that these fetuses were D-negative females, it cannot be ascertained that fetal DNA was present in these maternal plasma samples. The earliest pregnancy in which the presence of fetal DNA was confirmed in our study was 11 weeks.

Successful *RHD* genotyping was achieved despite the presence of maternal anti-D in 16% of the pregnant women studied. Our results indicate that fetal *RHD* genotyping can be performed with a high degree of accuracy when using maternal plasma as a source of DNA, despite of maternal anti-D. In conclusion, noninvasive fetal *RHD* genotyping by targeting exons 4, 5, and 10 with real-time PCR is highly sensitive and accurate in a mixed population and can be sufficient for clinical application.

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