Determination of Fetal *RHD* Genotype Including the *RHD* Pseudogene in Maternal Plasma

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Objective: To examine the accuracy of fetal *RHD* genotype and *RHD* pseudogene determination in a multiethnic population. **Methods:** Prospective study involving D-negative pregnant women. Cell-free DNA was extracted from 1 ml of maternal plasma by an automated system (MagNA Pure Compact, Roche) and real-time PCR was performed in triplicate targeting the *RHD* gene exons 5 and 7. Inconclusive samples underwent *RHD* pseudogene testing by real-time PCR analysis employing novel primers and probe. **Results:** A positive result was observed in 128/185 (69.2%) samples and negative in 50 (27.0%). Umbilical cord blood phenotype confirmed all cases with a positive or

negative PCR result. Seven (3.8%) cases were found inconclusive (exon 7 amplification only) and *RHD* pseudogene testing with both conventional and real-time PCR demonstrated a positive result in five of them, while two samples were also *RHD* pseudogene negative. **Conclusion:** Real-time PCR targeting *RHD* exons 5 and 7 simultaneously in maternal plasma is an accurate method for the diagnosis of fetal D genotype in our population. The *RHD* pseudogene real-time PCR assay is feasible and is particularly useful in populations with a high prevalence of this allele. J. Clin. Lab. Anal. 31:e22052, 2017. © 2016 Wiley Periodicals, Inc.

Key words: DNA/analysis; genotyping techniques; prenatal diagnosis/methods; RhD Blood group system; sensitivity and specificity

What is already known about this topic?

Fetal *RHD* genotype can be determined with accuracy by real-time PCR from cell-free plasma DNA.

What does this study add?

It presents results from routine application of the method in an admixed population. In addition, it describes a novel set of primers and probe for real-time PCR analysis of the *RHD* pseudogene.

Time PCR (RT-PCR) analysis targeting different combinations of *RHD* exons presents high accuracy for fetal genotyping in D-negative women (1–6).

Currently, this test is routinely performed in several countries to assess the need for anti-D immunoglobulin administration during pregnancy (7) and help identify those women at risk for hemolytic disease of the fetus and newborn (HDFN) (8).

Nowadays, the incidence of HDFN by anti-D in European countries is about 1 in 1,000 births (9) while in Brazil it is five times higher (10). The high rate of alloimmunization is correlated with failures in

INTRODUCTION

Fetal *RHD* genotype detection from circulating cell-free DNA in maternal plasma is a noninvasive test. Several studies have now demonstrated that Real-

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monitoring of prenatal care, and also incorrect administration or absence of prophylaxis.

The *RHD* gene is located in chromosome 1, and it encompasses ten exons contiguous to the *RHCE* gene to form the *RH* locus (11).

In Caucasians, the most common genetic mechanism observed in phenotypic D-negative individuals is the complete deletion of *RHD* gene characterized by a “hybrid” Rhesus box, probably by an unequal crossing over (11, 12).

In other ethnicities such as D-negative Black Africans, the most common mechanism is the presence of an *RHD* pseudogene, associated with the *RHCE**ce alleles, which contains a duplicated sequence of 37 bp committing the last 19 nucleotides of intron 3 and the first 18 nucleotides of exon 4 in addition to four missense mutations in exon 5 (609G>A, 654G>C, 667T>G, 674C>T) and a nonsense in exon 6 (807T>G) in which creates a sign of a stop codon (11–14).

In Caucasians and Black Africans other important phenotypic D-negative are hybrid alleles. The high similarity of both genes (*RHD* and *RHCE*) and their opposite orientation, proximity on the same chromosome, favors gene conversion in “Cis” and the formation of a loop of “hairpin” where parts of one gene are exchanged by the corresponding parts of the other resulting hybrid alleles such as *RHD-CE-D* and *RHCE-D-CE* (11–14). The Brazilian population is of heterogeneous ethnical origin and is unevenly distributed within this country of continental dimensions (15). In multiethnic populations, *RHD* gene variants due to polymorphisms are found more often and may be related to false-positive results in pregnancies with a phenotypically D-negative fetus (16).

The present study aims to examine the accuracy of fetal *RHD* genotype diagnosis by RT-PCR analysis in a multiethnic population and riddle *RHD* variants in mothers and fetus.

MATERIALS AND METHODS

Population and study design

This prospective study was carried out between May 2012 and June 2014. Two hundred and twenty D-negative pregnant women with 8–28 gestational weeks and no history of organ or bone marrow transplantation, attending a tertiary referral center for fetal medicine at Hospital das Clínicas, São Paulo University Medical School, Brazil were enrolled. D-negative pregnant women could be seeking for routine antenatal care or referred for specialist assessment. Institutional ethics committee approved the study (CAPPesq 0575/11) and all women who participated gave informed

consent. Twin pregnancies were also included in the study.

Samples

For this study, 10–12 ml peripheral blood samples was collected and distributed in two tubes with ethylenediaminetetraacetic acid anticoagulated. One tube (BD Vacuatainer®, Franklin Lakes, NJ), was used to perform maternal D phenotype while the second tube (Plasma Preparation Tube – PPT Vacuatainer®) was the source of maternal plasma. Blood was centrifuged within 2 h of sampling at $2,200 \times g$ for 10 min and stored at -70°C . At the time of extraction 1 ml of maternal plasma was centrifuged at $16,000 \times g$ for 10 min at 4°C . Supernatant was submitted to DNA extraction, as below.

DNA extraction

DNA was extracted from 1-ml samples of maternal plasma using the Large Volume kit in the MagNa Pure Compact DNA/RNA automated system (Roche Diagnostics, Mannheim, Germany), lowering the final elution volume to 50 μl . About 6 μl of the eluate was used as PCR template.

Real-Time PCR

All samples were tested using a duplex RT-PCR containing primers and probes for *RHD* exons 5 and 7 according to the Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence protocol (17) and, as a control for total DNA, a parallel assay targeting the CC chemokine receptor 5 (CCR5) was also run (18).

All RT-PCR amplifications were performed in a final volume of 25 μl using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA) for 50 cycles in the StepOnePlus™ (Applied Biosystems) RT-PCR equipment. One DNA extraction was performed for each sample and submitted to (exon 5 and 7 co-amplification) in triplicates while CCR5 was tested in duplicates. A positive and a negative control consisting of plasma, respectively, from *RHD*-positive and a *RHD*-negative individuals were included in all extraction routines, in addition to water for contamination control at the amplification step.

Data analysis

Fetal *RHD* was assigned as positive when both exons 5 and 7 presented cycle threshold (C_t) values ≤ 43 in at least two replicates and CCR5 displayed a

$C_t \leq 38$. Samples were considered *RHD* negative when only *CCR5* was detected in duplicate, both with a $C_t \leq 38$. Samples that presented *CCR5* $C_t \leq 38$ and $C_t \leq 43$ for exon 5 or 7 only were considered inconclusive. Positive results with a $C_t > 43$ for any target or samples with detectable amplification in only one of the replicates were deemed inconclusive and confirmed on a second DNA extraction.

Inconclusive cases

The exclusive amplification of exon 5 or 7 indicated the presence of an *RHD* variant. Investigation of the *RHD* pseudogene variant was carried by two techniques: conventional PCR according to the methodology described by Singleton et al. (19) and RT-PCR with novel sequence of primers and probe (Table 1). This novel *RHD* pseudogene primers/probe set was designed by placing the probe in the unique sequence junction characteristic of this variant created by the 37 bp duplication (underlined in Table 1). This assay was validated by testing 100 randomly selected D-positive subjects, all negative and 5 *RHD* pseudogene nuclear DNAs previously characterized.

In those cases that *RHD* pseudogene testing was negative, other variants were confirmed in maternal buffy coat by a conventional multiplex PCR analysis for *RHD* exons 3–7 and 9 (20). When no amplification was verified it indicated a *RHD* variant from the fetus. In those cases a sample was collected from the newborn cheek and submitted to the *RHD* partial BeadChip™ assay (Immucor, Norcross, GA). In those cases where the BeadChip revealed more than one *RHD* variant, it was performed standard Sanger sequencing of exons 3–10 of the *RHD* gene with the ABI BigDye Terminator v3.1 kit (Applied Biosystems), subsequently run on an automated sequencer equipment (ABI 310; Applied Biosystems).

Statistical analysis

The sensitivity, specificity, positive and negative predictive value of antenatal *RHD* genotyping were

TABLE 1. Novel *RHD* Pseudogene Primers and Probe Sequences Developed for PCR-RT

	5'–3' sequence
Forward primer	TCA CTG CTC TTA CTG GGT
Reverse primer	CGT AGA TGT GCA TCA TGT T
Probe	TET CAG ACC ACA TG <u>AAC TTA</u> CTG TTT - MGB

The underlined six nucleotide sequence is present only in the *RHD* pseudogene allele since it is created by the 17 bp duplication, double-underlined are the first three nucleotides of the duplication.

compared to conventional D serotyping, routinely carried on umbilical cord blood after birth, considered the gold-standard.

RESULTS

Blood samples were collected from 220 D-negative pregnant women. Thirty-five (15.9%) cases were excluded: exhausted in the standardization of methods ($n = 19$), fetal death ($n = 11$) and missing pregnancy outcome ($n = 5$). Table 2 summarizes maternal and pregnancy characteristics in the final study group ($n = 185$).

RHD exons 5 and 7 RT-PCR analysis were positive in 128 (69.2%) samples, negative in 50 (27.0%) and inconclusive in 7 (3.8%) cases (Table 3).

All cases that were inconclusive (7/185) showed amplification only for *RHD* exon 7 in the absence of exon 5, compatible with *RHD* pseudogene or *RHDVI* variant. All seven inconclusive cases were submitted to *RHD* pseudogene genotyping both by conventional PCR and the novel RT-PCR *RHD* pseudogene assay and demonstrated concordant results in five positive and two negative samples. Buffy-coat DNA from these seven mothers was obtained and confirmed the above result verified in plasma DNA.

In five mothers confirmed presence of a *RHD* pseudogene, and a genomic maternal DNA from those two

TABLE 2. Maternal Characteristics in 185 D-Negative Women who Underwent Fetal D DNA Testing

	Mean $\pm$ standard deviation/ n (%)
Maternal age, years	30.1 $\pm$ 6.4
Nulliparous	56 (30.3)
Twin pregnancy	8 (4.3)
Anti-erythrocyte antibody	
None	120 (64.9)
One	43 (23.2)
Two or more	22 (11.9)
Gestational age at blood collection, weeks	20.4 $\pm$ 5.6
1st trimester	32 (17.3)
2nd trimester	136 (73.5)
3rd trimester	17 (9.2)

TABLE 3. Comparison of *RHD* Exons 5 and 7 PCR-RT Results and Umbilical Cord Blood Phenotype in 185 D-Negative Pregnant Women. Values in Brackets are Percentages

<i>RHD</i> exons 5 and 7 PCR	Umbilical cord blood D phenotype	
	Negative, $n = 55$	Positive, $n = 130$
Negative	50 (90.9)	–
Inconclusive	5 (9.1)	2 (1.5)
Positive	–	128 (98.5)

TABLE 4. Comparison of Results Prenatal and Postnatal of Inconclusive Cases

Prenatal				Posnatal	
<i>RHD</i> exon 5	<i>RHD</i> exon 7	<i>RHD</i> ψ	RhD Phenotype	Beadchip <i>RHD</i> partial	Sequencing
Negative	Positive	Positive	Negative	–	–
Negative	Positive	Positive	Negative	–	–
Negative	Positive	Positive	Negative	–	–
Negative	Positive	Positive	Negative	–	–
Negative	Positive	Positive	Negative	–	–
Negative	Positive	Negative	Positive	DV type I, DAU5 or DBS2	DAU5
Negative	Positive	Negative	Positive	DV type I, DAU5 or DBS2	DAU5

inconclusive samples testing for *RHD* pseudogene and positive on *RHD* exon 7, were further submitted to a multiplex PCR targeting *RHD* exons 3–9 which confirmed that these women were truly *RHD* negative since no amplicon was obtained.

In both cases, the BeadChip assay performed with DNA from cheek cells of the newborns disclosed results compatible with DV type I, DAU5, and DBS2. These samples were submitted to exons 5 and 8 amplification as described, and further sequenced, revealing two mutations in exon 5 (667T>G, 697G>C) and one mutation in 8 (1136C>T), compatible with the variant DAU5 (Table 4).

In this study, there were no false positives nor false negatives. Excluding the inconclusive cases, there was 100% agreement between genotyping and phenotyping.

DISCUSSION

The present study confirms that RT-PCR targeting *RHD* gene exons 5 and 7 simultaneously in maternal plasma is an accurate method for the diagnosis of fetal D status in our population. Due to the great amount of polymorphisms involved in the Rh system, simultaneous analysis of at least two exons is recommended and helps in the reduction of false-positive results (1, 21, 22). On the other hand, false-negative results are mainly related to early gestational age at sampling, technical aspects related to maternal blood collection (23) and insufficient amount of DNA (24–26).

Previous studies involving the Brazilian population have tested different combinations of exons (4 + 10, 7 + 10, 4 + 5 + 10 and 5 + 7) and showed accuracies ranging from 97.3% to 100% (27–29). However, these studies employed manual DNA extraction methods, whereas automated systems allow better performance with high-throughput and reproducibility (30–32). It is also important to highlight that DNA was extracted from 1 ml of plasma in the present study, thus providing higher amounts of cell-free DNA which certainly contributed to the accuracy verified, including (32/185) first trimester pregnancies.

Interestingly, inconclusive results came from *RHD* variants that are typically found in mixed ethnic population. The SAFE assay is designed in order to avoid amplification of the *RHD* pseudogene and other variants stemming from exon 5 polymorphisms at the same time covering the main *RHD* variants. However, one disadvantage of this assay is that it does not identify the hybrid genes *RHD/RHCE*, another frequent rearrangement leading to the D-negative phenotype.

In the Brazilian population, the frequency of D negatives is about 10–11% (33, 34), in which the hybrid gene accounts for 0.16% (35) to 2% (36) and the pseudogene is described in approximately 3.5% (35, 36).

In this heterogeneous ethnical population it is particularly important to identify such individuals who are truly D negative, meaning that their erythrocytes do not carry any D antigen, so no further action is warranted in terms of antenatal clinical care.

Nevertheless 7/185 (4%) of our results were considered inconclusive because only exon 7 was amplified. A similar rate of inconclusive results was observed in another study (7/284, 2.5%) and those were compatible with DVI and *RHD* pseudogene (16). However, if we have not sought to identify the *RHD* pseudogene by this new assay, we could have missed the recognition of two DAU5 children, with obvious implications for their life either as eventual blood donors or recipients. Their mothers were informed of this rare status and they received a letter detailing how they shall be eventually transfused in the future, if needed, preventing alloimmunization. One child is female and the other is male.

Our study demonstrates that 5/7 inconclusive cases were *RHD* pseudogene. Understanding that we have a highly mixed population and that the presence of *RHD* pseudogene may lead to false-positive results, we developed a specific PCR-RT assay for the detection of this variant. The specific assay is more rapid and less labor intensive compared to conventional PCR, since it does not require the electrophoresis step,

making *RHD* pseudogene detection more practical. Next step will be the incorporation of these primers/probe set in the exon 5/7 assay, by using a third fluorophore, that does not interfere with VIC and FAM emissions, respectively, from exons 5 and 7 probes, making a triplex to be used in this context but also in the identification of D variant carriers among phenotypic D-negative blood donors (37).

Further studies with larger sample size are necessary to validate the performance of the primers and probe described in the present study. If proven to be accurate, inclusion of *RHD* pseudogene analysis will help avoid unnecessary administration of anti-D immunoglobulin to D-negative pregnant women in which the fetus is found to carry the *RHD* pseudogene.

In Brazil, the official policy is to provide RhD immunization to all D-negative pregnant women which may be costly since there is a high frequency of D negative in our population (approximately 10 (33)–11.9% (34)). In this context, we expect that fetal genotyping from maternal plasma to be cost-effective, as, in this study, it would have prevented the use of RhD immunoglobulin by at least 27% of the patients. It shall be included in the economic analysis of other costs associated with the close follow-up of women at risk of alloimmunization, such as frequent ultrasounds and antibody titer determinations.

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