Non-Invasive Prenatal *RHD* Genotyping Using Cell-Free Fetal DNA from Maternal Plasma: An Italian Experience

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Keywords

Fetal DNA · Hemolytic disease of the fetus and newborn · Prenatal *RHD* genotyping

Summary

Background: This study assessed the diagnostic accuracy of a non-invasive approach to fetal *RHD* genotyping using cell-free fetal DNA in maternal plasma and a combination of methodological strategies. **Methods:** Real-time PCR (qPCR) was performed on 216 RhD-negative women between weeks 10+0 and 14+6 of gestation (1st qPCR). qPCR was repeated (2nd qPCR) to increase the amount of each sample for analysis, on 95 plasma aliquots that were available from first trimester blood collection (group 1) and on 13 samples that were collected between weeks 18+0 and 25+6 of gestation (group 2). qPCR was specific for exons 5 and 7 of the *RHD* gene (*RHD5* and *RHD7*). The results were interpreted according to the number of positive replicates of both exons. **Results:** 1st qPCR: diagnostic accuracy was of 93.3%. Diagnostic accuracy increased from 90.5% (1st qPCR) to 93.7% (2nd qPCR) in group 1 and from 84.6% (1st qPCR) to 92.3% (2nd qPCR) in group 2. These increments were not statistically significant. **Conclusion:** Our approach to *RHD* genotyping in early pregnancy yielded high diagnostic accuracy. Increasing the amount of DNA analyzed in each sample did not improve significantly the diagnostic accuracy of the test.

Introduction

Fetal RhD inheritance is a crucial issue in pregnant women who, by virtue of maternal anti-D antibodies, are at risk of hemolytic disease of the fetus and newborn (HDFN). HDFN may be asymptomatic, may trigger mild jaundice or hydrops requiring in-utero transfusions, and may even lead to perinatal death. Therefore, evaluation of fetal *RHD* genotyping during pregnancy is essential to improve the management of gestation and prevent any complications.

Currently, this immune response is largely treated prophylactically by maternal injection of anti-D antibodies, before invasive procedures, during the 3rd trimester of gestation and immediately following the birth.

Since 40% of RhD-negative pregnant women are estimated to receive unnecessary antenatal anti-D prophylaxis while carrying a RhD-negative fetus, knowledge of the fetal blood group is crucial for targeted prophylaxis [1].

Amniocentesis or chorionic villi sampling accurately determine fetal RhD status but carry a risk of pregnancy loss and transplacental hemorrhage which increase maternal antibody titers. Many researchers have been focusing on the study and development of non-invasive diagnostic tests for *RHD* genotyping based on analysis of cell-free fetal DNA (cffDNA) from peripheral maternal blood and real-time PCR (qPCR) [2–6]. Although the most promising results were achieved when the test was performed during the 2nd and 3rd trimesters of pregnancy [7–11], a few approaches reached similar results in the 1st trimester [3, 12–15].

RHD genotyping using cffDNA is becoming increasingly reliable due to low costs and almost complete lack of invasiveness [16]. It was introduced into routine service in the UK in 2001; Denmark, the Netherlands and France followed later.

Our research group set up an accurate, non-invasive fetal *RHD* genotype approach as participating member of the Special Non-Invasive Advances in Fetal and Neonatal Evaluation' (SAFE) Network of Excellence [17] and while collaborating on a proposal for the 'WHO Reference Reagent RHD/SRY Plasma DNA Sensitivity Standard 07/222' [18].

The present study first aimed at assessing the diagnostic accuracy of our non-invasive approach to *RHD* genotyping by applying it to a group of RhD-negative pregnant women in the first trimester of gestation. The second aim was to determine whether diagnostic accuracy could be improved by increasing the sample to be analyzed by qPCR.

Material and Methods

Patients

Among pregnant women who attended the Obstetrics and Gynecology Outpatient Clinic of the University of Perugia, Italy, for routine prenatal screening, 216 RhD-negative women were consecutively enrolled between 2010 and 2013. Peripheral blood (5 ml) was drawn and collected into tubes containing EDTA as anticoagulant. After being informed of the purpose and experimental nature of the study, the women provided informed consent. The study was approved by the local ethics committee. The first blood sample was collected between weeks 10+0 and 14+6 of gestation, as calculated from the women's last menstruation and confirmed by ultrasound. 13 pregnant women agreed to repeat blood sampling in the 2nd trimester of gestation (between 18+0 and 25+6).

Methods

All blood samples were stored at +4 °C and treated within 4 h of collection. They were centrifuged at $1,600 \times g$ for 10 min, and the supernatant was collected in a 1.5 ml tube and centrifuged at $16,000 \times g$ for 10 min to pellet any remaining cellular debris. The plasma samples were divided into aliquots of 1,100 μ l and stored at -20 °C until use. Sample preparation and analysis were performed in a blinded fashion by all personnel involved in the study.

Genomic DNA from 1,000 μ l of maternal plasma was extracted by using the QIAmp DSP Virus kit (Qiagen, Hilden, Germany). After elution into 60 μ l sterile and DNase-free water 7.5 μ l were used as a template for the qPCR analysis. The manufacturer's instructions were modified as previously described [19].

qPCR analysis was performed using Real-Time PCR 7300 detection system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Extracted DNA was analyzed for exons 5 (*RHD5*) and 7 (*RHD7*) of the *RHD* gene to establish the fetal *RHD* genotype. The telomerase gene (*TERT*) was used as reference gene to confirm the presence and quality of total (fetal and maternal) DNA in each sample.

Primers and hydrolysis probes for target gene amplification were selected from among the most utilized and promising in the literature [2, 9, 20, 21]. Primers and hydrolysis probes for the reference gene were those that had previously been used [19].

Reactions were set up in a total volume of 25 μ l, using 12.5 μ l TaqMan Universal 2X PCR Master Mix (Applied Biosystems, Life Technologies), 7.5 μ l extracted DNA as well as optimized primers and probes (Applied Biosystems, Life Technologies). In particular, to detect the *RHD* gene, the reaction was performed in duplex with primers and probes at final concentrations of 900 and 100 nmol/l, respectively for *RHD5* and for *RHD7*. Primer and probe concentrations for *TERT* gene amplification were 300 and 100 nmol/l, respectively; the reaction was performed in singleplex. Cycling conditions for all reactions consisted of 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

Amplification products had a length of 127 bp for *RHD7*, 82 bp for *RHD5*, and 98 bp for the *TERT* gene.

Each sample was analyzed in triplicate for *RHD5* and *RHD7* sequences and in a single well for the *TERT* gene. Each DNA sample was tested for *RHD5*, *RHD7*, and *TERT* sequences on the same reaction plate. A calibration curve, using serial dilutions of the reference human genomic male DNA (Promega, Madison, WI, USA), was present in each PCR run to evaluate PCR efficiency from the slope.

During blood sampling, plasma preparation, DNA extraction and qPCR analysis, all precautions were adopted to avoid contaminations [19]. To verify potential contaminations, negative controls with DNA extracted from RhD-negative samples and no template control were included in each qPCR analysis. Positive controls, with DNA extracted from RhD-positive samples ascertained the acceptability of amplification reaction and the absence of PCR inhibitors.

Under these conditions two qPCR analyses were performed. In the 1st qPCR, plasma aliquots of all 216 samples which had been collected in the 1st trimester were analyzed to assess the diagnostic accuracy of the test. In the 2nd qPCR, a total of 108 samples were blindly re-analyzed. The results of the two analyses were combined considering each sample as tested in six replicates, 3 from 1st qPCR and 3 from the 2nd qPCR. Samples were randomly selected for the 2nd qPCR analysis: 95 plasma samples were selected as a second plasma aliquot was available from the first blood collection (1st trimester of pregnancy); 13 plasma samples were selected from the repeated blood samples that the women had agreed to provide at advanced gestational age.

After qPCR, fetal *RHD* genotype was established as positive or negative considering interpretation criteria of results based on the number of positive replicates [21]. A fetus was defined RhD-positive when both exons were positive. Otherwise the fetus was considered RhD-negative. For samples tested in one PCR assay, one single exon was defined as positive if 2/3 or 3/3 replicates generated amplification products; if no replicate or only 1/3 had a positive amplification, the exon assay was considered negative. For samples tested in two different PCR assays, an exon was defined as positive if at least 3/6 replicates generated amplification products; if no replicate or only 1/6 or 2/6 had a positive amplification, the exon assay was considered negative. A cycle threshold of <42 was interpreted as a positive signal. Figure 1 summarized our approach.

Statistical Analysis

Diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy of the test were determined on the basis of true- and false-positive/negative results.

ROC curves were elaborated in order to establish which analysis reached the best diagnostic accuracy, and their areas were compared with DeLong method [22]. All analyses were performed using MedCalc Software, Rel. 9.2.1.0 (Mariakerke, Belgium).

Results

Table 1 shows the clinical and demographic details of pregnant women at enrollment in the study.

Fetal *RHD* genotype determined by qPCR analysis was confirmed by postnatal serologic RhD typing. We obtained neonatal RhD blood group results of 193/216 enrolled pregnancies. Of these 193 pregnant women, 68 delivered a RhD-negative fetus and 125 a RhD-positive fetus.

Samples were considered to be from 1st trimester since 178/193 patients were at weeks 10–13 of gestation and 9 samples were at week 14 of gestation. Figure 2 reports the results of *RHD5* and *RHD7* amplifications (1st qPCR), in terms of number of positive replicates. Samples from pregnant women carrying RhD-negative fetuses gave mainly 0 (51% *RHD5* and *RHD7*) or 1 (34% *RHD5* and 38% *RHD7*) positive replicates. Samples from RhD-positive fetuses presented 3 (76% *RHD5* and 82% *RHD7*) positive replicates. Two

Fig. 1. A decision tree summarizing our approach to fetal *RHD* genotyping.

positive replicates occurred more frequently in RhD-positive samples (18% *RHD5* and 14% *RHD7*) than in RhD-negative samples (12% *RHD5* and 7% *RHD7*).

Table 2 summarizes the diagnostic performance of *RHD* testing: 178/193 samples were correctly classified, reaching a diagnostic accuracy of 93.3%.

To improve diagnostic accuracy of the test, we performed a 2nd qPCR analysis using two different strategies: 95/193 plasma samples (group 1) were re-analyzed using a second aliquot from the first blood collection (1st trimester of pregnancy); 13 new plasma samples (group 2) obtained from pregnant women who repeated blood sampling in the 2nd trimester of pregnancy (see 'Material and Methods') were analyzed.

Table 3 reports the results of 1st and 2nd qPCR in group 1 and group 2.

Group 1: 86/95 samples were correctly determined in the 1st qPCR analysis, reaching 90.5% diagnostic accuracy, with 2 false-positive (FP) and 7 false-negative (FN) results. Combining the results of the 2nd qPCR and the 1st qPCR (a total of 6 replicates for each group 1 sample) showed that 89/95 samples were correctly identified, and diagnostic accuracy increased to 93.7%; FP results decreased to 1 and FN results to 5. When the results of the 1st and 2nd qPCR were compared, a non-significant increase in diagnostic accuracy emerged from the difference of ROC curves areas (3.1%, 95% CI 0–8.4%, $p = 0.242$; AUC 91.3% with 95% CI 83.8–96.1% and AUC 94.5% with 95% CI 87.8–98.1%, respectively).

Fig. 2. *RHD5* and *RHD7* qPCR results expressed as number of positive replicates, obtained from the 193 samples analyzed at 1st qPCR analysis.

Table 1. Clinical data and demographics of pregnant women at enrollment in the study

Clinical data	RhD-negative fetus (n = 68)	RhD-positive fetus (n = 125)
Maternal age, median (range)	33 (22–44) years	32 (22–43) years
Gestational age, median (range)	12 (11–14) weeks	12 (10–14) weeks
First pregnancy	36	86
Physiological/complicated pregnancies		
Autoimmune diseases	6	3
Dysthyroidism	1	5
Hypertension	0	1
Ethnic origin		
Caucasian	66	122
South Asian	2	0
African	0	1
Mixed	0	2

Group 2: 11/13 samples were correctly determined at the 1st qPCR analysis reaching 84.6% of diagnostic accuracy, with no FP and 2 FN results. Combining the results of the 2nd qPCR and 1st qPCR (a total of 6 replicates for each group 2 sample) showed that 12/13 samples were correctly identified and diagnostic accuracy increased to 92.3% with no FP and 1 FN result. When the results of the 1st and 2nd qPCR of group 2 samples were compared, the increase in diagnostic accuracy was not significant as shown by the difference in ROC curves areas (8.3%, 95% CI 0–33.9%, $p = 0.522$; AUC 83.3% with 95% CI 53.1–97.2% and AUC 91.7% with 95% CI 63.1–98.7%, respectively).

These preliminary results suggest that increasing the amount of sample to be analyzed does not significantly improve the diagnostic accuracy of the test neither when it is repeated during the 1st

Table 2. Diagnostic performance of qPCR testing on 193 pregnant women during the 1st trimester of gestation

	%	95% CI	Number of results
Sensitivity	92.8	86.9–96.2	116 true-positive
Specificity	94.1	85.8–97.7	64 true-negative
FP results	5.9	2.3–14.2	4
FN results	7.2	3.8–13.4	9
PPV	96.7	93.5–99.9	
NPV	87.7	80.1–95.2	
Accuracy	93.3	88.8–96.0	

trimester of gestation nor when it is repeated during the 2nd. Lack of significance might have been due to the small number of samples that were available in both groups.

Table 3. Diagnostic performance of the test after the 2nd qPCR analysis performed on plasma samples of group 1 and group 2

	Group 1 (95 samples)				Group 2 (13 samples)			
	1st qPCR		2nd qPCR		1st qPCR		2nd qPCR	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
Sensitivity	88.9	78.8–94.5	92.1	82.7–96.6	66.7	30.0–90.3	83.3	43.7–97.0
Specificity	93.8	79.9–98.3	96.9	84.3–99.5	100.0	64.6–100	100	64.6–100
FP results	6.3	1.7–20.1	3.1	0.5–15.7	0	0–35.4	0	0–35.4
FN results	11.1	5.5–21.2	7.9	3.4–17.3	33.3	9.7–70.0	16.6	3.0–56.4
PPV	96.6	91.9–100	98.3	95.0–100	100.0	100–100	100	100–100
NPV	81.1	68.5–93.7	96.9	74.8–97.4	77.7	50.6–100	87.5	64.6–100
Accuracy	90.5	83.0–94.9	93.7	86.9–97.1	84.6	57.8–95.7	92.3	66.7–98.6
	Group 1 (95 samples), number of results				Group 2 (13 samples), number of results			
	1st qPCR		2nd qPCR		1st qPCR		2nd qPCR	
True positive	56		58		4		5	
True negative	30		31		7		7	
FP	2		1		0		0	
FN	7		5		2		1	

Discussion

In the present study, we describe our approach to non-invasive fetal *RHD* genotype testing by qPCR analysis of cfDNA that was extracted from maternal blood. The test was performed on 193 samples of peripheral maternal blood that was collected during 1st trimester of gestation. Test results by analysis of three qPCR replicates reached a high diagnostic accuracy of 93.3%.

The present study also evaluated whether the diagnostic accuracy of the test could be improved by increasing the amount of sample to be analyzed (2nd qPCR). For this purpose, 95 samples (group 1) containing cfDNA from plasma samples collected at the 1st trimester of gestation were analyzed by qPCR in six replicates (three replicates for each PCR run) while 13 samples (group 2) were analyzed by combining three qPCR replicates from plasma samples that had been collected during the 1st trimester and three replicates from 2nd trimester plasma samples. When the results of the 1st and 2nd qPCR of group 1 samples were compared, diagnostic accuracy increased from 90.5% to 93.7%. Diagnostic accuracy also increased from 84.6% to 92.3% when we compared the results of 1st and 2nd qPCR of group 2 samples. Therefore, analysis of larger sample (six qPCR replicates instead of three) seems to improve the diagnostic accuracy of the test slightly since it increases the probability of revealing even small quantities of cfDNA better, but this improvement is not significant.

The diagnostic accuracy of the test when performed on samples taken during the first trimester of gestation was comparable to other reports which analyzed samples that were collected up to the 3rd trimester [5, 9–11].

If we consider only studies which enrolled pregnant women at the 1st trimester of gestation [3, 12–15], diagnostic accuracy in fetal *RHD* genotyping ranged from 91.1% to 100%. Our results fall within this range.

Moreover, a meta-analysis of 37 publications and about 500 different methodological approaches for assessing fetal *RHD* genotyping in 3,261 samples from RhD-negative pregnant women showed a diagnostic accuracy of 94.8% [23].

Our results were reached through combination of multiple exons. This approach was also recommended by other authors [2, 24], who suggested including exons 4 or 5 and exons 7 or 10 so as to avoid wrong fetal *RHD* genotyping due to rare Rh blood group polymorphisms [25]. These exons depend on different mutations or DNA sequence exchanges between *RHD* and *RHCE*, the two homologous genes that are responsible for the RhD phenotype. For example, FP cases might be due to the hybrid *RHD-CE-D^S* that qPCR could wrongly reveal as the *RHD*-positive genotype, if exons 4–7 are not considered; nevertheless, it corresponds to a negative phenotype. In the Black population of African origin, the most common RhD-negative phenotype is due to the presence of the *RHD^ψ* pseudogene, which carries many mutations in exons 4, 5, and 6. If, for example, only exon 7 is considered, qPCR could incorrectly identify the sample as a RhD-positive phenotype [2, 26]. Other cases which may lead to FP results are due to the persistence of trophoblasts from a ‘vanishing twin’ or due to the placental chimerism [11]. However, a FP result is hardly critical since its effect would be administration of needless anti-D therapy to the mother just as if cfDNA-based technologies did not exist.

On the other hand, a FN finding would result in failure to give anti-D prophylaxis which would be a challengingly discordant outcome in terms of pregnancy management. A FN finding could be caused by rare *RHD* gene variants such as *DVI* and *DBT*, deriving from an exon 7 mutation, which correspond to a weak RhD-positive phenotype. It could also be linked to the absence of cfDNA or only a small quantity in the analyzed samples [2]. To overcome this problem and to improve the accuracy of fetal *RHD* testing, we decided to repeat qPCR analysis of different plasma aliquots from the same pregnant woman. We did not observe a significant improve-

ment in diagnostic accuracy when the test was repeated during the 1st trimester of gestation or when it was repeated during the 2nd trimester. Lack of significant improvement was probably due to the paucity of samples that were available for both analyses.

Our results are, therefore, very promising. They demonstrate the reliability of early routine prenatal *RHD* genotyping based on the detection of cffDNA in maternal blood during the 1st trimester of gestation. We are aware that the feasibility of reducing FN results needs to be evaluated in a future study. For this reason, the use of fDNA controls should be assessed, since they would identify samples without cffDNA or with cffDNA concentration that is too low.

Early fetal *RHD* genotyping could improve monitoring and treatment of all RhD pregnant women, particularly those who are alloimmunized to the D antigen. In fact, early knowledge of the fetal RHD status would avoid useless antenatal anti-D prophylaxis which is required in at risk situations such as amniocentesis, chorionic villus sampling, spontaneous or therapeutic miscarriage, or following prenatal bleeding. Antenatal anti-D prophylaxis would be reserved only for women carrying a RhD-positive fetus.

Consequently, early knowledge of the fetal *RHD* genotype could reduce health expenditure for an expensive drug that is in short supply worldwide. A pilot study evaluated the impact of this test on the cost of management of RhD-negative pregnant women and showed that the tests that are currently used in routine diagnosis across Europe cost between EUR 149.00 and 279.00 and do not bring any effective cost reduction [27]. Some authors suggested using low-cost accurate inhouse tests to reduce the final cost. However, not only financial but also medical concerns should, be taken into account. In fact, the use of a prenatal test for fetal *RHD* genotype assessment could reduce the anti-D donors that are exposed to blood products for hyperimmunization and production of anti-D.

Furthermore, RhD-negative pregnant women carrying a RhD-negative fetus would avoid unnecessary antenatal anti-D prophylaxis with its associated discomfort and risk of viral (hepatitis C) or prion (variant Creutzfeld-Jacob disease) contamination [28].

In conclusion, we believe that prenatal fetal *RHD* genotyping is reaching satisfactory goals. With only minor methodological adjustments to improve test performance, it could be introduced into routine clinical practice at the 1st trimester of gestation, also in Italy. We suggest the use of inhouse tests for prenatal fetal *RHD* genotyping, but we strongly recommend considering four focal points: i) repeated measurement of samples showing RHD-negative results, ii) use of internal controls such as exogenous DNA to verify the efficiency of DNA extraction and amplification steps [1], iii) use of reference material to monitor test performance compared with other laboratories [18], and use of fDNA controls, that could identify samples without cffDNA or with cffDNA concentrations being too small [29].

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Disclosure Statement

All authors declare that they have no conflicts of interest relevant to the present manuscript.

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