Reliable Determination of Fetal RhD Status by *RHD* Genotyping from Maternal Plasma

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Keywords

Noninvasive *RHD* typing · Fetal DNA · Maternal plasma

Summary

Background: Immunoprophylaxis with IgG anti-D is a standard prevention of hemolytic disease of the fetus and newborn. Fetal Rhesus D (RhD) blood group genotyping from maternal plasma of RhD-negative pregnant women allows targeted prophylaxis with IgG anti-D in RhD-positive pregnancies only. We set up a reliable protocol for prenatal *RHD* genotyping. **Methods:** 153 pregnant Caucasian RhD-negative women were tested in the 27th week (range 7–38th week) of pregnancy. 18 of them were alloimmunized to the RhD antigen. The fetal *RHD* genotype was determined based on an automated DNA extraction and real-time polymerase chain reaction method. Intron 4 and exons 5, 7 and 10 of the *RHD* gene and the *SRY* gene were targeted. **Results:** The fetal RhD status and gender was 100% correctly predicted in all 153 pregnancies (55 RhD-positive males, 45 RhD-positive females; 23 RhD-negative males, 30 RhD-negative females). **Conclusion:** The accuracy and applicability of our protocol for non-invasive fetal RhD determination allows the correct management of RhD-incompatible pregnancies. Our protocol could prevent unnecessary immunoprophylaxis in 53 of 153 cases. We therefore recommend that non-invasive fetal *RHD* genotyping is introduced as an obligatory part of prenatal screening.

Introduction

Rhesus D (RhD) blood group incompatibility between an RhD-negative mother and an RhD-positive fetus followed by alloimmunization and production of maternal anti-D antibodies is still the major reason for hemolytic disease of the fetus and newborn (HDFN) [1–3]. The combination of routine antenatal and postnatal anti-D prophylaxis has reduced the risk of RhD alloimmunization to 0.05–0.4% [4–6]. In Slovenia, postnatal prophylaxis was introduced in 1970 and antenatal immunoprophylaxis in the 28th week of pregnancy in 1994, which has reduced the rate of alloimmunization in RhD-negative mothers to 0.2% (unpublished data). In our population, approximately 81% of individuals are RhD-positive and 19% are RhD-negative. Hence, around 41% of RhD-negative pregnant women unnecessarily receive antenatal prophylaxis because they bear an RhD-negative fetus, which is similar to other European populations [7, 8]. After Lo et al. [9] in 1997 successfully isolated cell-free fetal DNA (cff-DNA) from the plasma of pregnant women, the non-invasive determination of the fetal *RHD* genotype from maternal plasma was introduced into many laboratories. The methodologies are based on a variety of quantitative real-time polymerase chain reaction (qPCR) protocols [10–12]. In order to resolve numerous technical issues regarding the *RHD* genotyping from maternal plasma, several studies and workshops have been performed, such as the International Workshop on Molecular Blood Group Genotyping (testing since 2004) and workshops under the auspices of the Special Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence [13–15]. However, so far, only Denmark and the Netherlands have introduced fetal *RHD* typing in RhD-negative pregnant women as a routine screening program [16, 17]. The results of the studies and workshops mentioned have brought about certain improvements such as: i)

Table 1. Large scale fetal *RHD* genotype screening studies

Ref.	Samples, n	Gestation, median, week	Tested <i>RHD</i> exons	Replicates, n	cff-DNA control	cf-DNA control	Sensitivity, %
[8]	1,257	30	7	3	none	none	99.6
[38]	1,869	28	5, 7	3	<i>SRY</i>	<i>CCR5</i>	99.7
[20]	1,113	25	5, 7	2	none	β -globin	99.7
[39]	545	17	4, 5, 10	2	<i>SRY</i>	<i>CCR5</i>	99.8
[16]	2,312	25	5, 7 or 7, 10 or 5, 10	3–4	none	<i>CCR5</i> or <i>GAPDH</i> or <i>SOD</i>	99.9
[30]	893	7–40	7, 10	2	none	none	99.5

CCR5 = C-C chemokine receptor type 5; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *SOD* = superoxidase dismutase.

improvement of the fetal DNA yield, ii) selection of appropriate *RHD* regions, and iii) inclusion of appropriate controls and standards.

Improvements in the Yield of Fetal DNA from Plasma

Plasma is a better source of cff-DNA compared to serum [18], and EDTA is a better choice of anticoagulant compared to heparin or citrate [19]. The recommended time of blood processing is within 48 h of venipuncture and not more than 5 days [20–22]. Plasma removed from whole blood by centrifugation must be centrifuged again at high speed or filtrated to achieve cell-free plasma [23]. Manual methods for the extraction of cff-DNA are time-consuming with the possibility of contamination, and prone to inconsistencies due to human handling. Therefore automated methods are preferable. There are some recommended manual methods such as the QIAamp DSP Virus Kit (Qiagen, Hilden, Germany) when compared to the QIAamp Blood Mini Kit (Qiagen) [15, 24] or the recently released commercial QIAamp Circulating Nucleic Acid Kit (Qiagen) [25]. The recommended automated methods for the extraction of cff-DNA are the MagnaPure LC System (Roche Diagnostics, Basel, Switzerland) and the Magnetic Separation Module 1 (Chemagen, Baesweiler, Germany) [26], which allow high-throughput isolation.

Selection of Appropriate *RHD* Regions for PCR

Complete deletion of the *RHD* gene is present in the majority of RhD-negative Caucasians [27]. The majority of the RhD-negative African population (66%) has a non-functional *RHD* gene, named *RHD* Ψ , and approximately 15% of RhD-negative Africans also possess the RhD-negative hybrid allele *RHD-CE-D** [28]. Different regions of *RHD* (exons 3, 4, 5, 7, 10 and intron 4) have been tested for the determination of the correct fetal RhD status [12, 29]. In the last 5 years, several large studies have been described for antenatal fetal *RHD* genotype screening (table 1). Similarly, the SAFE Network has prepared recommendations for routine screening assays: testing the *RHD* exon 5 (a region which discriminates between *RHD* and *RHD* Ψ) and exon 7 is recommended [15, 26].

Proper Controls and Standards

In order to prevent false-negative results in fetal *RHD* genotyping and to confirm the presence of fetal DNA in the tested blood sample of alloimmunized pregnant women, it is recommended to use cff-DNA controls such as the *sex determination region Y (SRY)* gene as a marker of male DNA, various biallelic insertion/deletion polymorphisms as potential markers of paternally inherited alleles, or the *RASSF1A* or *Maspin* gene, based on their different epigenetic modifications (methylation) in the fetal and maternal DNA [17, 30]. The lack of such controls can lead to false-negative results in 0.1–0.2% of cases [17]. Similarly, in order to prevent false-positive results, the use of total DNA isolation as control is useful in women carrying a variant *RHD* gene [17, 31]. Very suitably, the International Reference Reagent for the detection of *RHD* and *SRY* DNA in plasma can be used as a minimum sensitivity reagent and has been available to laboratories to determine the performance of their tests since 2010 [29]. When setting up our protocol for non-invasive fetal *RHD* genotyping, we gave the above-mentioned workshops on molecular blood group genotyping, SAFE recommendations, availability of the International Reference Reagent, and assistance from expert laboratories serious consideration [32]. Here we present our strategy for non-invasive fetal *RHD* genotyping as well as the results of the first cohort of our routine antenatal HDFN diagnostic testing performed in our laboratory.

Material and Methods

Maternal Samples and Plasma DNA Extraction

EDTA blood samples (10 ml) from 153 pregnant Caucasian RhD-negative women between 7 and 38 weeks of pregnancy were randomly obtained from the pool of routine prenatal screenings by the Prenatal Laboratory of the Blood Transfusion Centre of Slovenia. 18 (11.8%) women were alloimmunized to the RhD antigen and at risk for HDFN. ABO, RhD, and Kell blood group determination as well as the Indirect Coombs Test were performed using a standard gel technique (DiaMed, Cressier, Switzerland). Fetal gender and RhD status were confirmed after delivery. Blood samples were processed for DNA analysis as previously described [11] within 48 h. Plasma was stored at –20 °C until required. The study was approved by the Slovenian Ethics Committee. Informed consent was

Table 2. Primer and probe sequences and final concentrations in qPCR reactions for *RHD* intron 4, exon 5, exon 7, *SRY*, and *ALB*

Specificity	Sequence (5'→3')	Concentration in qPCR, nM	Ref.
<i>RHD</i> intron 4			in-house
Forward	GCCCTTCCATCATGATTCATTT	800	
Reverse	ACAAGGAAACAAAGGCCAAGAG	800	
MGB probe	FAM-AAGCACTTCACAGAGCA-MGB	300	
<i>RHD</i> exon 5			[11]
Forward	CGCCCTCTTCTTGTTGGATG	200	
Reverse	GAACACGGCATTCTTCCTTTC	200	
TaqMan probe	FAM-TCTGGCCAAGTTTCAACTCTGCTCTGCT-TAMRA	50	
<i>RHD</i> exon 7			[33]
Forward	GTAACCGAGTGCTGGGGATT	800	
Reverse	CTCCAAGCAGACCCAGCAA	800	
MGB probe	FAM-ACAGCTCCATCATGG-MGB	350	
<i>RHD</i> exon 10			[33]
Forward	TGCCTGCATTTGTACGTGAGA	800	
Reverse	CCTGCGCGAACATTGGA	800	
MGB probe	FAM-ACGCTCATGACAGCAA-MGB	300	
<i>SRY</i>			[33]
Forward	CGTGCATCCACCAGCAGTAA	600	
Reverse	TGGTTGCTAAGGACTGGATGAA	600	
MGB probe	FAM-TCCCCACAACCTC-MGB	200	
<i>ALB</i>			[33]
Forward	GCTGTCATCTCTTGTTGGGCTGT	400	
Reverse	ACAACAATGCCAGGGAGAGATTT	400	
MGB probe	FAM-ACTCTTAAGCCTAGACGAT-MGB	150	

MGB = Minor groove binder.

obtained from all tested pregnant women. Cell-free DNA (cf-DNA) was extracted from 400 µl of plasma using the Biorobot EZ1 workstation and the commercial kit EZ1 Virus Mini Kit v2.0 according to the manufacturer's protocol (Qiagen) [33]. Cf-DNA was eluted in 60 µl of elution buffer. All DNA isolations were made in duplicate in the same run, and eluted cf-DNA from the same woman was merged. In parallel, maternal mononuclear cells (MNC) were isolated and stored at -20 °C until required. DNA from the MNC was isolated with the commercial QIAamp Blood Mini Kit (Qiagen) according to the manufacturer's protocol.

Strategy of Non-Invasive Testing and qPCR

All isolated cf-DNA samples were tested for the presence of the *RHD* gene (intron 4 and exons 5, 7, 10), so complete deletion of *RHD*, presence of *RHD*ψ, and partial *RHD* (i.e. *RHD* category VI, *RHD-CE-D*⁺) could be detected. The Y-linked *SRY* gene was tested to confirm the presence of male fetal DNA, while the presence of the human serum albumin (*ALB*) gene as a reference gene was tested to confirm the presence of cf-DNA.

qPCR was performed with the ABI PRISM 7900HT (Life Technologies, Foster City, CA, USA) using 96-well plates and TaqMan Gene Expression Master Mix (Life Technologies). qPCR reaction conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Primers and probes for *RHD* intron 4, exon 7 and exon 10 as well as for the *SRY* and *ALB* genes were designed in our laboratory using Primer Express software (Life Technologies). Primers and probes for *RHD* exon 5 have been previously published [11]. Sequences and final concentrations of primers and probes (purchased from Life Technologies) in qPCR reactions are given in table 2. Isolated cf-DNA samples were analyzed in triplicate for *RHD* and *SRY*, while *ALB* was tested in duplicate. The volume of the tested cf-DNA was 5 µl per well. The final

volume of the qPCR reactions was 20 µl. The standard curve was performed in each qPCR run for *ALB* using the human RhD-positive male genomic DNA standard (Promega, Madison, WI, USA) ranging from 22.7 pg/µl to 0.0227 pg/µl.

qPCR reactions were analyzed using the Sequence Detector System 2.3 software (Life Technologies). Automatic baseline and threshold line functions were used for calculating the threshold cycle number (Ct) for all qPCR reactions. Amplification results were reported as Ct values. PCR reactions with Ct values for *RHD* and *SRY* genes lower than 42 cycles were considered as positive. A fetus was predicted as RhD-positive if at least 2 of 3 replicates of each tested *RHD* regions were positive. In the case of 1 positive out of 3 replicates or where there were discrepancies between the results of 4 tested *RHD* regions, the qPCR assay was repeated with newly isolated cf-DNA from the same maternal plasma sample. In the case that the fetus was predicted as RhD-negative, the presence of fetal DNA was considered as confirmed if at least 2 out of 3 replicates were positive for *SRY*.

If the qPCR results for *RHD* and *SRY* gene amplification were negative, we assumed that the fetus was an RhD-negative female, and additional tests were performed to ascertain that fetal DNA was present in the maternal plasma. DNA from the maternal MNC was tested for 8 biallelic insertion/deletion polymorphisms (S01a, S03, S04a, S05b, S06, S08b, S10a in S11a) as described before [34]. Those alleles absent from the maternal MNC-derived DNA served as a marker for the confirmation of fetal DNA presence.

The IBM SPSS Statistics 19 Multilingual-EQUIINOX system (IBM SPSS Statistics, Chicago, IL, USA) was used for descriptive statistical analysis. Sensitivity, specificity, and 95% confidence interval (CI) were calculated for *RHD* and *SRY* genotyping.

Table 3. Results of fetal RhD status and gender prediction from maternal plasma samples in 153 pregnancies

Gestation, weeks	n	Predicted RhD-positive, n (%)		Predicted RhD-negative, n (%)		Accuracy of <i>RHD</i> and <i>SRY</i> genotyping, %
		male	female	male	female	
≤ 14	5	1	2	0	2	100
15–28	141	50	40	23	28	100
29–42	7	4	3	0	0	100
Subtotal		55	45	23	30	
Total	153	100 (65.4)		53 (34.6)		100

Fig. 1. Proportions of predicted RhD-negative/positive male/female neonates in 153 pregnancies, and usefulness of employing the 8 insertion/deletion alleles for confirmation of cff-DNA fetal DNA in RhD-negative mothers with an RhD-negative female fetus.

Results

The RhD status of the fetus was predicted in 153 pregnancies. The median gestational age at the time of blood sampling was 27 weeks (range 7–38 weeks). Serological tests on the infant's red blood cells (RBCs) were performed, and fetal gender was confirmed after delivery. 1 woman was carrying twins; after delivery, both newborns were confirmed as RhD-positive females.

When compared to post-partum serological results, an accuracy rate of 100% was achieved in our prenatal prediction of fetal RhD status and gender determination from the maternal plasma (table 3). No false-negative or false-positive results were obtained. The positive predictive value (PPV) and negative predictive value (NPV) of the test were 100%. Overall, among 153 tested cases, 100 RhD-positive and 53 RhD-negative fetuses were found. Proportions of predicted RhD-negative/positive male/female neonates in 153 pregnancies are shown in figure 1. One sample was negative for *RHD* intron 4 and exon 5, and was determined as partial D, *RHD* category VI, as described before [35]. The genotype of the baby was confirmed after delivery from an umbilical blood sample using the RBC Ready Gene CDE PCR-SSP commercial kit (Inno-Train Diagnostik GmbH, Kronberg, Germany). As expected, no *RHD*Ψ was found in any of the tested samples.

RHD genotyping exhibited 100% sensitivity and specificity (95% CI 95.3–100%; 91.2–100%). *SRY* genotyping exhibited 100% sensitivity and specificity as well (94.2% CI 53.3–100%; 93.9–100%). Among the 153 cases, we predicted the fetal RhD status and gender in 144 samples from the first qPCR testing. In the remaining 9 cases, qPCR had to be repeated from the same samples, leading to an accurate prediction of fetal RhD status and gender (table 3).

The standard curves for the quantitation of cf-DNA and cff-DNA for all tested genes were linear in the range of over 4 log units, indicating a wide dynamic range and high reliability. The calculated median values and ranges of detected genomic equivalent/ml (GE/ml) of cf-DNA and cff-DNA in maternal plasma are presented in table 4.

Confirmation of cff-DNA Presence in the Case of Negative qPCR Results for *RHD* and *SRY*

In 19 of the 30 samples in which qPCR reactions were negative for *RHD* and *SRY*, implying that the fetuses were RhD-negative females, additional DNA from maternal MNCs was isolated and tested for 8 insertion/deletion alleles by qPCR in order to confirm the presence of fetal DNA. In the remaining 11 cases, this was not done because the samples were no longer available. The alleles, absent from the maternal genomic DNA, were then tested for their presence in the freshly isolated cf-DNA from the maternal plasma. In 14 out of 19

Table 4. Comparison of qPCR efficiencies, linear correlations (R^2) for the human RhD-positive male genomic DNA standard, and median values and ranges of detected genomic equivalent (GE)/ml (GE = 6.6 pg human DNA) of cf-DNA and cff-DNA in maternal plasma for the tested genes

Gene	qPCR efficiency, %	R^2	Ct value, median (range)	Detected cf-DNA and cff-DNA in maternal plasma, median (range), GE/ml
<i>ALB</i>	84.36	0.96	34.17 (31.92–34.77)	2,611 (522–357,051)
<i>RHD</i> intron 4	83.78	0.94	38.20 (34.25–41.34)	78 (11–858)
<i>RHD</i> exon 5	77.50	0.99	37.51 (33.70–41.93)	214 (17–1,901)
<i>RHD</i> exon 7	84.40	0.94	36.65 (33.05–41.08)	201 (13–1,822)
<i>RHD</i> exon 10	92.12	0.95	38.24 (33.14–41.36)	147 (19–4,120)
<i>SRY</i>	74.61	0.98	37.57 (32.58–41.91)	239 (21–3,851)

samples (74%), we confirmed the presence of cff-DNA, i.e. the alleles only possessed by the fetus. The approach was not informative in the remaining 5 cases (26%). The usefulness of employing the 8 insertion/deletion alleles for confirmation of cff-DNA in RhD-negative mothers with an RhD-negative female fetus is shown in figure 1. In all 30 cases in which the qPCR results were negative for *RHD* and *SRY*, thus predicting an RhD-negative female child, the newborn was indeed an RhD-negative female.

Discussion

In the last decade, non-invasive fetal *RHD* typing has been introduced in many countries [12]. This is extremely important in alloimmunized RhD-negative women for the correct management of their pregnancies. Furthermore, based on this method, unnecessary antenatal immunoprophylaxis with IgG anti-D can be avoided in approximately 40% of all RhD-negative pregnant women [8, 10, 20, 30, 31].

Before non-invasive fetal *RHD* genotyping was finally offered as a routine diagnostic test in 2011 by our laboratory of the Blood Transfusion Center of Slovenia, we had to adapt several steps in the protocol according to various recommendations in order to obtain optimal results. The following steps were taken into account: i) Manual isolation protocol of cf-DNA from 800 µl of maternal plasma using the commercial QIAamp DNA Blood Mini kit which was compared to automated isolation of cf-DNA from 400 µl of maternal plasma using the Biorobot EZ1 workstation and the commercial EZ1 Virus Mini Kit v2. This comparison showed that automated extraction improved the yields of cf-DNA, so we consider it as a reliable method of cff-DNA isolation from maternal plasma for non-invasive fetal *RHD* genotyping (data not published); ii) In order to assess the quality of our approaches, we have participated in the International Workshop on Molecular Blood Group Genotyping since 2004 [13, 14, 36, 37], as well as in the evaluation of the International Reference Reagent for the detection of *RHD* and *SRY* DNA in plasma performed by The National Institute for Biological Standards and Control (NIBSC) from UK plasma [29]; iii) Similarly, we have followed the recommendations for routine screening of the

SAFE Network [15, 26] and have included a reaction for *RHD* exon 5 which distinguishes between *RHD* and *RHD* Ψ in our previously used qPCR protocol; iv) All samples were also tested for the presence of *RHD* intron 4 in order to confirm the presence of *RHD* category VI. This approach is suitable for discriminating between the presence of *RHD* category VI and *RHD* Ψ .

After validation of the protocol, our non-invasive fetal *RHD* genotyping was used for the prediction of fetal RhD status in 153 Caucasian RhD-negative pregnant women in the 27th week (range 7–38th week) of pregnancy, among whom 18 were alloimmunized to the RhD antigen. All samples were tested for the presence of *RHD* intron 4 and exons 5, 7, 10, as well as for *SRY* as a control for the presence of male fetal DNA. *ALB* was used to confirm the presence of cf-DNA.

In our cohort, the fetal RhD status and gender were correctly predicted in all 153 pregnancies (55 RhD-positive males, 45 RhD-positive females; 23 RhD-negative males, 30 RhD-negative females). In the case of negative *RHD* and *SRY* qPCR reactions, an additional 8 insertion/deletion polymorphisms were tested. Using this approach, we were able to confirm the presence of cff-DNA in 14 of 19 tested samples. Overall, we were unable to confirm the presence of cff-DNA in only 16 (10%) of the 153 samples. Testing of insertion/deletion polymorphisms is essential in cases of alloimmunized pregnant women in order to avoid false-negative results followed by incorrect management of the pregnancy. In all 30 pregnancies where the fetus was predicted as an RhD-negative female, RhD-negative females were born. Finally, our protocol had an accuracy rate of 100% in the prediction of fetal RhD status. No false-positive or false-negative results were obtained.

Compared to other large scale screening studies (table 1), our protocol is also reliable for the prediction of fetal RhD in alloimmunized RhD-negative pregnant women. The confirmation of cff-DNA was not done in most of the previously mentioned studies [8, 16, 20, 30]. The weakness of our study is the low number of tested samples, which could be the reason for the 100% sensitivity and specificity. A further prospective study with a large number of samples will be performed in the future to confirm the reliability of this protocol.

Conclusion

We have developed a protocol for non-invasive fetal *RHD* determination from the maternal plasma, which accurately predicts the RhD status of the fetus and enables the correct management of immunoprophylaxis in RhD-incompatible pregnancies. In addition, it allows an appropriate management of pregnancies in RhD-negative women who are alloimmunized to the RhD antigen. In our first cohort of 153 RhD-negative pregnant women, our protocol could theoretically prevent unnecessary immunoprophylaxis in 53 cases. We therefore recommend that non-invasive fetal *RHD* genotyping is introduced as an obligatory part of prenatal screening.

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Disclosure Statement

The authors declare no conflict of interest.

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