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

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Introduction

Rhesus D (RhD) blood group incompatibility between an RhD-negative mother and an RhD-positive fetus followed by allosensitization 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in the 28th week of pregnancy in 1994, which has reduced the rate of alloimmunization in RhD-negative mothers to 0.2% (unpublished data).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)
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 ofcff-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 ofcff-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 ofcff-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 RHD4, 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 RHD4) 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 usecff-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
nuclear cells (MNC) were isolated and stored at –20 °C until required. Cf-DNA from the same woman was merged. In parallel, maternal mono-

All DNA isolations were made in duplicate in the same run, and eluted er's protocol (Qiagen) [33]. Cf-DNA was eluted in 60 μl of elution buffer. the commercial kit EZ1 Virus Mini Kit v2.0 according to the manufactur-

extracted from 400 μl of plasma using the Biorobot EZ1 workstation and 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.

Strategy of Non-Invasive Testing and qPCR

All isolated cf-DNA samples were tested for the presence of the RHD (intron 4 and exons 5, 7, 10), so complete deletion of RHD, presence of RHDΨ, 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 bial-lelic 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-EQUiNOX 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 2. Primer and probe sequences and final concentrations in qPCR reactions for RHD intron 4, exon 5, exon 7, SRY, and ALB

<table>
<thead>
<tr>
<th>Gene</th>
<th>Intron/Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>MGB Probe</th>
<th>Concentration in qPCR, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHD</td>
<td>intron 4</td>
<td>GCCCTTTCCATCATGATTCATT</td>
<td>ACAAGGAAACAAAGGCCAAGAG</td>
<td>FAM-AAGCAGCTTCAGAGA-MGB</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>exon 5</td>
<td>CGCCCTCTCTTGTGGGATG</td>
<td>GAACAGGCGATCTCCTTTC</td>
<td>FAM-TCTGGCAAGTTTCAACTGCTGCT-TAMRA</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>exon 7</td>
<td>GTAAACCGAGTGTGGGGATT</td>
<td>CTCAACAGCGACCCCGCAA</td>
<td>FAM-ACAGCTCATGACAGCAA-MGB</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>exon 10</td>
<td>TGCCCTGATTTGACTGGA</td>
<td>CCGCGGCAACATTGGA</td>
<td>FAM-ACGCTCATGAGACAGCAA-MGB</td>
<td>300</td>
</tr>
<tr>
<td>SRY</td>
<td></td>
<td>CGTGCACTCCACCGACGTA</td>
<td>TGGTGTCCTAAGGACCTG {TA}</td>
<td>FAM-TCCCCCAACAACCT-MGB</td>
<td>200</td>
</tr>
<tr>
<td>ALB</td>
<td></td>
<td>GCTGTCATCTTCTGTGGGCTGT</td>
<td>ACAACATTGCGGACAGATT</td>
<td>FAM-ACCTTAAAGCTTAGACGAT-MGB</td>
<td>150</td>
</tr>
</tbody>
</table>

MGB = Minor groove binder.
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 were 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 RHDΨ 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 3. Results of fetal RhD status and gender prediction from maternal plasma samples in 153 pregnancies

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

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.
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 cf-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 RHDΨ 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 RHDΨ.

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 cf-DNA in 14 of 19 tested samples. Overall, we were unable to confirm the presence of cf-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 cf-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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>qPCR efficiency, %</th>
<th>R²</th>
<th>Ct value, median (range)</th>
<th>Detected cf-DNA and cff-DNA in maternal plasma, median (range), GE/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB</td>
<td>84.36</td>
<td>0.96</td>
<td>34.17 (31.92–34.77)</td>
<td>2,611 (522–357,051)</td>
</tr>
<tr>
<td>RHD intron 4</td>
<td>83.78</td>
<td>0.94</td>
<td>38.20 (34.25–41.34)</td>
<td>78 (11–858)</td>
</tr>
<tr>
<td>RHD exon 5</td>
<td>77.50</td>
<td>0.99</td>
<td>37.51 (33.70–41.93)</td>
<td>214 (17–1,901)</td>
</tr>
<tr>
<td>RHD exon 7</td>
<td>84.40</td>
<td>0.94</td>
<td>36.65 (33.05–41.08)</td>
<td>201 (13–1,822)</td>
</tr>
<tr>
<td>RHD exon 10</td>
<td>92.12</td>
<td>0.95</td>
<td>38.24 (33.14–41.36)</td>
<td>147 (19–4,120)</td>
</tr>
<tr>
<td>SRY</td>
<td>74.61</td>
<td>0.98</td>
<td>37.57 (32.84–41.91)</td>
<td>239 (21–3,851)</td>
</tr>
</tbody>
</table>
Conclusion

We have developed a protocol for non-invasive fetal RHD determination from 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.

Acknowledgement

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Disclosure Statement

The authors declare no conflict of interest.

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