

Usefulness of Non-Invasive Fetal *RHD* Genotyping towards Immunoprophylaxis Optimization

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Keywords

Non-invasive prenatal diagnosis · Molecular blood group typing · Fetal RhD genotyping · Antenatal immunoprophylaxis · Hemolytic disease of the newborn

Summary

Introduction: Since anti-D immunoprophylaxis given to D-negative pregnant women is a blood product, blood donations have an impact on the availability of prophylactic doses. The Pan American Health Organization reported, in June 2017, that less than half of blood donors are volunteers in Latin America and the Caribbean. In these countries, guidelines for use of anti-D prophylaxis are still controversial. The aim of this study was to demonstrate the convenience of a simple and cost-effective non-invasive prenatal diagnostic assay for anti-D prophylaxis optimization in multiethnic populations.

Methods: Cell-free fetal DNA from plasma samples of D-negative pregnant women were analyzed by real-time PCR for simultaneous amplification of sequences of exons 5 and 10 of the *RHD* gene. Fetal *RHD* genotype was determined in 111 pregnant women. Neonates' phenotype was determined 72 h after birth. **Results:** Genotyping predicted fetal phenotype with 100% accuracy. Prenatal diagnosis showed 78% RHD-positive and 22% RHD-negative neonates. **Conclusion:** We demonstrated that, beyond the large genetic variation of the Rh system and the numerous D variants present in multiethnic groups, non-invasive fetal *RHD* genotyping using two sequences of the gene can be enough for clinical application in an admixed population. This robust technique of simple implementation allows to determine fetal RHD in

maternal blood with high sensitivity, specificity, and accuracy. The introduction of fetal *RhD* genotyping as part of an antenatal screening program constitutes a reliable manner to optimize anti-D prophylaxis; however, it has not been implemented so far in most American countries.

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Introduction

The introduction of postnatal prophylactic treatment with RH immunoglobulin (IgRH) in the 1960s allowed to reduce the prevalence of hemolytic diseases in fetuses and newborns (HDFNs) due to incompatibility in the RH system from 16 to 2% [1, 2]. The implementation of antenatal prophylaxis at weeks 28–34 of gestation in the 1970s, in addition to postnatal prophylaxis, reduced such values to 0.1–0.3% [3, 4].

Anti-D immunoglobulin is a limited biological product that cannot be manufactured; it is obtained from apheresis donations from voluntary donors who have high titers of anti-D antibodies. This situation has an impact on the availability of IgRH doses, which are frequently scarce. In many countries, it is necessary to import IgRH because it is not produced locally and in other countries such as Argentina, still having blood product companies, not only the product but also the raw material have to be imported for fractionation due to shortage of donors and the lack of implementation of national programs for obtaining plasma with high anti-D titers [5, 6]. For this reason, guidelines for the use of IgRH prophylaxis are still a controversial issue in some countries, especially if volunteer blood donors are not enough to guarantee sufficient and safe blood supplies to manufacture blood products. Since this is a

current matter in Latin America and the Caribbean, there is no uniformity of criteria regarding the indication of prophylaxis and management of D-negative pregnant women. In Argentina, some local public health authorities do not adhere to the national recommendations that suggest to administer prenatal prophylaxis between weeks 28 and 32 of gestation and only provide IgRH to D-negative pregnant women at risk of fetal-obstetric hemorrhage and to D-negative women after the birth of a D-positive child.

Another issue that should be taken into consideration in terms of the rational use of IgRH is that since it is a biological product prepared from pooled plasma of immunized donors, it involves all the risks associated with human blood products, including transmission of different infections. There are several antecedents in this respect. Kenny-Walsh et al. [7] found batches of IgRH used in Ireland between 1977 and 1978 that were contaminated with hepatitis C virus (HCV) and detected this virus in a group of women that had received IgRH. Smith et al. [8] investigated the infectivity for HCV of IgRH batches prepared in Ireland between 1991 and 1994 and detected the virus in 44 women who had received IgRH. In addition, in Germany, hepatitis G virus (GBV-C) sequences were detected in batches of anti-D immunoglobulin contaminated with HCV, responsible for a HCV outbreak in 1979 [9]. Even though technology has evolved significantly in recent decades, and plasma manufacturing plants have pursued strategies to reduce the risk of transfusion-transmitted infections, a residual risk still persist, and emerging diseases are a constant challenge in transfusion practice.

Since the molecular basis of the gene encoding D antigen was recognized, techniques for prenatal *RHD* genotyping have been applied for the management of D-negative pregnant women previously sensitized or at risk of immunization. The accurate prediction of fetal D status allows to administrate prenatal prophylaxis to D-negative women with D-positive fetuses and avoids unnecessary administration in cases of D-negative fetuses, optimizing the available resources. Thus, the assessment of the risk of HDFN by determining the *RHD* genotype from the cell-free fetal DNA (cffDNA) in maternal blood implicates an improvement in IgRH prophylaxis, only comparable to that of the introduction of the prophylaxis itself at that time. Therefore, non-invasive genotyping of fetal *RHD* status by analyzing cffDNA in maternal plasma has already been incorporated into routine clinical practice of many countries, causing a great impact on management protocols of D-negative pregnant women [10]. In Belgium, since 2002, fetal *RHD* genotyping has been used during the follow-up of D-negative pregnant women for an accurate indication of prophylaxis, and, in parallel, prevention policies have been implemented, allowing to avoid IgRH injection in 39% of the women who carry D-negative fetuses [11]. In Denmark, a national routine antenatal anti-D prophylaxis program implemented in 2010 guaranteed that administration of IgRH is based on the results of antenatal screening of fetal *RHD* gene and allows to avoid unnecessary use of prophylaxis in 37.3% of D-negative pregnant women [2, 12]. In the Netherlands, since July 1, 2011, both antenatal and postnatal prophylaxis are administered only to women in whom fetal *RHD* genotyping at gestational

week 27 predicts a D-positive fetus [13, 14]. As additional screening methods to guide IgRH prophylaxis, fetal *RHD* genotyping has also been implemented regionally in France, England, Finland, and Sweden [15, 16] and will probably be used for diagnosis by many other countries in the future [17].

Currently, in Argentina, like in other Latin American countries, there are no consensus guidelines for the management of D-negative pregnant women that include non-invasive genotyping of fetal *RHD*. Their availability would make a great impact on regional public health policies both in the management of D-negative pregnant women and with regard to the optimization of the scarce resources available for prophylaxis. In other countries, it is well known that between 20 and 40% of D-negative pregnant women carry D-negative fetuses [11], and therefore in these cases prophylaxis is not necessary.

Recently, a protocol for non-invasive prenatal *RHD* genotyping in an Argentinean admixed population has been published [18]. We agree with Clausen [19] when he comments, in reference to the latest research that it 'is a very good example of how an admixed population was first studied comprehensively and then the results of the population study provided the basis on which an algorithm was designed' ([19] p. 5). However, we also agree with the idea that 'it is possible that the overall algorithm may be simplified to become more suitable for routine analysis when implemented into clinical practice' ([19] p. 5). Herein, we report our experience on the use of a molecular assay for *RHD* genotyping in the screening of fetal *RHD* in another very mixed population of pregnant women in Argentina using two exons. We demonstrated a good performance of this simple protocol for clinical application to optimize immunoprophylaxis.

Material and Methods

This research was designed following the principles established in the Declaration of Helsinki of 1975 and revised in 2013. It was approved by the Training and Teaching Committee of the Fundación Banco Central de Sangre and by the Ethic Committee on Human Research of the Hospital Materno Provincial Dr. Raúl Felipe Lucini in accordance with local and national regulations.

Sample Preparation

Peripheral venous blood was obtained from D-negative pregnant women. Plasma was separated from the cellular component within 72 h of sampling. Initially, the blood was centrifuged at $1,600 \times g$ for 10 min, and the maternal plasma fraction was separated from the red blood cells (being careful not to contaminate the sample with maternal cells). The plasma was then centrifuged at $11,000 \times g$ for 10 min to ensure that all remaining cells were removed. Finally, the supernatant was transferred to 1.5-ml tubes. Plasma samples were stored at -20°C to -30°C until used.

cffDNA Extraction

cffDNA extraction was performed with the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, with some modifications. The initial volume of plasma used was 800 μl , and the extracted nucleic acid was eluted in 50 μl of elution buffer.

Real-Time Polymerase Chain Reaction

To determine the presence of fetal *RHD* gene in cffDNA, we performed real-time polymerase chain reaction (PCR) using TaqMan fluorogenic probes and

ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) for simultaneous amplification of sequences of exons 5 and 10 of the *RHD* gene. Amplification and detection of exons 5 and 10 were carried out using primers Exo5-F (5'-CGCCCTCTTCTGTGGATG-3'), Exo5-R (5'-GAACACG-GCATTTCTCCTTC-3'), Exo10-F (5'-TGCCTGCATTGTACGTGAGA-3') and Exo10-R (5'-AGTGCCTGCGCAACATT-3') to a final concentration of 300 nmol/l and probes Exo5 (VIC-TCTGGCCAAGTTTCAACTCTGCTCT-GCT-TAMRA) and Exo10 (FAM-CATGACAGCAAAGTC-MGB) at a final concentration of 200 nmol/l, respectively. Primers and probe for *RHD* exon 5 testing are selected according to Finning et al. [20]. Primers and probes for *RHD* exon 10 as well as for *SRY* testing were kindly provided by Nuria Nogues, of the Immunohematology Laboratory, BST, Barcelona, Spain.

To confirm the presence of cfDNA, a sequence of the *Homo sapiens* sex determining region Y (*SRY*, GenBank Accession number NC_000024.10, region 2787803...2787865) was amplified and detected using primers *SRY*-F (5'-GAGCAGTCAGGGAGGCAGAT-3') and *SRY*-R (5'-GCAAAACATGG-TAATTCAGTAACGTT-3') at a final concentration of 300 nmol/l and the *SRY* probe (FAM-AGCAGGGCAAGTAGT-MGB) at 200 nmol/l. The reactions were performed in a final volume of 20 µl using Taqman Universal PCR Master Mix (Applied Biosystems). After initial incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min and 45 2-step cycles of 95 °C for 15 s and 60 °C for 1 min was performed. Each sample was assayed in duplicate in a uniplex reaction for exon 10 and in triplicate in a multiplex reaction for the exon 5 sequence of the *RHD* gene and the Y chromosome sequence. The last sequence was limited to male fetuses as a control to detect cfDNA. This approach is used since 2004 at the Immunohematology Laboratory, Banc de Sang i Teixits, Barcelona, Spain, for prenatal *RHD* genotyping in Catalonia.

Samples Included in the Study

For implementation and regional validation of the molecular technique, a 10-month prospective study was carried out. Samples from 120 D-negative pregnant women who attended the Department of Hemotherapy and Immunohematology of the Hospital Materno-Provincial Dr. Raúl Felipe Lucini, Córdoba, Argentina, were obtained. Gestational age at time of blood collection ranged between 16 and 42 weeks. The number of plasma samples of pregnant women necessary and sufficient to validate the sensitivity and specificity of the method was defined based on the average number of D-negative pregnant women who attended the mentioned department in a period of 12 months prior to the beginning of the study, in addition to the frequency of D-negative and D-positive infants born from that group during the same period. The determination of fetal *RHD* genotype was performed in samples collected during prenatal routine controls. Neonates' phenotype was determined in a blood sample from heel prick, 72 h after birth.

Interpretation of the Results

The fetuses were considered D-positive (detection of fetal *RHD* gene) when the two replicates of exon 10 and at least two of the three triplicates of exon 5 were positive. D-negative fetuses (negative detection of *RHD* gene) were defined when none of the replicates of exons 5 and 10 amplified. In case of male fetuses, at least two of the triplicates of the *SRY* exons had to turn out positive to confirm the presence of fetal DNA.

Due to the fact that in the presence of *RHD* pseudogene, *RHD-CE-D* hybrid or some D variants (DVI type III or DBT), exon 5 does not amplify [17], in cases of positive amplification of both replicates of exon 10 under absence of amplification of exon 5 the samples should be classified as inconclusive. In such cases, additional studies with other techniques are required to elucidate diagnosis.

When both exons amplified but there was a considerable difference (≥ 4 cycles) between the threshold cycles (Ct) of the replicates of exon 10 with respect to exon 5, the fetus was considered D-positive as well. Even though in these cases complementary assays are required to characterize possible *RHD* variants, the unequivocal diagnosis of D-positive fetus makes this unnecessary in the context of clinical management of D-negative pregnant women. In case of discordance in the analysis of the results (discordance in amplification of duplicates of exon 10 or non-concordance in amplification of the two exons), the re-

sult was considered inconclusive, and a new sample was requested. When it was not possible to obtain a new sample, the result was defined as inconclusive and excluded from the final analysis.

Neonatal Phenotype

The results obtained in maternal plasma were compared to the newborns' phenotype using a blood sample from heel prick obtained within 72 h after birth. Detection of D antigen in neonates was performed by Grifols DG Gel Newborn (Diagnostic Grifols S.A., Barcelona, Spain). In pregnant women who discontinued routine controls or delivered in a different institution, it was not possible to match maternal results with the offspring phenotype; for this reason these data were not included in the final analysis.

Analytical Evaluation

The analytical performance of the assay was evaluated by accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) according to Agresti [21]. Confidence intervals (CI) were estimated according to Haldane [22] and Anscombe [23] correction.

Results

Real-time PCR was implemented and applied in plasma samples of 120 pregnant women for fetal *RHD* genotyping and detection of *SRY* sequence. Nine of the 120 samples studied presented non-conclusive results and were excluded from final analysis. Among these, 4/120 (3.33%) corresponded to women who did not give birth at the Hospital Materno Provincial; therefore, the phenotype of these newborns could not be determined. In another 4/120 (3.33%) cases, samples had pre-analytical problems, and it was not possible to obtain new samples before delivery. The remaining inconclusive case, 1/120 (0.8%), presented repeated reactive results for the amplification of exon 10 (Ct 35) but negative detection of exon 5. It was not possible to obtain a new sample of this patient because she did not attend to subsequent medical consultations neither before nor after delivery.

In the remaining 111 samples, the concordance between the obtained genotype and the fetal phenotype could be compared and analyzed. The prediction of fetal phenotype, both for *RHD* factor and fetal sex was accurate in all samples, showing 87 (78%) *RHD*-positive neonates and 24 (22%) *RHD*-negative ones; 57 (51%) were males and 54 (49%) females.

The analytical performance of the assay to determine *RHD* status was as follows: sensitivity 100% (95% CI 98.85–100%), specificity 100% (95% CI 95.83–100%), and accuracy 100%. The PPV for *RHD* status was 100% (95% CI 98.85–100%). The accuracy of the assay for detection of *SRY* sequence was 100%, sensitivity and specificity was 100% (95% CI 98.148–100%) and 100% (95% CI 98.246–100%), respectively. The PPV to detect the *SRY* sequence of Y chromosome in male fetuses was 100% (95% CI 98.148–100%). 53% of the 120 D-negative pregnant women included in the study were in the third trimester of pregnancy while 47% were in the second trimester. All pregnant women were born in Argentina and had a multiethnic descent profile: Asian 2.5%, European 34%, Argentine 42.5%, Native population 8% and unknown 13%.

Of the total number of pregnant women, 36 (30%) were pregnant for the first time, and 84 (70%) had been pregnant before. In

the group with previous pregnancies, 22 (26%) had had only 1 previous pregnancy, 28 (33.2%) 2 gestations, 14 (17%) 3, 10 (12%) 4, 6 (7%) 5, 1 (1.2%) 6, 1 (1.2%) 9, and 2 (2.4%) 10 gestations. 35 (42%) had previous history of spontaneous abortions; 24 (69%) of them reported one previous abortion, 7 (20%) two, and 4 (11%) three miscarriages, while 8 pregnant women (9.5%) referred history of a newborn died at birth and 5 (6%) had had a newborn with different clinical symptoms (from cholestasis to mental retardation and heart failure). Among all D-negative pregnant women carrying *RHD*-negative fetuses, 10 were pregnant for the first time and 14 reported history of previous pregnancies.

Among the 111 pregnant women with the defined fetal *RHD* genotype, 2 showed a significant difference between Ct amplification in exon 10 (Ct 32) and exon 5 (Ct 41). In these two cases, the fetuses were considered D-positive. Even though complementary studies would be necessary to assess the possibility that a gene is not being expressed in the mothers, the unequivocal diagnosis of fetuses as D-positive is enough to guide prenatal prophylaxis. Thus, complementary assays for *RHD* variants were not necessary since it exceeded the objective of this study.

Discussion

We demonstrated that noninvasive fetal *RhD* genotyping using two sequences of the gene can be enough for clinical application in an admixed population. Thereby, a simple and cost-effective prenatal diagnostic technique can be used to detect and restrict IgRH use to women carrying D-positive children. Fetal *RhD* testing can also improve the use of postnatal IgRH.

In this study, a simple protocol was applied using exons 5 and 10, and the results showed that this robust technique of simple implementation allows to determine fetal *RHD* in maternal blood with high sensitivity, specificity, accuracy, and precision. This technique follows the consensus recommendation to include exon 5 [12, 17, 20, 24], which, in combination with exon 10, helps to identify *RHD*-positive / D-positive subjects and to distinguish of *RHD*-positive / D-negative cases, mostly due to presence of the *RHD* pseudogen (*RHD* ψ) or cases of hybrid *RHD*-*CE*-*D*^s. Thus, this combination avoids false-positive results and also identifies some *RHD* variants [15].

For the purposes of clinical management of D-negative pregnant women and decision-making regarding prophylaxis, fetal genotyping should be able to predict fetal phenotype with high accuracy. This study demonstrates that in a multiethnic population the use of exons 5 and 10 allows to appropriately differentiate *RHD*-positive / D-positive individuals from *RHD*-positive / D-negative ones. Thus, management of D-negative pregnancies and target antenatal prophylaxis could be successfully achieved.

Even though *RHD* ψ or *RHD*-*CE*-*D*^s in *RHD*-positive / D-negative individuals could not be differentiated with the study of exons 5 and 10 and required complementary tests, these variants of the *RHD* genotype have no impact in clinical routine since only the expressed phenotype is relevant to clinical management of these patients.

The usefulness of the analysis of these two exons in a heterogeneous population to identify the fetal phenotype has been corroborated by a recent study of Trucco Boggione et al. [18], in which exons 4 and 7 were also studied. In spite of the inclusion of more than two exons in the suggested algorithm, it was necessary to carry out complementary studies to characterize *RHD*-positive / D-negative individuals. In the Argentinean population, approximately 2% of D-negative individuals harbor an *RHD* allele [25, 26]. This information has been recently corroborated in a study by Trucco Boggione et al. [18] and in our results, supporting that the assessment of noninvasive prenatal *RHD* using exon 5 and 10 is capable to appropriately predict fetal phenotype and guide target antenatal prophylaxis. For this reason, we propose that this technique should be routinely implemented as alternative diagnostic method for prenatal *RHD* typing in the management of D-negative pregnancies due to its high accuracy and cost-effectiveness, in order to reduce unnecessary administration of antenatal prophylaxis.

In many countries, IgRH is an expensive and limited resource, mainly because it is a biological product obtained from plasma pools of donors with high anti-D titers. According to a report released by the Pan American Health Organization in June 2017 [27], less than half of blood donors are volunteers in Latin America and the Caribbean. The report also reveals that in Latin America only 33.4% of the volunteers donate blood or blood components at least twice a year, and in Mexico only 2.7%. In these countries, including Argentina, where altruistic repeated donations are the exception and not the rule and where there are no public policies for implementation of programs to obtain plasma with high anti-D titers, the raw material for IgRH production is very scarce [5, 6]. This is the main limitation for IgRH availability, and therefore the administration of prenatal IgRH has been suspended in some cases and in different regions. Thus, in these countries, it is currently necessary to change the cultural habits of reposition and single donation motivated by urgency to a culture based on repeated altruistic donations. In this context, the routine use of fetal *RhD* genotyping in all D-negative pregnant women as part of an antenatal screening program would be a good measure towards immunoprophylaxis optimization.

On the other hand, it has been recently discussed that routine administration of biological products, such as IgRH, would not be ethically acceptable when molecular methodologies for fetal *RHD* genotyping are available to identify those women that do not need their administration [10].

Biological products prepared from pools of various donors carry the risk of infection transmission, as it has already been demonstrated in different studies [7–9]. There is a residual risk of viral contamination and a potential risk of prion transmission, which corroborates that IgRH, like any other biological product, should be administered only when it is really necessary [28]. It is necessary to bear in mind that this residual risk also involves the possibility of new emerging viral diseases, as it has occurred during the last 10 years. Emerging pathogens could potentially be transmitted since

detection techniques or screening programs may not always be available at the time of emergence.

The precautions to consider when using IgRH are various, and, given the current situation, it seems necessary to establish a prophylactic program to optimize the available resources; in terms of fetal-maternal alloimmunization, fetal *RHD* genotyping allows to identify those cases in which administration of IgRH is really necessary, making the management of D-negative pregnancies more suitable in order to optimize immune prophylactic recourses in clinical practice.

In European countries, 40% of D-negative pregnant women bear negative fetuses, while in countries like India the frequency is 25% [17]. Therefore, recognition of fetal *RHD* status of European and Indian D-negative pregnant women allows to avoid unnecessary administration of IgRH in 40 and 25% of women carrying D-negative fetuses, respectively. In our studied population, 22% of D-negative pregnant women carried D-negative fetuses; therefore, they were not at risk of immunization. These results, in line with what has been recently described by Trucco Bogionne et al [18], corroborate the need of updating antenatal screening programs in regions without consensual guidelines for the use of antenatal prophylaxis.

A simple and cost-effective prenatal diagnostic method for fetal *RHD* screening should be the base of the prophylactic program for a rational use of IgRH. The high accuracy of fetal genotyping using two sequences of the gene proved that it can be sufficient for clinical application in an admixed population. Thereby, owing to the good performance of this assay, we recommend that prevention policies for admixed populations, like those of America, consider

its incorporation for prophylaxis optimization of fetal-maternal alloimmunization. Noninvasive fetal *RhD* genotyping is highly reliable and can be used to optimize resources targeting antenatal and postnatal IgRH usage.

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Contribution to Authorship

SB and SVG conceived and designed the study. SB, SVG, and MCF processed the samples. VSG, LGS, LHC, GEF, and JMC recruited and followed the patients. All authors analyzed the data. SB and SVG designed and wrote the manuscript.

Disclosure Statement

The authors declare no conflicts of interest relevant to the publication of this article.

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