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ORIGINAL ARTICLE

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Single-exon approach to non-invasive fetal *RHD* screening in early pregnancy: An update after 10 years' experience

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Abstract

Background and Objectives: Anti-D prophylaxis, administered to RhD-negative women, has significantly reduced the incidence of RhD immunization. Non-invasive fetal *RHD* screening has been used in Stockholm for more than 10 years to identify women who will benefit from prophylaxis. The method is based on a single-exon approach and is used in early pregnancy. The aim of this study was to update the performance of the method.

Materials and Methods: The single exon assay from Devyser AB is a multiplex kit detecting both exon 4 of the *RHD* gene and the housekeeping gene *GAPDH*. Cell-free DNA was extracted from 1 ml of plasma from EDTA blood taken during early pregnancy, weeks 10–12. The genetic *RHD* results were compared with serological typing of newborns for a determination of sensitivity and specificity.

Results: In total, 4337 pregnancies were included in the study; 44 samples (1%) were inconclusive either due to maternal *RHD* gene variants ($n = 34$) or technical reasons ($n = 10$). Of the remaining 4293 pregnancies, a total number of nine discrepant results were found. False positive results ($n = 7$) were mainly ($n = 4$) due to *RHD* gene variants in the child. False-negative results were found in two cases, of which one was caused by a technical error. None of the false-negative cases was due to *RHD* gene variants. Overall, the sensitivity of the method was 99.93% and specificity 99.56%.

Conclusion: The single-exon assay used in this study is correlated with high sensitivity and specificity.

Keywords

fetal *RHD*, NIPT *RHD*, single-exon

Highlights

- Assay sensitivity was 99.93% and specificity 99.56%.
- The single-exon approach is safe and accurate for fetal *RHD* screening.

INTRODUCTION

Haemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloantibodies directed against fetal antigens on the red cell surface [1]. The RhD antigen is most frequently involved in HDFN, and therefore, potentially life threatening for the fetus. Antenatal and postpartum anti-D prophylaxis has reduced the incidence of RhD immunization to low levels, 0.2%–0.4% [2–4]. Previously, antenatal anti-D was often administered to all RhD-negative women, resulting in unnecessary treatment of those, approximately 40%, carrying an RhD-negative fetus. In an increasing number of countries, antenatal anti-D immunoglobin is used as prophylaxis only in RhD-negative women with an *RHD*-positive fetus [5].

Non-invasive prenatal test (NIPT) for fetal *RHD* has been used for more than 20 years. Initially, it was used to determine the fetal RhD status in immunized pregnancies but is now an established screening method in many laboratories to identify women who will benefit from prophylaxis [6–9]. The methodology is based on the purification of free circulating DNA from plasma samples and subsequent PCR amplification of one or several *RHD*-specific exons. It is essential that a fetal RhD assay has a high diagnostic sensitivity to avoid false-negative results, which may result in missed anti-D prophylaxis and risk of immunization. A positive control to estimate the amount of fetal DNA in the sample is desired, but it has been challenging to find suitable controls, and therefore, most assays lack a specific control for fetal DNA [10]. For DNA purification, virus isolation kits or specific kits for circulating DNA are used. For PCR analysis, different target exons and combinations of exons can be used in most available real-time instruments. Until recently, most assays have been using in-house methods; however, several CE-approved commercial kits for *RHD* PCR amplification are now available.

In Stockholm, a screening study for NIPT of fetal *RHD* in combination with targeted antenatal anti-D prophylaxis was introduced in 2009, and the first evaluation was published in 2012 [11]. Using a single-exon approach in early pregnancy, we showed that the assay was safe and accurate for samples obtained after week 8. In a clinical follow-up study, we showed that the immunization rate was reduced to a comparable level, 0.26%, to that in programs providing antenatal prophylaxis to all RhD-negative women [12]. Since then, routine blood sampling have been recommended after gestational week 10, and most samples are taken during gestational weeks 10–12. Since then, a number of changes in the procedure have been implemented, for example, extended automation and minor technical modifications and improvements in the method. Before abolishing routine serological typing of newborns, we decided to perform a new validation.

MATERIALS AND METHODS

Included samples

Samples from pregnant RhD-negative women analysed from May 2019 to October 2020 were included.

Whole blood samples, 5 or 7 ml EDTA tubes, were collected at antenatal or blood sampling centres and sent to the laboratory by regular post or transportation service. Only blood samples no older than 5 days were accepted for analysis. The samples were centrifuged for 15 min at 1500*g*. The blood tubes, were in most cases, put directly onto the QIAsymphony (QIAGEN, Hilden, Germany) instrument for DNA extraction but for samples close to 5 days before they could be analysed, plasma was transferred to cryotubes and frozen at −20°C until further analysis. As shown by others, the use of short-time frozen plasma does not have a major effect on results [13].

DNA extraction and PCR analysis

DNA extraction was performed using the QIAsymphony instrument in combination with the Virus/Pathogen kit (QIAGEN, Hilden, Germany). For each sample, 1 ml plasma was used for extraction, and the elution volume was 85 µl. A total of 24 samples were extracted in each run. DNA samples were usually stored in the freezer at −20°C until PCR analysis, since samples were needed to be collected for a total number of 31 before PCR analysis. The storage time at −20°C was less than 3 days.

PCR analysis was performed using a CE-labelled single exon kit (Devyser, Stockholm, Sweden). The PCR assay is designed for multiplex detection of *RHD* exon 4 and the endogenous control gene *GAPDH*. In May 2019, the composition of the PCR kit was updated to improve the signal intensity and decrease background signals (communication with Devyser).

For PCR analysis, 31 samples in triplicate + positive and non-template control controls were run in a 96-well PCR plate using the ABI 7500 Real-Time PCR instrument. The total PCR volume was 50 µl and consisted of 30 µl of PCR-mix + 20 µl of the extracted DNA sample. The PCR program was 95°C for 10 min and 50 cycles of 95°C for 15 s and 62°C for 1 min.

The plasma-equivalent per PCR reaction was 235 µl (1000 µl * 20 µl/85 µl).

For a positive result, at least two-thirds of replicates should be positive. Samples that were positive in one-third of replicates were reanalyzed if an extra aliquot of plasma was available; otherwise, a new sample was requested. For *RHD*-negative samples, the Ct values of the *GAPDH* gene needed to be in a specific range as determined by the manufacturer for the specific PCR-kit batch. Too low or too high *GAPDH* Ct-values indicated the presence of too high maternal DNA concentration or too low total-DNA concentration, respectively. In samples with too high *GAPDH* Ct-value, a reanalysis was performed if an extra aliquot of plasma was available, but in samples with too low *GAPDH* Ct-value, a new sample was requested directly. After two consecutive inconclusive results, no additional samples were requested, and anti-D-prophylaxis was recommended. The specific Ct value ranges for *GAPDH* used to approve samples correspond to approximately 150 (lower limit) to 40.000 (higher limit) genomic equivalents per 1 ml plasma.

For *RHD*-negative samples that showed a strong *RHD* signal, that is, Ct value for *RHD* was lower than the Ct value for *GAPDH*, a maternal *RHD* variant was suspected. These samples were further investigated for *RHD* variants with the Fluogene genomic typing system (Innotrain, Kronberg, Germany). Samples with unclear results were also analysed with an in-house developed NGS method for blood group typing (not published). In short, the NGS method was designed to analyse the most common blood groups by sequencing only the most informative exons. For *RHD* and *RHCE*, all 10 exons were sequenced, including intron sequences in the junction. *RHD* zygosity was determined by copy number variation analysis.

Cord blood type

ABO and RhD blood typing was performed after birth on EDTA-anticoagulated umbilical cord blood samples or citrate samples from the newborns. Blood typing was done in the automated system Vision using the BioVue cassettes ABO-Rh/DAT (Ortho Clinical Diagnostics Raritan, NJ, USA). The blood group serology results were used as the gold standard to assess the performance of the antenatal fetal *RHD* genotyping.

Sensitivity and specificity calculations

For determination of diagnostic sensitivity and specificity, genetic fetal *RHD* typing was compared to serological typing performed at birth. Sensitivity was calculated as a proportion of true PCR positives in comparison to serology of the newborn = true positive/(true positive + false-negative).

Specificity was calculated as a proportion of true PCR negatives in comparison to serology of the newborn = true negative/(true negative + false-positive).

The study was approved by the Stockholm Regional Ethics Committee (no 2022-01282-02).

RESULTS

Samples

In total, 7579 samples in 7066 pregnancies were analysed. After exclusion of samples from pregnancies outside Stockholm, terminated pregnancies and pregnancies with missing blood group serology results in the newborn, 4337 pregnancies were included in this study. After a first analysis, a total number of 151 samples (3.5%) showed inconclusive results. Of these samples, maternal *RHD* variants were suspected in 34 cases. Of the remaining 117 samples, the reason for inconclusive results were one-third positive replicates in 49 samples (42%), too low *GAPDH* Ct-value in 44 samples (38%) or too high *GAPDH* Ct-value in 24 samples (20%). After reanalysis of the extra aliquot of plasma or resampling, conclusive results were obtained for most samples, but in 10 pregnancies (0.2%) the results remained inconclusive (Figure 1). The reasons for inconclusive results for these 10 cases were no re-sampling ($n = 7$), too low *GAPDH* Ct-value ($n = 2$) or too high *GAPDH* Ct-value ($n = 1$). The RhD status of these 10 newborns was positive in two cases (both in the group of '1/3 pos') and negative in eight cases.

Excluding the 34 pregnancies with suspicion of maternal *RHD* variants and the 10 pregnancies with inconclusive results, a total number of 4293 pregnancies were included for determination of sensitivity and specificity.

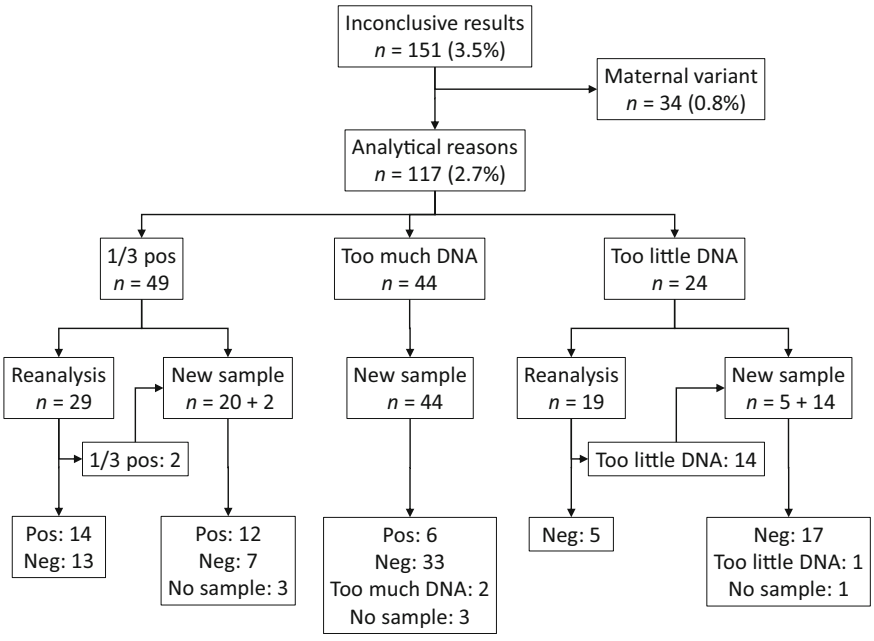

FIGURE 1 Reasons and outcome for inconclusive results

Sensitivity and specificity

Of the 4293 pregnancies, 2692 showed *RHD*-positive results and 1601 *RHD*-negative results. A total number of nine discrepant cases were found when comparing fetal *RHD* genotyping and serological typing at birth (Figure 2).

False-positive results were detected in seven cases (0.16%). In four out of seven cases, *RHD*-gene variants were found in the children after genotyping (two with RHD*Psi, one with K409K and one with c.635-1G>A). In two other cases, maternal *RHD* variants were suspected since the children lacked the *RHD* gene when genotyped. In both cases, the *RHD* signal was strong at the time of fetal *RHD* analysis but not strong enough to report as a suspected maternal *RHD* gene. Unfortunately, no sample was available from the mothers for genotyping. The last case showed a positive *RHD* signal in two-thirds of replicates at the time of analysis. This is the only case that shows a 'real' false-positive result caused by the analysis and not due to *RHD* gene variants.

False-negative results were detected in two cases. One case was probably caused by insufficient mixing of PCR-mix. Amplification curves were not optimal, and the RHD signals were not strong enough to cross the threshold for a positive result. In the other case, the total amount of DNA was lower than normal, Ct-value for *GAPDH* was high but still within the accepted range. It could be that a combination of low DNA amount and a low fraction of fetal DNA made it difficult to detect fetal *RHD* in this sample.

Overall, the sensitivity of the method was 99.93% (95% CI 99.73%–99.99%) and specificity 99.56% (95% CI 99.08%–99.82%).

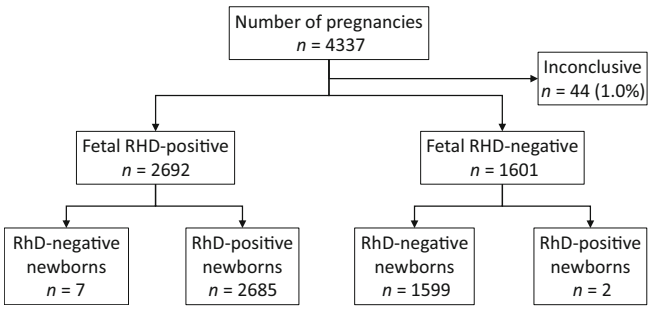

FIGURE 2 Summary of the results. Genomic versus serological typing

Maternal RHD variants

In 34 cases, the *RHD* status of the fetus could not be determined due to the suspicion of a maternal *RHD* variant. These cases were further analysed with genomic typing if possible. In Table 1, the results of the genomic typings are shown. The most common variant was RHD*Psi followed by DAU. A variant, not previously described, was found in one case. This was a frameshift mutation caused by a 1-bp deletion at position c.1178 (or c.1179) in exon 9. In two cases, analysis of maternal DNA detected no *RHD* gene. In both cases, the fraction of fetal DNA was likely high enough to produce a strong *RHD* signal.

DISCUSSION

In this study, we have evaluated the performance of a single-exon screening assay for non-invasive fetal *RHD* determination during early pregnancy, weeks 10–12. In the previous study from 2012, we observed a sensitivity of 99.3% when excluding samples before gestational week 10 [11]. Sensitivity was 99.93% in the current study. During the past 10 years we have gained better experience in running the tests, stepwise improvements have been made in the procedures, and instruments and the *RHD* assay has been optimized.

The method we have used is a single-exon assay for the detection of *RHD* exon 4 in combination with the housekeeping gene *GAPDH*. In many other studies, a combination of different exons, for example, exons 4, 5, 7 and 10, have been used [6, 8, 14]. The major advantage of using several exons is to minimize the risk of false-negative results due to *RHD* variants. However, as shown from this and our previous

TABLE 1 Summary of pregnancies with suspicion of maternal *RHD* variants ($n = 34$)

RHD change	*n*	DNA changes	RhD consequence	ISBT allele name	*RHCE*-alleles
RHD*Psi	8	Several	Null allele	RHD*08N.01	ce
DAU	5	c.1136C>T	p.Thr379Met	RHD*10	ce
Weak 11	4	c.885G>T	p.Met295Ile	RHD*11	Cce
Weak 15	3	c.845G>A	p.Gly282Asp	RHD*15	cEe
RHD-RHCE(3)-weak D type 4.0	2	Exon 3 hybrid on a weak 4 background	Null allele	RHD*01N.72	ce
Weak 2	1	c.1154G>C	p.Gly385Ala	RHD*01W.2	CcEe
Weak 92	1	c.1145T>C	p.Leu382Pro	RHD*01W.92	Cce
New	1	c.1178delG (or 1179delG)	Frameshift mutation	—	Cce
Not known[a]	5				
Not analysed	2				
No RHD gene found	2				

[a]RHD gene detected but not further analysed for variants.

study, false-negative results have usually been due to technical reasons and not at all due to undetected *RHD* variants in the fetus. A sensitivity of 99.93% also shows the accuracy of this method. This could, of course, reflect the population included in the study and may differ in other populations where other *RHD* variants are present, although today, we have a diverse population in the Stockholm area, with many ethnicities represented [15–17].

Another advantage of using the multi-exon approach is that maternal *RHD* variants like RHD psi can be identified if one of the exons is designed not to amplify this variant. In these cases, the RHD status of the fetus can be determined, and the number of inconclusive and false-positive results may be reduced.

One of the false-negative results was probably caused by low amount of free circulating DNA. The amount of free circulating DNA, both total and fetal DNA fraction, usually varies from one individual to another. In addition, different factors, for example, body weight, can affect the DNA amount [18, 19]. Also, lysis of the blood during shipping and/or storage can cause leakage of genomic DNA from the mother and decrease the sensitivity of the method. Using an internal control gene like *GAPDH* can provide information about the total amount of DNA present and serve as an indicator of the quality of the sample. In six samples, the first result showed a negative result, but the test was considered inconclusive since the *GAPDH* signal indicated too much DNA. Re-sampling of these cases showed positive results. Without the presence of an internal control gene, these samples would have been reported as false-negative.

In samples with a high Ct value for *GAPDH* (too little DNA), a re-analysis of the extra aliquot of plasma showed inconclusive results again in 14/19 samples. New samples were requested for these patients. Because of low success rates in the re-analysis of the same sample, we have decided to request a new sample directly for these patients.

False-positive results were found in seven cases, and four of them were due to *RHD* variants in the fetus. In two cases, probably maternal *RHD* variants caused false-positive results. The diagnosis of maternal *RHD* variants is based on the signal strengths from *RHD* versus *GAPDH* and may not always be 100% accurate. This was also shown in two other cases where maternal *RHD* variants were suspected, but further investigation showed that the signals were likely due to high fetal-DNA content in the sample. Whether it is an *RHD* variant in the fetus or mother does not matter for clinical decision-making since anti-D prophylaxis is recommended in both cases. Altogether the rate of false-positive results was 0.16%, which means that a relatively small number of pregnant women are administered unnecessary anti-D prophylaxis. In addition, 34 women (0.78%) were given anti-D prophylaxis since the genotype of the fetus could not be determined due to a possible maternal *RHD* variant.

The quality of the blood sample and the extracted DNA is of utmost importance for reliable analysis of fetal *RHD*. The best approach is a fully automated workflow and minimizing freeze-thawing of plasma and/or DNA samples [20, 21]. In this study, we worked mainly on fresh plasma samples from primary blood tubes. However, since the number of samples used for DNA extraction using QIAsymphony ($n = 24$) did not match with the previous size of the PCR kit ($n = 31$), we needed to freeze DNA samples in order to sum up to 31 samples before PCR analysis. Since the completion of this study, the PCR kit format has been updated for the analysis of 24 samples, and the QIAsymphony instrument has been upgraded with an AS module for PCR setup. The workflow from sample preparation to PCR assay setup is now fully automated. We have already seen effects on the number of inconclusive samples. The fraction of inconclusive results due to analytical reasons is now around 2% as compared to previously 3.0%–3.5%.

In summary, the single-exon approach used in this study for fetal *RHD* determination during early pregnancy is correlated with high sensitivity and specificity. As of November 2020, we have discontinued serological typing of newborns.

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CONFLICT OF INTEREST

Mehmet Uzunel was employed by Karolinska University Hospital when this study was initiated but is currently working at Devyser AB. Eleonor Tiblad, Anette Mörtberg and Agneta Wikman do not have any conflicts of interest/competing interests to declare.

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