Original article

Evaluation of an automated platform for non-invasive single-exon fetal *RHD* genotyping early in pregnancy

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Background - RhD immunization is still the major cause of hemolytic disease of the fetus and newborn. Fetal *RHD* genotyping during pregnancy followed by tailored anti-D prophylaxis for pregnant RhD-negative women carrying an *RHD*-positive fetus to prevent RhD immunization is a well-established practice in many countries. This study aimed to validate a platform for high-throughput, non-invasive, single-exon, fetal *RHD* genotyping consisting of automated DNA extraction and PCR set-up, and a novel system for electronic data transfer to the real-time PCR instrument. We also investigated the effect of storage conditions of fresh or frozen samples on the outcome of the assay.

Materials and methods - Blood samples from 261 RhD-negative pregnant women collected in Gothenburg, Sweden, between November 2018 and April 2020 during gestation week 10-14 were either tested as fresh after storage for 0-7 days at room temperature or as thawed plasma samples previously separated and stored for up to 13 months at −80°C. Extraction of cell-free fetal DNA and PCR set-up were performed in a closed automated system. Fetal *RHD* genotyping was determined by real-time PCR amplification of the *RHD* gene exon 4.

Results - The outcome of *RHD* genotyping was compared with either the results obtained with serological RhD typing of newborns or with the results of *RHD* genotyping performed by other laboratories. No difference was observed in genotyping results when using fresh or frozen plasma during short- and long-term storage, revealing high stability of cell-free fetal DNA. The assay has shown high sensitivity (99.37%), specificity (100%), and accuracy (99.62%).

Discussion - These data confirm that the proposed platform for non-invasive, single-exon, *RHD* genotyping early in pregnancy is accurate and robust. Importantly, we demonstrated the stability of cell-free fetal DNA in fresh and frozen samples after short- and long-term storage.

Keywords: *cffDNA, non-invasive fetal RHD genotyping, hemolytic disease of the newborn.*

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INTRODUCTION

The Rh blood group system is one of the most clinically significant in transfusion medicine, second only to ABO. During pregnancy, RhD incompatibility between an RhD-negative mother and an RhD-positive fetus may result in maternal RhD immunization. Production of maternal anti-D antibodies that cross placenta and bind fetal red cells is still one of the main causes of severe hemolytic disease of the fetus and newborn[1-4].

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Since the discovery of cell-free fetal DNA (cffDNA) in maternal plasma in 1998[5,6], many countries have introduced non-invasive determination of fetal *RHD* genotype from maternal plasma in combination with tailored antenatal administration of anti-D prophylaxis to RhD-negative women carrying an RhD-positive fetus[7-11]. This practice has reduced the incidence of RhD immunization to 0.2-0.4%, preventing unnecessary treatment of RhD-negative women carrying an RhD-negative fetus[12].

Quantitative real-time polymerase chain reaction (qPCR) is still the most common technology applied for the determination of fetal *RHD* type[13,14]. The workflow may differ depending on whether one or more exons are to be investigated, the system used for DNA extraction and qPCR analysis, and the timing of the screening during pregnancy[15-17]. One of the challenges of these assays can be the possibility of false negative results. These may be due to technical issues such as low levels of cffDNA in maternal blood (especially if the sample is acquired early in pregnancy) or loss of cffDNA. Another important aspect is how storage conditions may affect the levels and quality of cffDNA.

A study previously performed in Sweden for non-invasive, single-exon, determination of fetal *RHD* genotype at the end of the first trimester was shown to be highly specific and sensitive[18]. The aim of our study was to validate a robust and easily applicable platform for clinical use of high-throughput, single-exon, fetal *RHD* screening in a closed automated platform for plasma preparation, DNA extraction and PCR set-up, including a novel information technology (IT) infrastructure for electronic data transfer to a qPCR instrument to minimize manual handling and any possible errors. Moreover, we investigated the appropriate storage time for fresh samples at room temperature (RT) and whether long-term storage of frozen plasma samples would impact the results of downstream testing.

MATERIALS AND METHODS

Sample processing and storage

Pregnant women whose blood group was RhD-negative or previously unknown were invited to participate in a prospective study on fetal *RHD* genotyping at the time of their first routine visit to the maternity care center in Gothenburg, Sweden, between November 2018 and April 2020. Informed consent was required and a total of 836 women were recruited for the study. Two 6 mL peripheral blood samples were collected in tubes containing ethylenediaminetetraacetic acid (K2EDTA) during gestational week 10-14. The first blood tube from each pregnant woman was tested for ABO and RhD blood group and red blood cell antibody screening using an automated instrument (NEO; Immucor, Rödermark, Germany). The RhD blood group was determined by direct agglutination using two monoclonal blend reagents: ImmuClone Anti-D duo IgM+IgG, clones TH28 and MS26 (Immucor), and Novaclone Anti-D IgM+IgG, clones D175-2 and D415 1E4 (Immucor). The second sample was used for fetal *RHD* genotyping in women who had previously been typed RhD-negative. A total of 261 samples were analyzed. The samples collected for determination of fetal *RHD* status were stored unopened for 0-7 days at RT and thereafter centrifugated at 1,500 *g* × 10 minutes (min). Plasma from 47 primary collection tubes was tested immediately after centrifugation. Plasma from 214 samples was separated in two 1.2 mL aliquotes and stored for up to 13 months at –80°C. The study was performed at the Immunohematology Laboratory of the Department of Clinical Immunology and Transfusion Medicine, Sahlgrenska University Hospital, Sweden. The study project was approved by the regional ethical committee in Gothenburg (reference No. 1022-17).

Extraction of cffDNA

The extraction of cffDNA was carried out using an automatic magnetic beads-based technique according to the manufacturer`s instructions. Briefly, cffDNA was extracted from either thawed or fresh plasma using the QIAsymphony SP/AS instrument in combination with QIAsymphony DSP Virus/pathogen Kit (Qiagen, Hilden, Germany). For each sample, purified DNA from 1.2 mL plasma was eluted to 70 µL and dispensed in triplicate in a 96-well plate.

Fetal *RHD* genotyping

Fetal *RHD* genotyping was performed by qPCR analysis using a commercially available *RHD* screening kit detecting exon 4 (Devyser, Hägersten, Sweden) on a QuantStudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The thermoprofile was 95°C for 10 min and 50 cycles of 95°C for 15 seconds and 62°C for 1 min. The total PCR volume of 50 µL consisted of 30 µL PCR

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mix + 20 µL extracted sample. Extracted DNA samples were analyzed in triplicates. One *RHD*-negative and two *RHD*-positive controls (PC1 and PC2) were analyzed in parallel with the samples in every run, according to the manufacturer`s instructions. The Devyser RHD kit included primers and probes specifically designed for *RHD* exon 4 and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), used as an internal control. The cycle-threshold (Ct) of *RHD* in PC1 and PC2 was adjusted close to 28.5 and the reference values for Ct *GAPDH* were calculated as follows:

average of Ct *GAPDH* in PC1 and PC2 –1.5/+6.5.

Fetal *RHD* results were accepted or not depending on the internal control *GAPDH* and *RHD* Ct values in every replicate of each sample. The interpretation criteria are described in **Table I**. The results were either compared with those obtained from serological RhD typing of newborns or from *RHD* genotyping performed by two laboratories in Sweden, using an aliquot separated from the same primary tube. Both laboratories used qPCR reagents from the same manufacturer but different platforms for the performance of the test.

IT infrastructure

A novel plate layout was created for data conversion, using the XSLT technology, to enable electronic data transfer from a QIAsymphony SP/AS to a QuantStudio 7 Flex Real-Time PCR instrument and for complete traceability of all generated data. The QIAlink software (Qiagen) was used to connect QIAsymphony SP/AS and QuantStudio 7 Flex. These instruments and the QIAlink server running the QIALink software were connected in a Virtual Routing and Forwarding (VRF) closed network (**Figure 1**).

Table I – *Criteria for interpretation of the results*

RHD	***GAPDH****	**Results**
2/3 or 3/3 positive **Ct *RHD* later than Ct *GAPDH***	3/3 positive Within reference values	**Fetal *RHD* type positive**
3/3 negative	3/3 positive Within reference values	**Fetal *RHD* type negative**
1/3 positive	3/3 positive Within reference values	Inconclusive
1/3 or 2/3 or 3/3 positive **Ct *RHD* earlier than Ct *GAPDH***	3/3 positive Within reference values	Inconclusive Possible maternal *RHD* gene
3/3 negative	3/3 positive Later or earlier than reference values	Inconclusive
3/3 negative	No signal	No DNA amplification

**GAPDH* signal must be detected in all replicates to approve the results.
Fetal *RHD* results were accepted or not depending on the internal control *GAPDH* Ct and *RHD* Ct values in the sample according to the criteria in the table.

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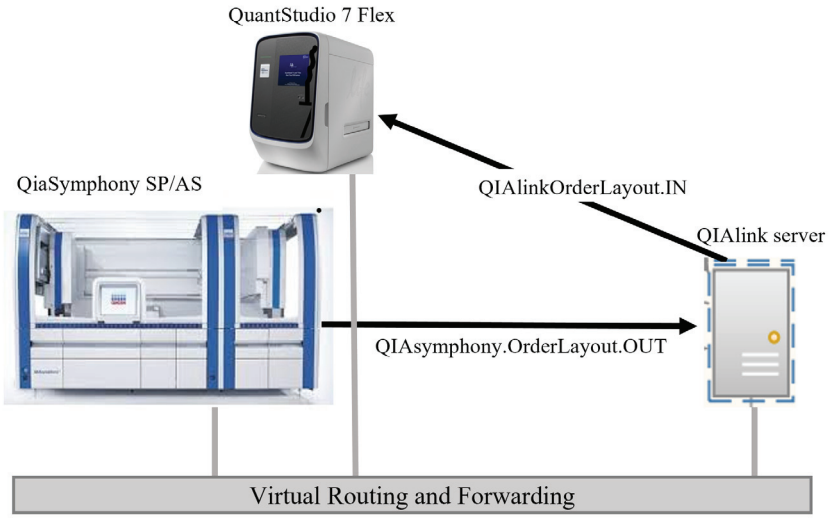


Figure 1 - Overview of the automated platform for DNA extraction, PCR set-up and IT infrastructure for electronic data transfer to qPCR instrument
qPCR: quantitative real-time polymerase chain reaction.

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Statistical analysis

A MedCalc Software Ltd. Diagnostic (Ostend, Belgium) test evaluation calculator (version 20.106) was used for statistical analysis.

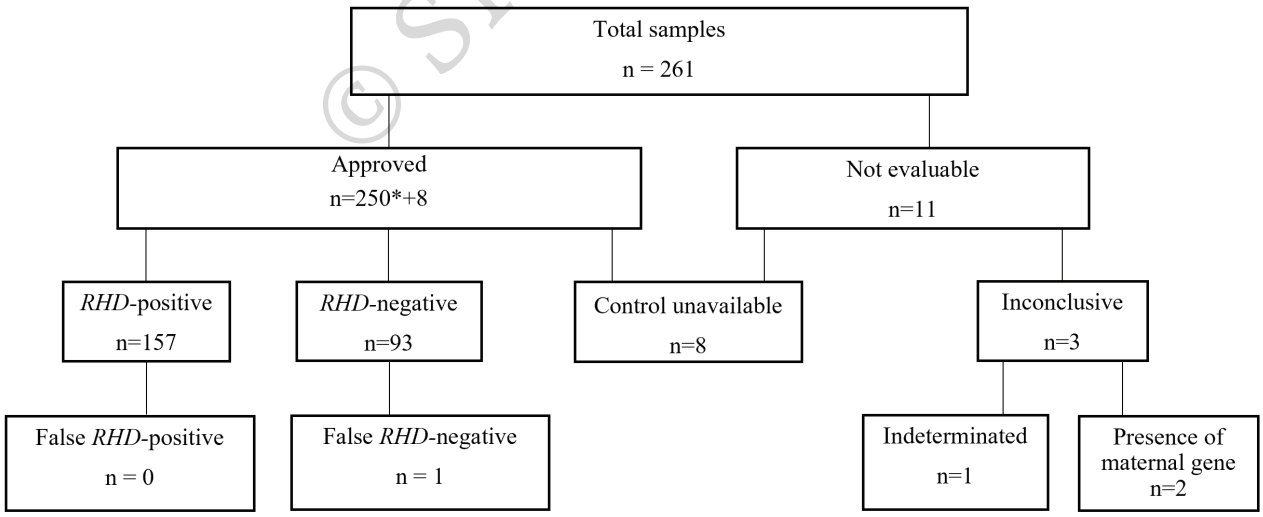
RESULTS

Overall, in this study, 261 plasma samples were analyzed for fetal *RHD* genotyping. Sufficient levels of cffDNA were extracted and dispensed in the PCR-plate from all samples using a fully automated system. In 250/261 cases, the results of fetal *RHD* genotyping were consistent with either serological blood typing of newborns (187 samples) or with genotyping performed by another laboratory (61 samples) or both (2 samples). In 5/250 cases, the results were at first inconclusive, but were approved after reanalysis by qPCR of a frozen aliquote from the original sample. A total of 207/250 samples were tested as previously frozen and 43/250 as fresh blood samples. Based on the interpretation criteria (**Table I**), fetal *RHD* type was positive in 157 pregnancies (62.8%) and negative in 93 pregnancies (37.2%). No false positive result was detected; however, one false negative result was observed. The fetal *RHD* type was negative on a sample tested at gestational week 10, while the newborn was serologically RhD-positive. *GAPDH* values were approved according to the criteria in **Table I**, indicating sufficient cffDNA extraction. The discordant result was shown to be related to the D antigen variant DVI detected when performing serological blood group typing of the newborn.

Genotyping results were not evaluable in 11 samples. In 8/11 cases, the results were approved but were not evaluable in this study because of lack of controls. In 6/8 of these cases, serology was missing, possibly due to abortion, miscarriage, or delivery elsewhere, and plasma volume was too low to be sent to another laboratory for genotyping; 2/8 samples showed inconclusive results at another laboratory (low Ct *GAPDH*). In 3/11 cases, the results of fetal *RHD* type were inconclusive; in one sample the reason was Ct *GAPDH* too low and plasma volume insufficient for reanalysis. In 2 samples, fetal *RHD* type could not be determined due to the presence of a maternal gene. These samples were shown to give clear positive agglutination reaction when performing maternal serological RhD typing by indirect antiglobulin test. All results are summarized in **Figure 2**.

Importantly, the electronic data transfer between the QIAsymphony and the QuantStudio 7 Flex Real-Time PCR systems was shown to work correctly and to be robust. The validation of the novel IT infrastructure was, therefore, approved, contributing to increased patient safety since no manual data processing was needed.

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*5/250 results were at first inconclusive, but approved after reanalysis

Figure 2 - Overview of analyzed samples and fetal *RHD* genotyping results

Storage of fresh blood samples for up to 7 days at RT, before testing or plasma aliquoting for freezing, was shown not to affect the quality of DNA nor, consequently, the accuracy of the results. Moreover, frozen plasma aliquoted and stored for up to 13 months at −80°C was shown not to influence DNA stability.

Overall, these results have shown high sensitivity (99.37%; 95% CI: 96.52-99.98%), specificity (100%; 95% CI: 96.07-100.00%) and accuracy (99.62%; 95% CI: 97.83-99.99%). When comparing fetal *RHD* genotyping results of 63/250 samples (25%), full concordance with those obtained by fetal *RHD* determination performed at another laboratory was observed and is indicative of high reproducibility (100%).

DISCUSSION

Over the last decade, many countries have introduced non-invasive fetal *RHD* genotyping in the clinical setting for targeted antenatal anti-D prophylaxis. A combination of two or more exons has often been used with the aim to minimize false negative results due to *RHD* variants possibly causing RhD immunization[14,19]. It has previously been reported that the *RHD* exons 7 and 10 contain several specific sequences that differ between the *RHD* and *RHCE* genes revealing the possible presence of hybrid genes[13]. Another common combination is exon 5 and 7, where exon 5 does not amplify *RHD*08N.01* (*RHD*Ψ*, present in 66% of Black African) and *RHD*06* (*RHD*DVI*, concerning around 0.02% of Europeans) while exon 7 detects both[8,20-22]. Other reports have shown that a single exon strategy has demonstrated a similar, or even a better performance, than multiple exon designs with respect to false negative and false positive results[15,17,23]. In Sweden, following a study evaluating *RHD* exon 4, fetal *RHD* screening in combination with targeted antenatal anti-D prophylaxis has been recommended as part of a national screening program for maternal care centers since 2015. The single-exon amplification approach was shown to enable high-throughput testing and to have high sensitivity and specificity.

The first aim of our study was to validate a platform for routine detection of *RHD* exon 4 from maternal plasma early in pregnancy, consisting of automated steps for DNA extraction, dispensing of DNA and PCR reagents in a closed system (QIASymphony SP/AS), and a novel IT infrastructure for electronic data transfer to the QuantStudio 7 Flex Real-Time PCR System. Each sample was analyzed in triplicate and the Ct of the *RHD* gene (presumed to be of fetal origin) was compared to the endogenous internal control of amplification, the reference gene *GAPDH*. A common challenge is to differentiate the *RHD* gene of maternal from that of fetal origin; however, this approach can make the distinction, preventing false positive results due to maternal *RHD* gene variants and thereby increasing the specificity of the assay. CffDNA consists of a low amount of small fragments that increase in concentration during pregnancy and rapidly disappear after delivery. *RHD* Ct values lower than *GAPDH* (*RHD* signals appear earlier than *GAPDH* ones) indicate a high DNA concentration of maternal origin leading to unpredictable fetal *RHD* genotyping results. In our study, no false positive result was observed. One false negative result was reported and shown to be related to the presence of the D antigen variant DVI in the newborn by serological RhD typing. This variant, which has a very low frequency in the European population, is currently analyzed in newborns from RhD-negative mothers as a standard routine at our department.

A limitation of the assay is that fetal *RHD* status is predictable only for women with negative RhD blood group due to a total deletion of the *RHD* gene. The presence of maternal *RHD* gene variants causing lack or partial expression of D antigen leads to discrepancies in maternal molecular and serological results and to inconclusive fetal *RHD* genotyping results[24]. However, these variants were an absolute minority in our population, showing that this strategy is widely applicable in our setting. Pregnant women whose fetal *RHD* type is inconclusive are recommended to receive antenatal anti-D prophylaxis at the beginning of the third trimester of pregnancy and in the case of any event possibly triggering RhD immunization.

The second aim of our study was to evaluate the impact of storage time for fresh samples at RT and long-term storage of frozen plasma samples as pre-analytical factors possibly influencing the performance of the assay. Optimal sample storage conditions are essential for non-invasive prenatal testing of cffDNA. Previous data have demonstrated that fresh samples can be stored for up to 5 days at RT before extraction of DNA using exon

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4 assay to determine fetal *RHD* genotype. However, our data demonstrate that fresh samples are stable and can be stored for up to 7 days at RT. As an alternative to fresh samples, we investigated the effect of long-term storage of frozen plasma samples separated after collection and stored for up to 13 months at –80°C. Interestingly, all samples showed approved results according to the criteria in **Table I**. We observed that cffDNA was readily detectable in previously frozen plasma as well as in samples that were immediately processed and analyzed. This is important when preparing a pool of samples to be used as internal controls to standardize the procedure. Another possible application is storage of frozen aliquots of previously analyzed samples in a biobank for further studies, such as investigation of *RHD* gene variants or evaluation of new Rh-related diagnostics.

In the Swedish population, approximately 15% of pregnant women are RhD-negative and around 40% of them carry an *RHD*-negative fetus, therefore they might receive unnecessary antenatal prophylaxis in case of miscarriage, abortion or amniocentesis. Based on our results, in our clinical setting, antenatal prophylaxis would have been recommended for 158 (including women with an inconclusive result) of the 261 women (63%) while 93 women (37%) would have avoided unnecessary antenatal prophylaxis.

The test was introduced in clinical practice at Sahlgrenska University Hospital at the beginning of November 2020 for samples from Västra Götaland County, Sweden, with high stability in the workflow. Approximately 5,300 samples have been analyzed so far. A long-term follow up including 2,300 samples has been performed, reaching an even higher sensitivity (99.99%) (*data not shown*) when considering a larger scale of samples.

Follow up over time will determine the frequency and type of *RHD* variants in the population of western Sweden.

CONCLUSIONS

In conclusion, we confirm the advantage of non-invasive fetal *RHD* genotyping based on single-exon determination. The performance of the platform for automated DNA extraction and PCR set-up including electronic data transfer to the qPCR instrument was optimal. Moreover, our study has revealed high test reliability using cffDNA extracted from fresh and frozen samples after short- and long-term plasma storage. Our platform is appropriate for high-throughput *RHD*-screening of RhD-negative women taking advantage of tailored treatment with antenatal anti-D prophylaxis.

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AUTHORSHIP CONTRIBUTIONS

Both Authors contributed equally to the study conception, data collection and evaluation, and to writing the manuscript.

The Authors declare no conflicts of interest.

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