Noninvasive Single-Exon Fetal *RHD* Determination in a Routine Screening Program in Early Pregnancy

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OBJECTIVE: To develop a simple and robust assay suitable for fetal *RHD* screening in first-trimester pregnancy and to estimate the sensitivity and specificity of the test after its implementation in an unselected pregnant population.

METHODS: Pregnant women attending their first antenatal visit were included, and fetal *RHD* determination was performed for all women who typed RhD-negative by routine serology. DNA was extracted by an automated system and quantitative polymerase chain reaction was done by an assay based on exon 4. Reporting criteria were simple and strict.

RESULTS: Four thousand one hundred eighteen pregnancies, with a median gestational age of 10 weeks, were included. After 211 (5.1%) reanalyses, fetal *RHD* was reported positive in 2,401 (58.3%), negative in 1,552 (37.7%), and inconclusive in 165 (4.0%) based on the first sample. After a second sample in 147 of 165, only 14 remained inconclusive, all resulting from a weak or silent maternal *RHD* gene. Using blood group serology of the newborns as the gold standard, the false-negative rate was 55 of 2,297 (2.4%) and the false-positive rate was 15 of 1,355 (1.1%). After exclusion of samples obtained before gestational week 8, the false-negative rate was 23 of 2,073 (1.1%) and the false-positive rate was 14 of 1,218 (1.1%). Both sensitivity and specificity were close to 99% provided samples were not collected before gestational week 8. From gestational week 22, sensitivity was 100%.

CONCLUSION: Fetal *RHD* detection in early pregnancy using a single-exon assay in a routine clinical setting is feasible and accurate.

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See related editorial on page 219.

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In 1997, Lo and coworkers[1] reported that approximately 3–6% of cell-free DNA in the plasma of pregnant women is of fetal origin. Unlike fetal cells, which can persist for many years, cell-free fetal DNA is rapidly cleared from the maternal circulation and undetectable within hours of delivery.[2] The first assay of cell-free fetal DNA was based on the detection of the Y chromosome in a female background. Shortly thereafter, it was demonstrated that fetal *RHD* genes could be detected in *RHD*-negative women.[3] Many studies have been published since then that confirm the safety aspects of this technology.[4–6] Initially the focus was on the diagnosis of *RHD* status of the fetus in a small number of immunized women, but recent screening studies have included several hundred to several thousand nonimmunized pregnant women.[7–9] In these studies, the majority of maternal blood samples were taken in the second or third trimester of pregnancy and only a few published studies have focused on detection in early pregnancy.[10,11] The benefits of

determining the fetal *RHD* status in the first trimester of pregnancy include knowing which RhD-negative women should have repeated samples for antibody screening and being able to avoid unnecessary exposure to immunoglobulins at invasive procedures such as amniocentesis and chorionic villus sampling and other situations with risk of fetomaternal hemorrhage. Various assays have been developed for the detection of cell-free fetal DNA using different target *RHD* exons or combinations of exons in the quantitative polymerase chain reaction (PCR) with an accuracy of 95–100% in most of the studies and no clear evidence of a superior strategy.[12]

Immunization against the RhD antigen is the most common cause of hemolytic disease of the fetus and newborn. When postnatal anti-D prophylaxis was introduced in the 1970s, the incidence of RhD immunization decreased from 14% to 1.5% in RhD-negative pregnant women.[13,14] In many countries, routine antenatal anti-D prophylaxis is offered in the beginning of the third trimester of pregnancy[15] and this has resulted in further reduction in the rates of RhD immunization to approximately 0.2–0.4%.[16,17] At present, routine antenatal anti-D prophylaxis is offered to all RhD-negative women in these countries, although approximately 40% of these women carry an RhD-negative fetus and are thus not at risk of immunization. Because anti-D immune globulin is derived from pooled human plasma, every effort should be made to avoid unnecessary exposure resulting from the risk of infectious agents, known or unknown.[18] Furthermore, the supply of anti-D immune globulin is not unlimited and should be given on strict medical indications.

In Sweden, a decision was taken by the authorities in the early 1990s that routine antenatal anti-D prophylaxis would not be given to RhD-negative women. In September 2009, the antenatal centers in the Stockholm area, which account for 25% of the pregnancies in Sweden, introduced a new screening program that included routine cell-free fetal DNA *RHD* genotyping in early pregnancy followed by targeted routine antenatal anti-D prophylaxis to all RhD-negative pregnant women carrying an *RHD*-positive fetus. This article describes the development of a simple and robust assay based only on exon 4 and its implementation in the screening program demonstrating its suitability for fetal *RHD* screening in early pregnancy in an unselected pregnant population.

MATERIALS AND METHODS

All 83 maternity care centers in the Stockholm area participated in this prospective, population-based cohort study on fetal *RHD* DNA testing starting in September 2009. There are approximately 26,000 deliveries per year in Stockholm where 27.6% of the population has a first- or second-generation immigrant background (European Union 10.4%, Europe outside the European Union 3.2%, Africa 2.8%, South America 2.0%, North America 0.7%, Asia and Middle East 8.2%, unknown 0.3%).[19]

Nonimmunized RhD-negative pregnant women were invited to participate in the study at their first visit to the maternity care center and more than 95% of the RhD-negative women in the Stockholm area were enrolled. The 4,118 pregnancies included in our study sample had a known pregnancy outcome by May 1, 2011.

After informed consent was obtained, two 4.5-mL ethylenediaminetetraacetic acid-anticoagulated blood samples were obtained at the maternity care centers as part of routine blood sampling at the booking visit. One sample was used for routine ABO RhD blood typing and red blood cell antibody screening and the other was used for fetal *RHD* determination in women who typed RhD-negative. The blood samples for fetal *RHD* determination were transported to the central laboratory where they were centrifuged for 5 minutes at 1,000 g and plasma was separated, aliquoted in 2×1-mL tubes, and frozen at −30°C until DNA extraction. Blood typing and antibody screening were done according to established routines on an automated blood grouping instrument.

DNA extraction was performed using the MagNA Pure LC instrument in combination with MagNA Pure LC Total Nucleic Acid Isolation Kit–Large Volume according to the manufacturer's instructions. For each sample, 1 mL of thawed plasma was used and the purified DNA was eluted to 70 microliters. The elution volume of 70 microliters was chosen to be able to test triplicates from each extracted sample.

Polymerase chain reaction amplification was performed using an ABI 7500 Real-Time PCR System with thermoprofile 95°C 10 minutes and 50 cycles of 95°C for 15 seconds and 62°C for 1 minute. The total PCR volume of 50 microliters consisted of 30 microliters PCR mix and 20 microliters extracted sample. The PCR assay was designed for the multiplex detection of *RHD* exon 4 and *GAPDH* DNA as an internal control. The PCR products were 136 and 188 bp, respectively. The primer sequences were based on those described earlier.[20] The limit of detection was calculated on generated data using PriProbit software.[21] Limit of detection was determined to be 4 *RHD* DNA copies per PCR corresponding to 13.6 pg per PCR assuming 7.5 pg of genomic DNA per cell.

Extracted DNA samples taken from the women in our study cohort were analyzed in triplicates on the

same 96-well plate. Both *RHD*-positive and *RHD*-negative controls were analyzed on the plate in parallel with 31 tested samples. A positive control for fetal DNA was not used. To ensure that high concentration of maternal DNA did not hamper the detection of fetal DNA in the analysis, the total DNA content in each sample was estimated by comparing the obtained *GAPDH* cycle threshold value in the sample with the *GAPDH* cycle threshold value obtained in the positive control with known DNA concentration. Strict criteria for the cycle threshold values in the positive control and for the required DNA concentration in the sample were used. A fetal *RHD*-negative result was not accepted if the *GAPDH* cycle threshold value was less than 27 (DNA concentration approximately 200 ng). Because it is also important that total DNA concentration is not too low, results with glyceraldehyde-3-phosphate dehydrogenase cycle threshold value of more than 35 (DNA concentration approximately 0.5 ng) were also considered unacceptable. The criteria for the interpretation and reporting of results are described in Table 1.

Because the transport time of the samples from the maternity units varies, we also evaluated the effect of the time from specimen collection to plasma separation using triplicate ethylenediaminetetraacetic acid samples requested from each of 25 women in early pregnancy. These samples were centrifuged (5 minutes, 1,000 g) and plasma separated and frozen after 1) less than 24 hours; 2) 36–48 hours; and 3) 60–72 hours storage as whole blood at room temperature.

ABO and RhD blood typing was performed after birth on ethylenediaminetetraacetic acid-anticoagulated umbilical cord blood samples or citrate samples from the newborns. Blood typing was done using the DiaClon ABO/Rh for Newborns DVI+ gelcards. The blood group serology results were used as the gold standard to assess the performance of the antenatal fetal *RHD* genotyping.

The pregnancies included in our study were described using percentages for categorical variables and medians and ranges for continuous variables. The sensitivity and specificity of the fetal *RHD* genotyping were calculated from the rate of fetal *RHD* detection in the samples with positive and negative serology, respectively. Sensitivity was recalculated excluding specimens collected earlier than successive cutoff values for gestational age and the results presented graphically. The effect of gestational age on the average cycle threshold values of fetal *RHD* positive pregnancies was examined by stratifying by trimester (week 3–12, week 15–28, week 29+) presenting the results graphically on a box plot and comparing the distributions using a Kruskal-Wallis test. The study was approved by the Stockholm Regional Ethics Committee (no. 2009/479-31/4).

Table 1. Interpretation Criteria of Quantitative Polymerase Chain Reaction Results After 50 Cycles (Analysis of Triplicates)

RHD	*GAPDH*	Result
3/3 or 2/3	3/3 or 2/3	Fetal *RHD*-positive
1/3	NA	Reanalysis second aliquot
No signal	3/3 C_t 27–35	Fetal *RHD*-negative
No signal	C_t >35	Reanalysis second aliquot
No signal	C_t <27	New sample

NA, not applicable; Ct, cycle threshold.

RESULTS

Samples from 4,118 pregnancies were analyzed for fetal *RHD*. Initial analysis of triplicate plasma samples from 25 volunteers, separated and frozen within 24 hours (day 1), 36–48 hours (day 2), and 60–72 hours (day 3) of sample collection, indicated that valid analysis could be done after 1–3 days storage of whole blood with reproducible results (*RHD* cycle threshold mean 37.1, 38.8, and 38.4 for days 1, 2, and 3, respectively and *GAPDH* cycle threshold mean 32.6, 32.6, and 31.3, respectively). In the majority of samples included in the study, plasma was separated and frozen within 2 days and DNA extraction and PCR analysis were done within 1 week. Samples that were more than 3 days old were rejected if the DNA concentration was too high and *GAPDH* cycle threshold less than 27 based on our criteria for acceptance.

After reanalysis of the second aliquot of the samples in 211 cases (5.1%) with inconclusive results in the first analysis, a positive or negative fetal *RHD* result was reported for 96% of the samples with 165 (4%) remaining inconclusive (Fig. 1). After a second sample was obtained from 147 of the 165 pregnancies with inconclusive results, 2,485 (60.3%) of the 4,118 pregnancies were reported fetal *RHD*-positive, 1,601 (38.8%) fetal *RHD*-negative, and 32 (0.8%) remained inconclusive. In 14 of these 32 inconclusive samples, the fetal *RHD* could not be determined as a result of a maternal *RHD* gene. In the remaining 18 with inconclusive fetal RHD status, we did not receive a second sample, mostly as a result of spontaneous abortions and miscarriages (n=13).

The pregnant women included in our study had a median age of 31 years (range 14–51 years) and for three fourths of the pregnancies, the first sample for fetal DNA testing was obtained in the first trimester (Table 2). There were 61 twin pairs with eight of these

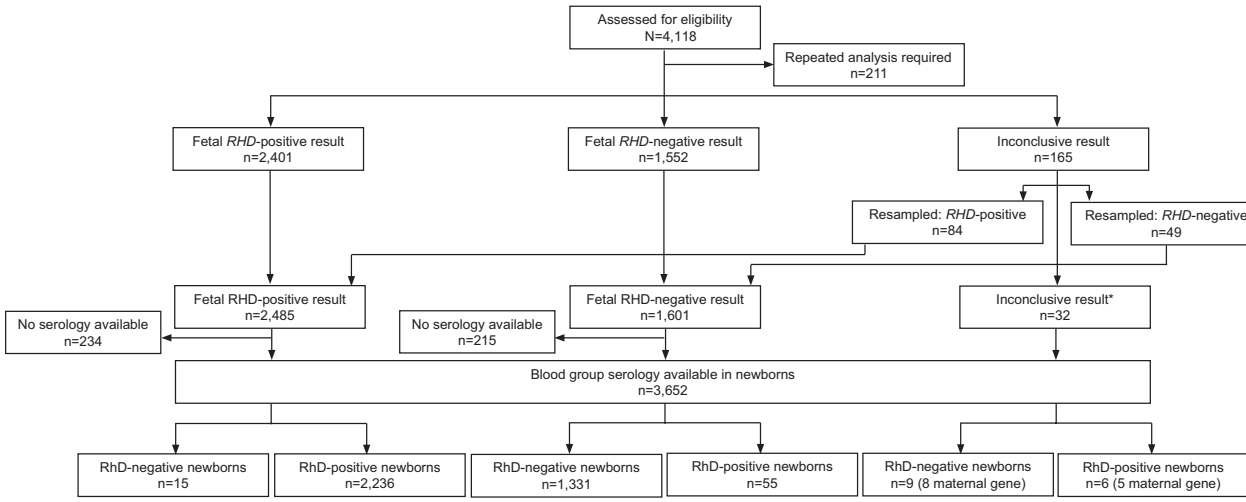

Fig. 1. Summary of the results of fetal *RHD* DNA tests after the first and second samples and the blood group serology results of the newborns. *14 maternal *RHD* gene, 18 not resampled.

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being discordant for RhD type. In all twin pairs, *RHD* genotype was correctly determined, ie, *RHD*-positive if at least one fetus carried the *RHD* gene. The cycle threshold values for fetal *RHD* when analyzed in first, second, and third trimesters are shown in Figure 2. A comparison of the cycle threshold values using a Kruskal-Wallis test indicated a significant effect of trimester (P=.001).

Blood group serology results for the newborns were missing for 466 pregnancies, leaving 3,652 newborns for whom the validity of the *RHD* genotyping could be assessed. Based on these, the false-negative rate was 55 of 2,297 (2.4%; 95% confidence interval [CI] 1.8–3.1%) and the false-positive rate was 15 of 1,355 (1.1%; 95% CI 0.6–1.8%) (Table 3). After exclusion of samples obtained before gestational week 8, the false-negative rate was 23 of 2,073 (1.1%; 95% CI 0.7–1.7%) and the false-positive rate was 14 of 1,218 (1.1%; 95% CI 0.6–1.9%), giving a sensitivity and specificity of the test close to 99%. After further exclusion of samples analyzed before gestational week 10, the sensitivity was 99.3% and from gestational week 22 the sensitivity was 100% (Fig. 3).

There were 14 samples for which the fetal *RHD* type could not be determined, as a result of an early *RHD* signal in the assay, which was suspected to be caused by a maternal gene. All of these were confirmed as having a maternal *RHD* gene, either by serologic retyping only (n=4) or by serologic and genomic typing at the Nordic reference laboratory in Lund, Sweden (n=10), where seven different molecular backgrounds for the weak RhD expression were defined, two of which are new (Table 4). For samples in which a maternal gene was suspected and later confirmed, the fetal *RHD* was reported as undetermined and Rh prophylaxis was recommended.

Table 2. Characteristics of the Pregnancies and Study Samples (n=4,118)

Characteristic	
Age (y)	31 (14–51)
Gestational week at first test	10 (3–40)
Twin pregnancies	61
Trimester of analysis	
First trimester (weeks 3–12)	3,108 (75.5)
Second trimester (weeks 13–28)	774 (18.8)
Third trimester (week 29 and later)	177 (4.3)
Unknown	59 (1.4)
Fetal RhD after first sample (n=4,118)	
Positive	2,401 (58.3)
Negative	1,552 (37.7)
Inconclusive	165 (4.0)
Fetal RhD after second sample in 147 of 165 (n=4,100)	
Positive	2,485 (60.7)
Negative	1,601 (39.1)
Inconclusive	14 (0.34)
Of which maternal gene	14
Blood group serology	
Positive	2,297 (55.8)
Negative	1,355 (32.9)
Missing	466 (11.3)
Miscarriage or abortion	260
Moved	72
Delivered elsewhere	47
Stillbirth	4
Home delivery	3
Not done or unknown	80

Data are median (range), n or n (%) unless otherwise specified.

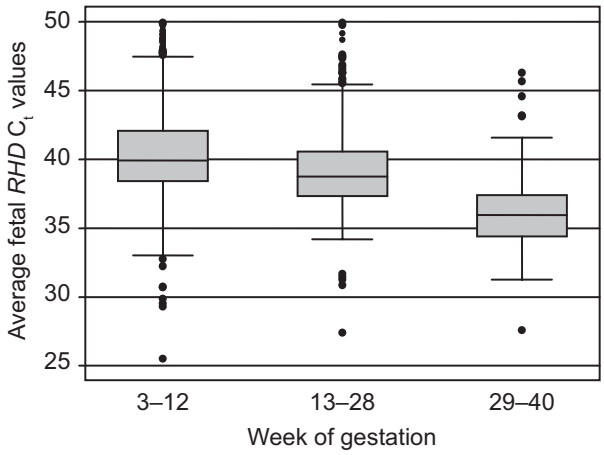

Fig. 2. Box plot of average of fetal *RHD* cycle threshold values in first, second, and third trimesters: upper and lower quartiles define the box, the *horizontal line* within the box indicates median, and distant values are plotted as individual points.

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DISCUSSION

We have evaluated the performance of a screening test for noninvasive fetal *RHD* determination in early pregnancy in the population of RhD-negative pregnant women in the Stockholm area. When used in a routine clinical setting at gestational week 10 (median) with high volumes of samples, the test had high sensitivity and specificity with a low frequency of reanalyses and few inconclusive results. Before 8 weeks of gestation, fetal *RHD* genotype could not always be reliably determined, and once this was recognized (August 2010), analysis for fetal *RHD* was not performed before the 8th week of pregnancy. After exclusion of samples taken before 8 weeks of gestation, the false-negative and false-positive rates were approximately 1% comparable to the diagnostic accuracy of cord blood serology. Among those defined as false-positive (relative to serology as gold standard), we expect some to be the result of weak RhD expression in the child. All discordant samples are currently being followed up.

With information about the *RHD* genotype of the fetus, exposure to a human plasma product can be avoided in approximately 40% of RhD-negative women who are not at risk of immunization. This applies not only to routine antenatal anti-D prophylaxis, but if known early also to miscarriages and terminations as well as amniocentesis and chorionic villus sampling, procedures that are requested by approximately 10% of pregnant women in Stockholm. Unlike other large-scale published studies on cell-free fetal DNA *RHD* genotyping,[6–8] we focused on fetal *RHD* determination in early pregnancy. This strategy also allows decisions to be made whether an RhD-negative woman should be followed with repeated blood samples for antibody screening. With testing in early pregnancy, there is time for the collection of a new sample at a subsequent routine visit in cases in which results are equivocal, thus reducing the burden of extra visits on the pregnant mother and the clinical staff. A consequence of our strategy of testing in early pregnancy is the rather high percentage (11%) of the maternal plasma samples that could not be followed up with cord blood serology, more than half of these resulting from termination of pregnancy or miscarriage. However, this neither reflects a problem with the test itself nor a clinical disadvantage, because all these women had the possibility to benefit from the screening. In Sweden almost all women (greater than

Table 3. Performance of Fetal Genotyping Against Serology as the Gold Standard

All Samples	n	% (95% CI)
Serology-positive (n=2,297)		
Fetal *RHD*		
Positive	2,236	97.3
False-negative	55	2.4 (1.8–3.1)
Inconclusive (with maternal gene)	5	0.02
Inconclusive (second sample not received)	1	
Serology-negative (n=1,355)		
Fetal *RHD*		
False-positive	15	1.1 (.6–1.8)
Negative	1,331	98.2
Inconclusive (with maternal gene)	8	0.06
Inconclusive (second sample not received)	1	
Samples from gestational week 8 onward		
Serology-positive (n=2,073)		
Fetal *RHD*		
Positive	2,045	98.6
False-negative	23	1.1 (.7–1.7)
Inconclusive (with maternal gene)	4	
Inconclusive (second sample not received)	1	
Serology-negative (n=1,218)		
Fetal *RHD*		
False-positive	14	1.1 (.6–1.9)
Negative	1,196	98.1
Inconclusive (with maternal gene)	7	
Inconclusive (second sample not received)	1	

CI, confidence interval.

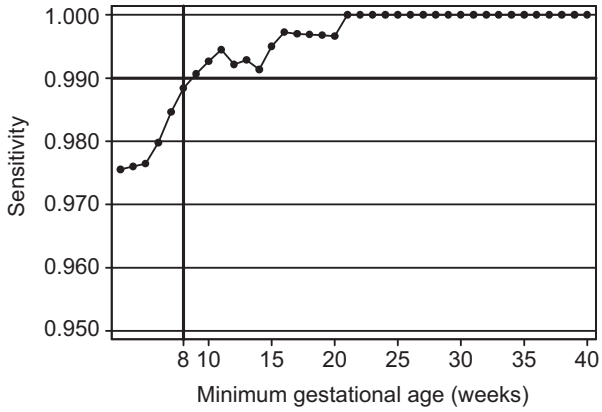

Fig. 3. Sensitivity compared with threshold of gestational age included in calculation.

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99%) book an appointment early in the first trimester for antenatal care, and they will undergo routine rubella screening. Thus, the present program can be included in the routine management and will not require any extra appointment for blood sampling. The argument against early screening is that cell-free fetal DNA concentration is lower than in later pregnancy with a slightly higher rate of false-negative results. The sensitivity of fetal *RHD* detection is 100% from gestational week 22. A limitation in our screening test is the lack of a positive control for fetal DNA. Until now no optimal controls for fetal DNA have been identified.[25] DNA sequences for the Y chromosome can be used only for male fetuses[26] and use of the methylated *RASSF1A* gene as a fetal DNA control adds extra steps, extra costs, and potentially more reanalyses.[27] Most centers do not include a positive control for fetal DNA in their screening assays.[25] An alternative strategy could be to retest all samples with a negative result, but this would add logistic challenges and increase costs. The sensitivity of the test, the costs of the program, and the logistics all need to be considered when fetal *RHD* screening is introduced in clinical routine.

The study reported here was performed to validate the performance of the screening test in a true clinical situation with routine staff taking care of the samples and separating and freezing the plasma and where blood sample transport times can be several days. Our experience is that after more than 4 days storage as whole blood at room temperature, the level of DNA is often too high in the sample with a consequent risk of missing a fetal *RHD* signal. This is especially important in first-trimester samples in which the amount of fetal DNA sometimes can be low and a high maternal background may prevent the detection of a weak fetal signal. It has previously been reported that transport time up to 5 days can be accepted in analysis of second-trimester samples.[28] In our study reanalyses had to be performed on 211 specimens (5.1%) as a result of uninterpretable results. In 46, the repeated analysis on the second aliquot after a new DNA extraction gave a conclusive result

Table 4. Summary of Results on 10 of the 14 Samples With Maternal *RHD* Genes*

RhD Serology	n	*RHD* Change	RhD Consequence	ISBT Allele Name	*RHCE* Genotype†	Reference
D_{weak}	1	1154G>C	Gly385Ala	*RHD∗01W.2*	*Ce/cE*	22
$D_{weak}/D_{partial}$	1	602 C>G	Thr201Arg	*RHD∗weak partial 4.0*	***ce**/ce*	22
		667 T>G	Phe223Val			
		819G>A	Silent			
$D_{weak}/D_{partial}$	2	885G>T	Met295Ile	*RHD∗weak partial 11*	***Ce**/ce*	22
D_{weak}	1	340C>T	Arg114Trp	*RHD∗01W.17*	***ce**/ce*	23
D_{weak}	1	1145C>T	Leu382Pro	—‡	*Ce/ce*	—
D_{el}	1	1217_1220delT	Phe407fs	—‡	*ce/ce*	—
D_{el}	1	IVS2-2a>g	Exon skipping	—§	***Ce/ce***	24
D_{el}	1	None detected	—	—	*ce/ce*	—
D neg‖	1	Not tested‖	—	—	—	—

ISBT, International Society of Blood Transfusion.

* Four samples were RhD-positive by serological methods, two that were strongly positive with the routine antiglobulin test, and two that were positive in adsorption and elution tests and were not investigated further. Testing for the *RHD* pseudogene was negative in all cases.

† For those *RHD* alleles reported previously, the *RHCE* haplotype found in *cis* is bolded. In the case of *RHD∗weak partial 11*, two different *RHCE* haplotypes have been reported.

‡ New genetic change, not previously reported according to the ISBT blood group allele nomenclature (www.isbt-web.org) or the dbRBC database (www.ncbi.nlm.nih.gov/projects/gv/mhc/xslcgi.cgi?cmd=bgmut/systems_alleles&system=rh).

§ New genetic change recently (November 2011) reported in four Danish individuals.

‖ Not available for follow-up, serological status according to routine tests only (ie, adsorption or elution not performed).

but in 165 (4%), a new sample had to be requested. In many cases this was the result of high levels of free DNA, after long transport time, extended storage time in the laboratories before the plasma was separated, or all of these. The logistics concerning transport times from the maternity care centers and handling of the samples in the laboratories have been steadily improved during the study to optimize flow.

In previously published studies, different exons or combinations of exons were used as targets for the PCR. The Special Non-Invasive Advances in Fetal and Neonatal Evaluation Network of excellence advocates the combination of exon 5 and exon 7 to discriminate the pseudogene (*RHDφ*) present in many individuals of African descent.[29,30] Recent studies[31,32] describe multiplex assays with exons 4, 5, and 7 or exons 4, 5, 7, and 10. Our study found a high accuracy using the assay described here, which was based only on exon 4 and designed specifically not to detect *RHDφ*. We believe it is desirable that neither a maternal nor fetal pseudogene be detected by a screening assay whose purpose is to provide routine antenatal anti-D prophylaxis only to women at risk for RhD immunization. A fetus with a pseudogene will be reported as *RHD*-negative, consistent with blood group serology. Until recently, the focus of the techniques for fetal *RHD* detection has been on the diagnosis of a few immunized women and analyses have been performed in specialized laboratories so that safety aspects have received a lot of attention with recommendations for analyses of at least two different exons and also for repeated analyses.[33] The purpose of our work was to develop a technique suitable for screening in a routine clinical setting where both the safety and practicality of the assay were acceptable. For example, although the use of more exons enables increased sensitivity, this also results in a more complicated interpretation of the results and the potential need for more reanalyses.

Hemolytic disease of the fetus and newborn is a serious disease that is potentially life-threatening. Antenatal Rh prophylaxis minimizes the risk for immunization and is considered to be cost-effective, but in many countries, this treatment has been offered to all Rhesus-negative women.[34] There are several convincing arguments in favor of introducing fetal *RHD* screening and targeted Rh prophylaxis to those carrying an *RHD*-positive fetus: with the availability of a noninvasive and reliable test, the fetal *RHD* status can be determined, thus avoiding administration of a human-derived plasma product to those who do not need it[35] and provision of prophylaxis to those where it is indicated. The latter consideration is of particular importance in countries such as Sweden where routine antenatal anti-D prophylaxis is not used. We have found that an assay targeting only exon 4 is safe and reliable for screening from gestational week 8 in our population and we have introduced routine Rh prophylaxis in gestational week 28–30 for RhD-negative women for whom we detect an *RHD*-positive fetus. Our conclusion that screening for fetal DNA should not be done before gestational week 8 is supported by a recently published meta-analysis on noninvasive fetal sex determination.[36]

Noninvasive screening in early pregnancy also opens up new possibilities for diagnosis of anomalies, ie, X-linked conditions or single-gene disorders in which early intervention is important. The cost-effectiveness of fetal *RHD* screening combined with targeted antenatal Rh prophylaxis is an important area for further study.

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