Report of the first nationally implemented clinical routine screening for fetal RHD in D– pregnant women to ascertain the requirement for antenatal RhD prophylaxis

Frederik Banch Clausen, Mette Christiansen, Rudi Steffensen, Steffen Jørgensen, Christian Nielsen, Marianne Antonius Jakobsen, Rikke Dyhrberg Madsen, Karina Jensen, Grethe Risum Krog, Klaus Rieneck, Ulrik Sprogøe, Keld Mikkelsen Homburg, Niels Grunnet, and Morten Hanefeld Dziegiel

BACKGROUND: A combination of antenatal and postnatal RhD prophylaxis is more effective in reducing D immunization in pregnancy than postnatal RhD prophylaxis alone. Based on the result from antenatal screening for the fetal RHD gene, antenatal RhD prophylaxis in Denmark is given only to those D– women who carry a D+ fetus. We present an evaluation of the first national clinical application of antenatal RHD screening.

STUDY DESIGN AND METHODS: In each of the five Danish health care regions, blood samples were drawn from D– women in Gestational Week 25. DNA was extracted from the maternal plasma and analyzed for the presence of the RHD gene by real-time polymerase chain reaction targeting two RHD exons. Prediction of the fetal RhD type was compared with serologic typing of the newborn in 2312 pregnancies, which represented the first 6 months of routine analysis.

RESULTS: For the detection of fetal RHD, the sensitivity was 99.9%. The accuracy was 96.5%. The recommendation for unnecessary antenatal RhD prophylaxis for women carrying a D– fetus was correctly avoided in 862 cases (37.3%), while 39 women (1.7%) were recommended for antenatal RhD prophylaxis unnecessarily. Two RHD+ fetuses (0.087%) were not detected, and antenatal RhIG was not given.

CONCLUSION: These data represent the first demonstration of the reliability of routine antenatal fetal RHD screening in D–, pregnant women to ascertain the requirement for antenatal RhD prophylaxis. Our findings should encourage the implementation of such screening programs worldwide, to reduce the unnecessary use of RhIG.

Pregnancy-related immunization against the red blood cell D antigen is the major cause of hemolytic disease of the fetus and the newborn (HDFN), a condition marked by fetal anemia, hydrops fetalis, jaundice, kernicterus, and intrauterine death. Since the late 1960s, postnatal prophylactic treatment with Rh immune globulin (RhIG) has prevented maternal immunization with great efficacy and has reduced the incidence of HDFN in D– women who are carrying a D+ fetus. Postnatal RhD prophylaxis has reduced the immunization risk from 16% to 2%. In the late 1970s, the combination of antenatal and postnatal RhD prophylaxis was then reported to reduce the immunization risk even further, and based on these promising results, antenatal prophylaxis was implemented in several countries. A large-scale study from 2008 showed that the

ABBREVIATIONS: HDFN = hemolytic disease of the fetus and the newborn; RAADP = routine antenatal anti-D prophylaxis.

From the Department of Clinical Immunology, Section 2034, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark; the Department of Clinical Immunology, Aarhus University Hospital, Skejby, Aarhus, Denmark; the Department of Clinical Immunology, Aalborg Hospital, Aalborg, Denmark; the Department of Clinical Immunology, Næstved Hospital, Næstved, Denmark; and the Department of Clinical Immunology, Odense University Hospital, Odense, Denmark.

Address reprint requests to: Frederik Banch Clausen, Department of Clinical Immunology, Section 2034, Rigshospitalet, Copenhagen University Hospital, Blegdamsvæj 9, DK-2100, Copenhagen, Denmark; e-mail: frederik.banch.clausen@rh.regionh.dk.

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combination of antenatal and postnatal RhD prophylaxis reduced the immunization risk by an additional 50%, with a similar reduction in the incidence of severe HDFN.9 Consequently, Pilgrim and colleagues1 have estimated the reduction in immunization risk after the introduction of antenatal prophylaxis to be 66%.

According to current practice of routine antenatal anti-D prophylaxis (RAADP), antenatal RhIG is given to all D− pregnant women, which is unnecessary for D− women who are carrying a D− fetus. This occurs in approximately 40% of pregnancies in Europe.8 Contrary to practice in North America, Canada, Australia, and many European countries, the standard care for D− Danish women before 2010 comprised antenatal antibody screening and postnatal anti-D only. Established practice was considered satisfactory. However, several international publications demonstrated a potential for optimization, and a thorough revision of antenatal care of D− women was initiated by the Danish National Board of Health.

On the basis of the discovery of cell-free fetal DNA circulating in maternal plasma,9 detection of the fetal RHHD gene offers a tool for noninvasive prenatal prediction of the fetal RhD type.2,10 Consequently, it is possible to identify D− women who are carrying a D+ fetus and to include only those women in the RAADP program. Unnecessary treatment with costly RhIG is thus avoided as was suggested more than a decade ago.11

Real-time polymerase chain reaction (PCR)-based detection of fetal RHHD is exceedingly reliable,2,12,13 and recent large-scale feasibility studies of antenatal RHHD screening have been conducted, reaching sensitivities of 95.6% to 99.8%.14-16 Fetal DNA detection has been improved by focused research activity,2,17 including research on improving DNA extraction.16,18,19 In addition, increased understanding of the genetic background of RhD has facilitated strategically designed assays to navigate among the variants of the RHHD gene in the highly polymorphic Rh blood group system.2,20 In Europe, a major contributor to this field has been the Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence.21

For fetal RHHD detection, false-negative results are typically due to low quantities of fetal DNA in maternal plasma, whereas false-positive results are primarily caused by the presence of pseudogenes or variant genes that do not produce D epitopes.22 The primary objective in a clinical screening program is to avoid false-negative results because women with undetected D+ fetuses will be at risk of immunization.

A national RAADP program was implemented on January 1, 2010, in Denmark. The program represents a transition from the former practice of postnatal RhD prophylaxis only (a single dose of 250 to 300 μg of RhIG) to a combination of antenatal and postnatal RhD prophylaxis (each with a single dose of 250 to 300 μg of RhIG). The antenatal RhIG dose is given at Gestational Week 29 to nonimmunized, D− women who were found to be positive in an antenatal screening for fetal RHHD. This program was accepted by the National Board of Health in Denmark, with the prerequisite that it should be cost-neutral compared to the administration of RhIG to all D− pregnant women. Antenatal RHHD screening is performed by targeting specific exon sequences in the RHHD gene in DNA extracted from maternal plasma obtained from a blood sample taken in Gestational Week 25. This screening is conducted via five different health care regions in Denmark, and each region employs its own method.

We present an evaluation of the first 6 months of the antenatal RHHD screening program in Denmark. To our knowledge, this is the first national clinical application of noninvasive prenatal detection of fetal RHHD to ascertain the requirement for antenatal RhD prophylaxis.

**MATERIALS AND METHODS**

According to regulations from the National Board of Health in Denmark, all pregnant women who test serologically D− at the primary pregnancy-related blood grouping in Gestational Weeks 6 to 12 are offered antenatal RHHD screening. For this purpose, ethylenediaminetetraacetate blood was sampled by the woman’s general practitioner at Gestational Week 25, and the sample was sent to one of five regional laboratories for DNA analysis. Each region applied its own method, because in-house methods existed in advance and were developed and validated based on in-house expertise and technology (see Table 1 for an overview of the methods from the five different health care regions). DNA was extracted from 1 mL of maternal plasma using automated DNA extraction systems. Fetal DNA was detected by real-time PCR that targeted two exons of the RHHD gene, Exons 5 and 7, Exons 5 and 10, or Exons 7 and 10. The plasma-equivalent per PCR (plasma volume × template volume/elution volume) ranged from 83 to 167 μL. Total DNA was assessed by detecting housekeeping genes (see Appendix S1, available as supporting information in the online version of this paper, for detailed descriptions of each of the methods).

Based on the result of the DNA analysis, the fetal RHHD status was categorized as follows: RHHD+, RHHD−, or inconclusive (the last category includes all samples that were inconclusive either due to methodologic issues or due to variant D types in either the pregnant women or the fetus). Only the outcome “RHHD−” resulted in exclusion from the recommendation to receive antenatal RhD prophylaxis. Results were reported electronically to both the general practitioner and the maternity ward, since administration of RhIG was conducted by midwives at a routine visit in Gestational Week 29.
The fetal RHD type based on the antenatal RHD screening result was systematically compared to the postnatal serological D typing of the newborn to evaluate assay variables, such as sensitivity, specificity, and accuracy (accepting the postnatally determined D phenotype as the true D type). Each variable and binomial 95% confidence intervals (CIs) were calculated using computer software (GraphPad Prism 5.02, GraphPad Software, Inc., San Diego, CA).

For the present evaluation, each laboratory was asked to supply data, including sample identification number, antenatal RHD screen result, newborn RhD type, and assigned recommendation for antenatal RhD prophylaxis. Subsequently, data from fetal typing without a newborn type were excluded from this report. Data from all the health care regions were analyzed centrally. A total of 3094 samples were tested between January and June 2010; 114 samples were retested. We obtained complete results from 2312 pregnancies. Ethnicity was not recorded, but the vast majority of the pregnant women were of European descent. The present evaluation was performed as part of a national quality assessment program.

**RESULTS**

We studied the results from 2312 pregnancies representing the first 6 months of routine antenatal RHD screening in Denmark. A total of 1411 (61%) newborns were D+, and 901 (39%) newborns were D−. For routine antenatal RHD detection, the national sensitivity was 99.9% (95% CI, 99.5%-100%). Table 2 summarizes the variables of sensitivity, specificity, and accuracy obtained in each region and nationally.

As a consequence of routine antenatal RHD screening, an unnecessary recommendation for antenatal RhD prophylaxis was avoided in 862 (37.3%) cases. Antenatal RhD prophylaxis was unnecessarily recommended for only 39 women (1.7%). In two (0.087%) pregnancies, a D+ fetus was not detected, and antenatal RhIG was not given; however, the women received postnatal RhIG. In addition, we obtained six (0.26%) false-positive results and 74 (3.2%) inconclusive results. For each of the inconclusive samples, it was recommended that the woman should receive antenatal RhD prophylaxis. Details on the false-positive, false-negative, and inconclusive results are presented in Table 2.

The national accuracy based on all data including the inconclusive results was 96.5% (95% CI, 95.6%-97.2%). The national accuracy for predicting the fetal RhD type based on the data excluding the inconclusive results was 99.6% (95% CI, 99.3%-99.9%). Notably, based on the number of women receiving an unnecessary recommendation for antenatal prophylaxis (URAP, Table 2), the probability of a correct recommendation for antenatal RhD prophylaxis was 98.3% (95% CI, 97.7%-98.8%). In regard to
the risk of immunization, a high sensitivity of minimum 99.5% was reached in all regions with different methods and different RHD exon targets.

TABLE 2. Performance of routine antenatal RHD screening in Denmark (January to June 2010)

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Accuracy (%)*</th>
<th>Accuracy (%)†</th>
<th>TP</th>
<th>TN</th>
<th>FP</th>
<th>FN</th>
<th>INC</th>
<th>URAP</th>
<th>URAP (%)</th>
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<tr>
<td>1</td>
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<td>99.5</td>
<td>98.7</td>
<td>99.1</td>
<td>99.1</td>
<td>186</td>
<td>149</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0.6</td>
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<tr>
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<td>558</td>
<td>99.7</td>
<td>100</td>
<td>99.8</td>
<td>95.2</td>
<td>344</td>
<td>187</td>
<td>0</td>
<td>1</td>
<td>26</td>
<td>14</td>
<td>2.5</td>
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<td>99.6</td>
<td>93.6</td>
<td>147</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>16</td>
<td>8</td>
<td>3</td>
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<tr>
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<td>6</td>
<td>2</td>
<td>74</td>
<td>39</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* Excluding inconclusive results.
† Including inconclusive results.
FN = false-negative results; FP = false-positive results; INC = inconclusive results; n = number of completed pregnancies; TN = true-negative results; TP = true-positive results; URAP = unnecessary recommendation for antenatal prophylaxis.

Detailed description of the FN, FP, and INC results.

FN
Region 1: One FN due to weak PCR amplification led to the conclusion, based on preliminary criteria, that the sample was RHD−; the criteria were subsequently changed. Region 2: One FN was RHD− (but RHD+ in later testing of frozen sample), due to either failed fetal DNA extraction or a sample mix-up.

FP
Region 1: One FP was positive for RHD Exons 7 and 10 and negative for Exons 4 and 5. One FP was most likely caused by a pipetting error during the processing of the plasma sample. Region 3: Two FPs were due to fetal D variants demonstrated by analysis of cord blood DNA, which was positive for Exon 10 but negative for Exons 5 and 7. One FP had an unknown cause. Region 4: One FP was from a pregnancy resulting from assisted reproductive technology; the sample was Exon 10 positive but Exon 5 negative (also in retesting), whereas a blood sample from the newborn was negative for both exons.

INC
Region 2: Twenty-four INCs with high levels of total DNA (>2 ng/μL). The samples were categorized as inconclusive based on the initial concern that excess total DNA might hinder the detection of fetal DNA; however, the fetal RhD type was correctly predicted in all 24 samples. Two INCs were due to a maternal variant D type and a fetal DVI type. Region 3: Six INCs were from samples with two of three positive PCR replicates (postnatally, five were determined to be D− and one D+). Thirteen INCs were maternal weak D. Two INCs were maternal D0 as determined by serology. Ten INCs were maternal D− but RHD+. Region 4: Fifteen INCs were from one failed PCR procedure, which caused uninterpretable results. One INC was due to the weak detection of Exons 5 and 10; the newborn was D−. Region 5: One INC was due to the weak detection of Exon 10; the newborn was D+.

DISCUSSION

We present the first national clinical application of noninvasive routine antenatal screening for fetal RHD in D− women using cell-free fetal DNA extracted from maternal plasma. We report an overall fetal RHD gene detection sensitivity of 99.9%, according to the postnatal serologic D typing of the newborn. This study is the largest to date detecting fetal RHD, and our results demonstrate the reliability of routine antenatal RHD screening in line with the reliability demonstrated in recent large-scale feasibility studies. Importantly, routine screening faces the challenge posed by blood sampling by general practitioners who have only few D− pregnant patients every year. Subsequently, a few GPs mistakenly draw blood from D+ women. Another challenge is the logistics concerning the transportation of blood samples from remote sites to collection sites and further transport to the five regional laboratories. Finally, it sometimes proved challenging to report the results to the correct maternity ward along with the general practitioner who requested the analysis.

In this study, the high sensitivity of our analyses was most likely due to automated DNA extraction with high plasma-equivalents per PCR and blood sampling at Gestational Week 25, when the concentration of cell-free fetal DNA in the maternal plasma is significantly higher than earlier in pregnancy. Overall, we observed few discrepancies, and there were only two with clinical relevance for the immunization risk. We identified the cause of one of the false-negative results; fortunately, this was an early mistake that will not be repeated. It signifies that the criteria for determining results should be chosen with great care, based on extensive knowledge of the assays. The false-positive results were due to fetal variant D types in three cases, a presumed pipetting error in one case, and unknown causes in two cases. One of the unknown causes could have been a vanishing twin or placental chimerism. One false-positive result should have been categorized as inconclusive, but the inconclusive category was not applied initially in that region at the time of the discrepancy.

There was a large group of inconclusive results. This outcome was expected, based on our use of strategies with the purpose of avoiding false-negative results. In Regions 2, 3, and 5, women with weak D types were included in the antenatal RhD prophylaxis program because such women, although rarely, may be immunized by a D− fetus. However, the fetal RHD type could not be determined due to masking by the maternal RHD gene. The inconclusive results were obtained from samples with maternal variant D types (weak D, D0, and pseudogenes),
weak PCR amplification, excess total DNA, and a failed PCR procedure. The presence of variant D types was consistent with observations from other studies.15,25

The concern that the detection of fetal DNA could be hampered in samples with excess total DNA proved to be unfounded because these samples were predicted correctly (n = 24). Therefore, the applied procedure of accepting only a maximum of 2 ng/μL total DNA was abandoned after August 2010. In addition, individual validation studies in three different regions revealed that fetal DNA was detectable even with very high levels of total DNA and after 8 days of transport (data not shown).

Finning and colleagues15 obtained similar results when detecting RHD Exons 5 and 7, which is equivalent to the testing performed in Region 1; they generated three (0.2%) false-negative results using a manual DNA extraction system and one (0.1%) false-negative result using an automated DNA extraction system. They obtained three (0.3%) and seven (0.7%) false-positive results for the two extraction methods; their sample population contained only a few maternal weak D types. Similarly, in a Dutch feasibility study with 1257 informative pregnancies, Müller and colleagues16 found two (0.2%) false-negative results using a manual DNA extraction system and one (0.1%) false-negative result using an automated DNA extraction system. They obtained three (0.3%) and seven (0.7%) false-positive results for the two extraction methods; their sample population contained only a few maternal weak D types. Similarly, in a Dutch feasibility study with 1257 informative pregnancies, there were three (0.2%) false-negative and five (0.4%) false-positive results, and 0.6% of the women carried a variant RHD gene.14 In our study, 0.5% of the women carried a variant RHD gene.

Our results demonstrate the reliability of routine antenatal screening for fetal RHD based on the detection of fetal DNA in maternal plasma samples obtained in Gestational Week 25. The data were obtained from five different laboratories representing the five health care regions of Denmark. The screening was performed satisfactorily in all regions, demonstrating that different assays targeting different RHD exons may be successfully applied. Due to the decentralized structure of the Danish health care regions, the details of the assays were subject to the decision of each region. Therefore, different solutions are used in some of the regions.

The rationale for performing antenatal RHD genotyping is to implement a directed administration of anti-D, avoiding unnecessary use of and exposure to RAADP. The central objective is the highest possible sensitivity of the assays to avoid undetected RHD+ fetuses. To reach this, it is even acceptable to suffer a lower specificity leading to unnecessary administration of anti-D in a small number of cases. Our collective sensitivity was 99.9%. Altogether, 37.3% of the women were correctly recommended not to receive antenatal RhD prophylaxis, as opposed to a standard RAADP program where antenatal RhD prophylaxis would be administered to each and every D− woman. In our study only 1.7% of the women were unnecessarily recommended to receive RAADP.

Despite the excellent sensitivity demonstrated here, we advocate that serologic cord blood typing of the newborn should be continued, at least for women with an antenatal RHD− screening. This is primarily based on a concern that a lack of participation in the antenatal RHD screening program could lead to lack of postnatal anti-D as well if postnatal serologic analysis is terminated.

We recommend that an RHD screening analysis is based on testing a single sample. Even a limited algorithm for further testing will influence cost-effectiveness and the timely reporting of results to clinicians. In addition, obtaining a second sample from a large number of pregnant women would be logistically impracticable, time-consuming, and economically highly unfavorable. Thus, the screening analysis and the corresponding recommendation for administration of anti-D should be safe, robust, and independent of retesting as far as possible.

A special challenge has been recommendations for women with variant D types. For these, the exact serologic practice and the distinction between D+ and D− are reflected in the number of RHD screenings that turn out with an unambiguous positive result as indicated by a low Ct value. We have chosen a pragmatic approach and use the diagnostic term inconclusive for these cases and recommend RAADP for these women. By doing this, we address the theoretical risk of a variant D woman being immunized by her D+ fetus. Different serologic practice in the five regions leads to different numbers in this category.

The RAADP regime was planned for by the national Danish health authorities, and information had been distributed to all general practitioners approximately half a year before the implementation of the program. Screening and RAADP is thus an integrated part of the present standard regime for antenatal care, and it is publicly funded. Our calculations show that the expenses for routine antenatal screening are covered by the reduction in anti-D usage.

The actual implementation of the program was slow with an initial number of samples far below the expected number. However, we did observe a significant improvement in compliance with daily numbers of samples received that gradually approached the expected numbers. For health care Region 5, this improvement occurred after the study period due to procedural obstacles for implementation. Disregarding health care Region 5, an estimated 80% of the expected number of samples reached the laboratories for DNA analysis. We also observed that the gestational ages of the samples received deviated from the scheduled 25 weeks. Results from a full compliance study are not available. After a recommendation of antenatal anti-D, it is also likely that full compliance of antenatal administration of anti-D is
not reached yet. A central issue is to assess the degree of compliance at several points in the program, as well as the improvement of compliance; such a compliance study is in progress.

The crucial issue for future investigation is the clinical effect of the RAADP program in Denmark. We estimate that approximately 10 years of data collection will be needed in Denmark (which has 5.6 million inhabitants and annually 9500 births by D− women) to assess the clinical effect of the program at a level comparable to the study from Koelewijn and colleagues. Preparation for such a long-term study is in progress.

In conclusion, noninvasive routine antenatal screening for fetal RHD in maternal plasma from Gestational Week 25 can be conducted with high sensitivity, predicting the fetal RhD type with high accuracy. In this study, 37.3% of the D− women were excluded from an unnecessary recommendation for antenatal RhD prophylaxis, thus avoiding unnecessary treatment and the use of costly RhIG.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest relevant to the manuscript submitted to TRANSFUSION.

REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods from each of five health care regions in Denmark.

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