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Effect of high throughput *RHD* typing of fetal DNA in maternal plasma on use of anti-RhD immunoglobulin in RhD negative pregnant women: prospective feasibility study

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ABSTRACT

Objectives To assess the feasibility of applying a high throughput method, with an automated robotic technique, for predicting fetal RhD phenotype from fetal DNA in the plasma of RhD negative pregnant women to avoid unnecessary treatment with anti-RhD immunoglobulin.

Design Prospective comparison of fetal *RHD* genotype determined from fetal DNA in maternal plasma with the serologically determined fetal RhD phenotype from cord blood.

Setting Antenatal clinics and antenatal testing laboratories in the Midlands and north of England and an international blood group reference laboratory.

Participants Pregnant women of known gestation identified as RhD negative by an antenatal testing laboratory. Samples from 1997 women were taken at or before the 28 week antenatal visit.

Main outcome measures Detection rate of fetal RhD from maternal plasma, error rate, false positive rate, and the odds of being affected given a positive result.

Results Serologically determined RhD phenotypes were obtained from 1869 cord blood samples. In 95.7% (n=1788) the correct fetal RhD phenotype was predicted by the genotyping tests. In 3.4% (n=64) results were either unobtainable or inconclusive. A false positive result was obtained in 0.8% (14 samples), probably because of unexpressed or weakly expressed fetal *RHD* genes. In only three samples (0.2%) were false negative results obtained. If these results had been applied as a guide to treatment, only 2% of the women would have received anti-RhD unnecessarily, compared with 38% without the genotyping.

Conclusions High throughput *RHD* genotyping of fetuses in all RhD negative women is feasible and would substantially reduce unnecessary administration of anti-RhD immunoglobulin to RhD negative pregnant women with an RhD negative fetus.

INTRODUCTION

In 2002 the National Institute for Health and Clinical Excellence (NICE) recommended that all RhD negative pregnant women should be offered anti-RhD immunoglobulin at 28 and 34 weeks' gestation.¹ It also

“endorsed studies into the feasibility of mass testing antenatally for fetal blood group by analysis of fetal DNA in maternal plasma.”¹ Benefits would be twofold. Firstly, a substantial reduction in the use of anti-RhD immunoglobulin, an expensive blood product in short supply and, secondly, women with an RhD negative fetus would be spared unnecessary exposure to this pooled human blood product. The antigens of the Rh blood group system are located on two proteins encoded by two homologous genes, *RHD* and *RHCE*.² The most immunogenic of the Rh antigens, RhD, is encoded by *RHD* and the RhD negative phenotype usually results from homozygosity for a complete deletion of *RHD* (see bmj.com for further details).

In 2001 the International Blood Group Reference Laboratory of the English National Blood Service introduced fetal *RHD* genotyping from fetal DNA in maternal plasma as a service. Maternal plasma has now almost replaced fetal cells, obtained by amniocentesis or chorionic villus sampling, as the source of fetal DNA. The method currently used routinely for fetal *RHD* genotyping is labour intensive and expensive and therefore not suitable for the mass screening of all RhD negative women. Recent developments in technology and the introduction of automated robotic techniques have brought down costs and increased the potential for higher throughput.

We validated a high throughput *RHD* fetal genotyping technique by comparing the results obtained with the RhD serological phenotype obtained from cord blood taken at delivery.

METHODS

Blood samples—Anticoagulated blood samples were chosen for fetal genotyping from any RhD negative pregnant women attending antenatal clinics that use the Birmingham and Sheffield centres of the National Blood Service for routine ABO and RhD blood grouping and antibody screening. This did not involve taking additional blood samples to those collected for routine testing. The blood samples were collected at the women's 28 week visit to the antenatal clinic. Ethnicity was 55% white, 8% Asian, 1.5% black, 0.5% Caribbean

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Results of testing 1869 DNA samples from plasma of RhD negative pregnant women for fetal *RHD* and comparison with serologically determined phenotype of her baby's cord sample*

Predicted phenotype from fetal DNA	Serological phenotype of cord sample	No (%)	Conclusion
RhD positive	RhD positive	1118 (59.8)	Correct
RhD negative	RhD negative	670 (35.9)	Correct
RhD positive	RhD negative	14 (0.8)	False positive
RhD negative	RhD positive	3 (0.2)	False negative
<i>RHD</i> variant	4 RhD positive/4 RhD negative	8 (0.4)	Inconclusive
Inconclusive	13 RhD positive†/18 RhD negative	31 (1.7)	Inconclusive
Inconclusive‡	18 RhD positive/7 RhD negative	25 (1.3)	Inconclusive

*If anti-RhD is given only when predicted phenotype from DNA is RhD positive, sensitivity of test is 96.7% (95% CI 95.5% to 97.6%) and specificity is 98% (96.7% to 98.8%). If anti-RhD is given when predicted phenotype from DNA is RhD positive, *RHD* variant, or inconclusive, sensitivity is 99.7% (99.2% to 99.9%) and specificity is 94% (92.0% to 95.5%).

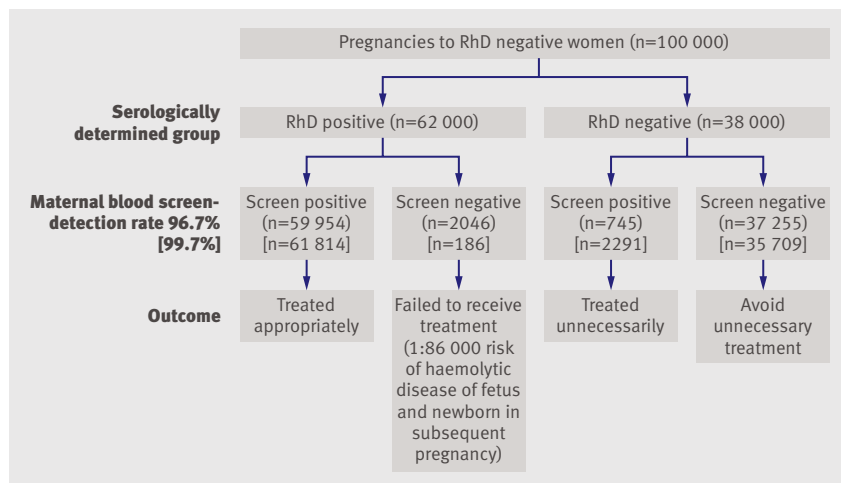
†One resulting from DNA extraction failure.

‡*RHD* detected in maternal DNA.

black, 1% mixed, 1% other, and 33% unknown or not given. We selected at least 1500 individuals on the basis of 80% power to detect an error rate of 2%.

Robotic isolation of DNA from maternal plasma and detection of *RHD*—DNA was robotically extracted from 0.56 ml of maternal plasma. The extracted DNA was used for real time quantitative polymerase chain reaction to detect the presence of fetal *RHD* gene sequences and to quantify total (maternal and fetal) DNA in the plasma. These tests were interpreted without knowledge of the RhD phenotype, which was subsequently determined on red cells from the cord blood sample. The procedure was carried out either immediately or the extracted DNA was stored at -30°C until testing was performed. See bmj.com for details of laboratory techniques.

Statistical analysis—We used Fisher's exact test to determine the sensitivity and specificity of the test and the Mann-Whitney rank sum test to compare the effect of total DNA concentration on the accuracy of the prediction of fetal RhD status and whether to recommend giving anti-RhD immunoglobulin.



Hypothetical flow diagrams of fetal *RHD* screening from maternal blood, with positive screen result classes as predicted phenotype from DNA of RhD positive only or with predicted phenotype from DNA of RhD positive, *RHD* variant, and inconclusive results

Serological RhD testing on cord bloods—The accredited testing laboratories in the National Blood Service and in hospital trusts performed RhD typing on cord blood samples using routine serological methods. At that time they did not know the genotype as determined from the fetal DNA.

RESULTS

Paired analyses—*RHD* genotyping was performed on DNA from 1997 pregnant women, with a mean gestational age of 27.9 weeks (median 28 weeks, range 8–38 weeks). Most (92%) samples were tested at 26–32 weeks' gestation. Cord blood RhD phenotypes were available from 1869 deliveries; 128 fetal phenotypes were not available for paired analysis because 124 cord samples were untraceable and there were four fetal deaths.

Prediction of fetal RhD phenotype from DNA—In 95.7% of cases the fetal RhD status predicted from the genotype was the same as the serologically determined phenotype from cord blood: 1118 (59.8%) were RhD positive and 670 (35.9%) were RhD negative. There were 14 false positive results (0.8%), and only three samples (0.16%) gave “false negative” results. Eight samples (0.4%) gave “variant *RHD*” results and inconclusive fetal genotyping results were obtained from 56 samples (3%) (table). See bmj.com for details.

Accuracy of test to determine requirement for anti-RhD administration—We compared two alternative models for determining the accuracy of the maternal plasma test. The fetus was categorised as “screen positive” if either the predicted phenotype from DNA was RhD positive, or the predicted phenotype from DNA was RhD positive, *RHD* variant, or inconclusive. With the first model, the detection rate was 96.7%, the false positive rate was 1.96%, and the odds of being RhD positive given a positive result were 80:1. By the second method, the detection rate was 99.7%, the false positive rate was 6.0%, and the odds of being RhD positive given a positive result were 27:1. The second, more cautious, definition is more beneficial (figure).

Discrepant results—Many samples that gave false negative or inconclusive results were more than 14 days old and had excessively high levels of maternal DNA, probably as a result of the breakdown of maternal leucocytes. Under usual clinical laboratory criteria these samples would not have been accepted for testing and repeat samples requested.

DISCUSSION

We have developed a high throughput technology for determining fetal *RHD* genotype from cell free fetal DNA in the plasma of RhD negative pregnant women. We tested almost 2000 samples from RhD negative women and compared the results with RhD phenotypes obtained by serological testing of red cells from the corresponding cord blood samples. There was concurrence between genotype and phenotype in 95.7% of the tests. In 0.8% of the tests the genotype falsely predicted an RhD positive phenotype and in 0.16% of the tests the genotype falsely predicted an

WHAT IS ALREADY KNOWN ON THIS TOPIC

Fetal RhD phenotype can be predicted from fetal DNA in the plasma of RhD negative pregnant women

About 38% of RhD negative pregnant women have an RhD negative fetus and might receive unnecessary antenatal anti-RhD immunoglobulin

WHAT THIS STUDY ADDS

An automated high throughput method developed for determining fetal RhD phenotype from fetal DNA in the plasma of RhD negative pregnant women is accurate

Screening of fetal *RHD* in all RhD negative pregnant women is feasible

RhD negative phenotype. In the remaining 3.4% of the tests the genotyping results were inconclusive.

Relevance of false positive results

The incidence of an *RHD* positive genotype associated with an RhD negative phenotype (0.8%) is consistent with the prevalence of *RHD* genetic variants associated with lack of phenotypic expression of an RhD antigen found in other studies.^{3,4} Such “false positives” would, however, be of limited importance. Only a few mothers would continue to receive anti-RhD immunoglobulin unnecessarily with *RHD* genotyping compared with the large numbers currently treated in the absence of fetal genotyping. The red cells of some of these fetuses probably express small quantities of RhD antigen, not detected by routine serological testing, and so administration of anti-RhD could be considered appropriate.

Relevance of false negative results

A false negative result could be more important because withholding treatment would be associated with potential alloimmunisation and morbidity or mortality from haemolytic disease of the fetus and newborn in subsequent pregnancies. The overall risk of antenatal alloimmunisation depends on the likelihood of a subsequent pregnancy (62%) and that the baby will be RhD positive (71.5%, assuming no change of partner). Based on the observed detection rate in this trial of 99.7%, the risk of a future pregnancy being affected by haemolytic disease of the fetus and newborn that would have been prevented if all women had received anti-RhD prophylaxis is 1:86 000 RhD negative pregnant women. About 5% of sensitisations result in fetal or neonatal death and another 5% of infants might experience mild to severe developmental problems, therefore a false negative result presents a 1:860 000 risk of additional fetal or neonatal loss or of developmental problems.

In our study only three samples gave an apparently false RhD negative result. These were delayed in transport. Strict implementation of a policy of testing only samples that were less than seven days old would reduce the risk of false negative results.

Variant *RHD* genes

Categorisation of a fetus predicted to carry a variant *RHD* gene as “screen positive” might be an overcautious

approach. Further validation of the test might result in such variants being treated as RhD negative and antenatal immunoglobulin therapy being withheld.

When the mother is predicted to carry an *RHD* gene, a cautious approach should be applied. In this study, 72% of women suspected of having *RHD* genes gave birth to RhD positive babies and treating such inconclusive cases as “screen positive” would be the most accurate approach.

A similar fully automated trial has been performed in Amsterdam.⁵ In 1257 cases researchers obtained three false negative results and five false positive results, giving a diagnostic accuracy of 99.4%.

Future studies

We are about to start feasibility trials on testing maternal blood samples obtained during the first visit to the antenatal clinic and at the visit for the anomaly scan at 20 weeks.

Conclusions

High throughput *RHD* genotyping performed on free fetal DNA in maternal plasma at about 28 weeks' gestation reliably predicts fetal RhD phenotype with an acceptably low rate of false negative results. The introduction of fetal genotyping followed by the withholding of antenatal anti-RhD prophylaxis from mothers with an RhD negative fetus would result in about 36% of women being saved from unnecessary exposure to human blood products, inconvenience, and discomfort.

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