Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience

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Objective To evaluate the diagnostic performance of noninvasive fetal blood group genotyping.

Design Descriptive analysis.

Setting Dutch national reference laboratory for pregnancies complicated by alloimmunisation.

Population All consecutive alloimmunised pregnant women for whom fetal blood group genotyping of rhesus D, c, E or of K in maternal plasma was performed from 2003 up to 2010.

Methods The test results of each individual assay were collected. Real-time polymerase chain reaction was performed for RHD exon 5 and RHD exon 7, or the specific allele of the RHCE or KEL gene. A stringent diagnostic algorithm was applied. In the case of a negative result, the presence of fetal DNA was ascertained by the analysis of the Y chromosome-specific SRY gene or other paternal genetic markers. Results were compared with available serology after birth or genotyping results of amniotic fluid cells.

Main outcome measures Percentage of conclusive test results and diagnostic accuracy.

Results A total of 362 tests was performed (D: n = 168; c: n = 49; E: n = 85; K: n = 60). The median gestational age was 17 weeks (range 7–38 weeks). In 351 women (97%), a test result was issued: in seven samples, the presence of fetal DNA could not be confirmed; in two samples, non-specific amplification in the K assay led to an inconclusive result; in two samples, a maternal silent RHD gene prevented fetal RHD genotyping. No false-positive or false-negative results were found among those women for whom cord blood serology or genotyping results of amniotic fluid cells were available (n = 212).

Conclusions Noninvasive fetal blood group genotyping is accurate and applicable in a clinical diagnostic setting.

Keywords Cell-free DNA, haemolytic disease of the fetus and newborn, maternal plasma, noninvasive prenatal diagnosis.

Introduction

Haemolytic disease of the fetus and newborn (HDFN) is caused by maternal alloantibodies directed against fetal red cell surface antigens that the mother herself lacks. The D antigen of the rhesus (Rh) blood group system is the most frequently involved antigen in HDFN and despite the widespread use of prophylactic antenatal and postpartum anti-D immunoglobulin, RhD alloimmunisation is still a significant cause of fetal and neonatal morbidity and mortality. In addition, alloimmunisation to the c antigen of the Rh blood group system and the K antigen of the Kell blood group system can cause severe HDFN. Antibodies against the C and E antigens of the Rh system or against antigens of other blood group systems rarely lead to clinical manifestations.

In alloimmunised pregnant women, knowledge of the fetal antigen status is beneficial to tailor pregnancy management. In general, blood group antigens are biallelic co-dominant systems and if the father is heterozygously
positive for a certain blood group antigen there is a 50% chance that the fetus does not carry the risk antigen. In these pregnancies, there is no risk of HDFN and no further follow-up is needed. If, however, the fetus does inherit the implicated antigen, careful monitoring for fetal anaemia with serial assessment of maternal antibody titres and activity, fetal Doppler ultrasound measurements of the peak systolic velocity in the middle cerebral artery, and, ultimately, intrauterine fetal blood sampling may be indicated. Traditionally, fetal blood group genotyping has been performed through amniocentesis. This invasive procedure carries a small risk of miscarriage and could potentially enhance maternal sensitisation. The discovery of cell-free immunoglobulin prophylaxis. Moreover, most data evaluate the use of this test to restrict antenatal anti-D ples from nonimmunised D-negative pregnant women, to DNA in case negative results were obtained. Few studies clinical setting and lacked a control for the presence of fetal accuracy.

genotyping in D-negative mothers with close to 100% RHD
K
have reported on noninvasive genotyping of fetal

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Methods
Sanquin Diagnostic Services is the national reference laboratory for pregnancies complicated by alloimmunisation in the Netherlands.

For this study, we collected the test data of all consecutive alloimmunised pregnant women for whom fetal blood group genotyping in maternal plasma was performed in our laboratory from 2003 up to 2010. Fetal D typing was offered from 2003, fetal K typing from 2006, and typing for C and E from 2007. Tests were offered in all alloimmunised pregnancies in which the father was heterozygously positive for the target antigen and was strongly advised if the antibody titre was ≥16 (≥2 for anti-K) or if the antibody-dependent cell-mediated cytotoxicity test result was ≥30%. Tests were performed at the request of midwives or gynaecologists throughout the Netherlands. We advised a minimum gestational age of 9 weeks for fetal D, C and E typing, and 12 weeks for fetal K typing, because of the lower sensitivity of the K assay.

Ethylenediaminetetraacetic acid anticoagulated blood was drawn from both the mother (30 ml) and, if possible, from the reporting father (10 ml) and was sent to our laboratory. Maternal blood samples were centrifuged at 1200 × g for 10 minutes within 48 hours of sampling. The plasma fraction was again centrifuged at 2400 × g for 20 minutes and the supernatant was collected and stored at −20°C until further processing. In the case of fetal K typing, the blood samples were sent by express courier and processed within 8 hours to prevent the increase of the proportion of maternal DNA caused by lysis of nucleated blood cells in the tube that could hamper the specificity of the assay. Both parental samples were typed serologically for D, C/c, E/e and K/k to identify paternal blood group antigens that could potentially serve as a genetic control marker to confirm the presence of fetal DNA.

DNA was extracted in duplicate from 2 × 2 ml plasma using the QIAamp Blood Mini Kit (Qiagen, Hilden, Germany), following the 'Blood and Body Fluid Protocol' recommended by the manufacturer. Volumes of the used reagents were increased proportionately to accommodate the 2-ml sample size. Adsorbed DNA was eluted with 60 µl water.

Real-time polymerase chain reaction (PCR) analysis was performed with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using Taqman chemistry. For RHD detection, RHD exon 5 and RHD exon 7 were analysed by duplex PCR. Both PCRs are positive when an intact RHD gene is present (Figure 1) but no product of RHD exon 5 is generated when a non-functional RHD pseudogene or an RHD-CE-D' gene is present, both genes commonly found in people from African descent. RHD exon 7 PCR is positive in almost all RHD genes, but not in RHD-CE-D'. The use of both of these targets prevents false-positive results in fetuses carrying only an RHD pseudogene or RHD-CE-D' gene, and, at the same time, allows for fetal RHD typing in D-negative mothers carrying these variant RHD genes. For detection of the C and E alleles of the RHCE gene, allele-specific primers from Finning et al. were used. These primer sets show a high specificity with DNA levels up to 50 ng (unpublished results). For detection of the K allele of the KEL gene, we used an in-house-developed allele-specific primer. To prevent mispriming of the K allele-specific primer on the antithetic k allele, we designed a k allele-specific peptide nucleic acid (PNA) probe. Clamping of this PNA
Figure 1. Diagrammatic representation of the RHD and RHCE genes in four haplotypes. (A) Normal RHD gene leading to a D-positive (D+) phenotype. (B) Complete deletion/absence of the RHD gene leading to a D-negative (D−) phenotype. (C) Mutations (white bands) in exons 4, 5 and 6 of the RHCE gene leading to a D− phenotype. (D) RHCE-derived exons (white boxes) in the RHCE-CE-Dγ hybrid gene leading to a D− phenotype. Asterisks in the RHD gene denote the sites used for polymerase chain reaction amplification (exons 5 and 7). Black bands in the RHCE gene represent the single nucleotide polymorphisms encoding either E or e and C or c. Black boxes, RHD exons; white boxes, RHCE exons; RHCEψ, RHD pseudogene.

probe to the k allele prevents non-specific amplification, ensuring allele-specificity of the reaction. To minimise the non-specific amplification of paternal cell-free DNA and to achieve optimal PNA clamping, the level of maternal cell-free DNA was aimed to be as low as possible. Therefore, plasma was separated preferentially within 8 hours of blood sampling in the case of fetal K typing. As a control marker to confirm the presence of fetal DNA, PCR analysis of the Y-chromosome-specific SRY gene was also performed (only applicable in male-bearing pregnancies).27 Part of the albumin gene was amplified as a control for DNA isolation.28 All primer and probe sequences are listed in Table S1. The reactions were set up in a volume of 50 μl, using 25 μl Taqman Universal PCR Master Mix (Applied Biosystems) and 10 μl extracted DNA (for the albumin PCR, 3 μl extracted DNA was used in a 25-μl reaction volume). Primers and probes were used at final concentrations of 300 and 100 nM (900 and 150 nM for SRY, respectively). The final concentration of the k-specific PNA probe was 2 nM. Cycling conditions for all PCRs were 2 minutes at 50°C and 10 minutes at 95°C, followed by 50 cycles of denaturation for 15 seconds at 95°C and primer annealing and elongation for 1 minute at 60°C.

For each of the two DNA isolations undertaken on each sample, PCR for the specific blood group antigen was performed in triplicate. Each replicate was judged for amplification according to previously defined cycle threshold (Ct) values (see Table S2). Interpretation of the combined results of both isolations led to a positive (i.e. antigen-positive fetus) or negative (i.e. antigen-negative fetus) test result. In the case of discrepant results between the two isolations, the test result was inconclusive. Discrepant results between RHD exon 5 and RHD exon 7 PCR (i.e. more than 2 Ct value difference) or Ct values outside the expected range for fetal DNA (i.e. <34) called for further serological and molecular parental RHD analysis. Because of potential non-specific amplification of maternal DNA in the K PCR (inherent to the gene/assay), Ct values >43 led to an inconclusive result. Also, because of the lower sensitivity of the K assay, when a negative K genotyping result was obtained before 18 weeks of gestation, repeat testing at 18 weeks was recommended.

The PCRs for SRY and albumin were performed in duplicate. SRY was scored positive when the Ct value was <42. For the albumin PCR, a standard of genomic DNA was used (15 000, 1500, 150 pg) to quantify the amount of total cell-free DNA in the sample.

In the case of a negative result for a specific blood group antigen and a positive result for SRY (thereby proving the presence of fetal DNA in the sample), a blood group antigen-negative fetus was reported. In the case of a negative result for a specific blood group antigen and a negative result for SRY, the presence of fetal DNA was ascertained through the use of a set of 24 biallelic insertion/deletion polymorphisms or another discrepant paternally inherited blood group antigen (for a detailed description, see Scheffer et al.29). Only when we were able to confirm the presence of fetal DNA (one or more paternal markers positive in maternal plasma), was a negative result for the specific blood group antigen issued. If the presence of fetal DNA could not be confirmed, the overall test was inconclusive.

As part of continuous test performance quality control, upon reporting the test result to the physician, we requested that we receive follow-up of the neonatal blood group phenotype, in the case that cord blood serology was performed. In the case of subsequent amniocentesis (e.g. because of an inconclusive PCR result in maternal plasma), we requested that we receive the fetal blood group
genotyping result. If no follow-up was received, the requesting physician was contacted by telephone after the expected date of birth to ask whether the blood group phenotype or genotype had been determined.

After collecting all results, descriptive statistics were generated using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The Fisher exact test (two-sided) was used to determine sensitivity and specificity with 95% confidence intervals (95% CI).

Results

From 2003 up to 2010, 362 tests were performed for a total of 346 alloimmunised pregnant women (Table 1). Sixteen women had both anti-c and anti-E alloantibodies and testing for both fetal blood group antigens was performed. The median gestational age at the time of blood sampling was 17 weeks (range 7–38 weeks) for all assays. Five women carried twins; two of these had anti-D alloantibodies, one had anti-E alloantibodies and two had both anti-c and anti-E alloantibodies. In 2009, when testing for all four blood group antigens was available, the majority of tests were requested when serology still indicated the absence of a clinical risk (i.e. antibody titres <16 and antibody-dependent cell-mediated cytotoxicity test results <30% for anti-D, anti-c and anti-E; see Table S3).

Fetal RHD typing was performed in 168 women. Conclusive results were obtained in 161 (96%). A positive test result was issued for 113 samples. In the majority of these (n = 104), both RHD exon 5 and exon 7 PCR were positive, with Ct values within the expected range for fetal DNA (between 34 and 39). In six other women (Table 2; cases 1–6), an abnormally high level of amplification of RHD exon 7 (Ct values between 29 and 32) indicated amplification of a maternal RHD pseudogene. In three further women (cases 7–9), a discrepant amplification of fetal DNA and non-specific amplification of maternal DNA was observed. Both of these pregnancies with a negative RHD exon 5 and RHD exon 7. Sequencing of the RHD gene in maternal genomic DNA demonstrated the presence of a so-called silent RHD gene, in which a mutation in the gene leads to the loss of function. Subsequent genotyping of amniotic fluid cells showed a D-positive and a D-negative child, respectively.

Fetal c typing was performed in 49 pregnancies. Conclusive results were obtained in all. The PCR was positive in 30 pregnancies and negative in 19.

Fetal E typing was performed in 85 pregnancies. All test results were conclusive. The PCR was positive in 52 pregnancies and negative in 33.

Fetal K typing was performed in 60 pregnancies. Conclusive results were obtained in 56 (93%). A positive test result was issued in 24 pregnancies; a negative test result in 32. In two pregnancies, negative for K, the presence of fetal DNA could not be confirmed and the test result was reported as inconclusive. In two other pregnancies, very high (>43) Ct values were obtained and we could not discriminate between the presence of a very low amount of fetal DNA and non-specific amplification of maternal DNA, leading to an inconclusive test result. Both of these samples were drawn at 16 weeks of gestation. Repeat testing at 17 weeks in one woman showed similar Ct values and the test result remained inconclusive. A K-negative child was born. In the other woman, no second maternal blood sample was obtained because of an intrauterine fetal demise. Cord blood serology showed that the child was K-positive. Of 17 pregnancies negative for K before 18 weeks of gestation, repeat testing was performed in seven. All were still negative the second time.

In 71 of the 139 pregnancies (51%) in which the PCR result for the specific blood group antigen was negative, the presence of fetal DNA could be confirmed by a positive

<table>
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result for SRY (Figure 2). In 61 other pregnancies, biallelic insertion/deletion polymorphism markers (n = 50; 36%) or another paternally inherited blood group antigen (n = 11; 8%) could be used. In seven samples (5%), the presence of fetal DNA could not be confirmed and the test results were reported as inconclusive (five for fetal RHD typing and two for fetal K typing, as described above).

Overall, a test result was issued in 97% (351 of 362). In general, test results were reported to the requesting physician within two (in the case of an antigen-positive fetus) to four (in the case of an antigen-negative fetus) working days after blood sampling (data not shown).

Cord blood serology (n = 204) or genotyping results of amniotic fluid cells (n = 8) was available in 212 of the 362 women (59%; Table 3). Fetal RHD typing was positive in 96 of 96 plasma samples from women reportedly giving birth to a D-positive child, resulting in a 100% sensitivity (95% CI 96.2–100%). In all 37 plasma samples from women reportedly giving birth to a D-negative child, fetal RHD typing was negative, resulting in a 100% specificity (95% CI 90.5–100%). Cord blood serology for c and E was performed in 19 and 21 women, respectively. No discrepancies with maternal plasma PCR results were found. Results for fetal K typing could be confirmed in 30 pregnancies. No false-positive or false-negative results were found.

In three of the five women carrying twins, postnatal cord blood serology was performed. In the first, the test result for RHD was positive and a D-positive girl and D-negative boy were born. In the second, the test result for RHD was negative and two D-negative boys were born. In the third, test results for c and E were positive and both boys born had a c-positive and E-positive phenotype.

Of the seven pregnancies with an inconclusive test result because of failure to confirm the presence of fetal DNA, amniocentesis was performed in two, showing a D-negative and a K-negative fetus, respectively. Postnatal cord blood serology in an additional three women showed all three infants to be D-negative. In the remaining two women, cord blood serology was not performed. In all seven pregnancies, a girl was born.

### Discussion

In this study we have evaluated the diagnostic performance of noninvasive fetal blood group genotyping performed over a 7-year period in a national reference laboratory in the Netherlands. We have shown noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised women to be accurate and applicable in a clinical diagnostic setting. Through the application of a stringent diagnostic algorithm on two separate DNA isolations and the confirmation of the presence of fetal DNA in the case of negative PCR results, we were able to report fully conclusive results in 351 of 362 tests performed. Moreover, as far as we could ascertain, no false-positive or false-negative results were found.
All test results were used by the referring physician to guide the clinical and laboratory management of the alloimmunised pregnancy. In our series, 126 women (including six with both anti-c and anti-E antibodies) could be reassured that their fetus was not at risk of haemolytic disease and extensive monitoring for fetal anaemia during pregnancy was not required. Two hundred and nine women (including ten with both anti-c and anti-E antibodies) were informed that their fetus was at risk of haemolytic disease and the appropriate pregnancy management could be arranged. None of these women had to undergo an invasive procedure to determine the fetal antigen status. Women whose test results were inconclusive (n = 11) were monitored as at risk of HDFN, at least until genotyping of amniotic fluid cells, if performed, proved otherwise.

The antigens of the Rh system are encoded by \( RHD \) and \( RHCE \), two almost homologous genes located on chromosome 1 (Figure 1). In the Caucasian population, homozygous deletion of the \( RHD \) gene is the predominant cause of the D-negative phenotype. In contrast, 82% of D-negative black Africans do not have a homozygous deletion of \( RHD \), but carry one or two \( RHD \) variant genes: the \( RHD \) pseudogene\(^{24} \) or the \( RHD-CE-D^\prime \) hybrid gene.\(^ {25} \) Neither produces any epitopes of D. When genotyping for fetal \( RHD \) in a multiethnic population, such as the Dutch, it is important that false-positive results do not result from the presence of these relatively common variant genes. Through the analysis of both exon 5 and exon 7 of \( RHD \) we avoided such false results and were able to predict the fetal D status in eight women in whom an \( RHD \) pseudogene variant gene was present in the mother (two also carrying \( RHD-CE-D^\prime \)), whether or not inherited by the fetus. At the same time, combining both exon 5 and exon 7 PCR results, more rare \( RHD \) genes producing variant D antigens could be detected, preventing false-negative results in three women. In one of the largest validation studies published on noninvasive fetal \( RHD \) genotyping, Rouillac-Le Sciellour et al.\(^ {31} \) amplified \( RHD \) exon 7 and exon 10 in 893 maternal plasma samples. They had to exclude 26 D-negative women carrying an \( RHD \) pseudogene and five carrying \( RHD-CE-D^\prime \), unable to predict the fetal phenotype with the combination of these two targets. Chinen et al.\(^ {32} \), tested 102 D-negative women in a Brazilian population and reported two false-positive results in women carrying an \( RHD \) pseudogene, using an exon 7–exon 10 approach. Other groups using these two targets did reach 100% accuracy,\(^ {33}–^{35} \) but study populations were presumably all-white.

Noninvasive fetal \( RHD \) genotyping is not only a valuable tool in the management of RhD-alloimmunised pregnancies, but also allows antenatal anti-D immunoglobulin prophylaxis to be reserved for only those non-immunised D-negative pregnant women that carry a D-positive fetus.\(^ {13}–^{16} \) In such a fetal \( RHD \) screening setting, typing would be performed upon automated DNA extractions to facilitate high-throughput screening, with an accepted certain loss of sensitivity and specificity at a more advanced gestational age and with a primary aim to avoid false-negative results, whereas false-positive results would be less important.\(^ {36}–^{37} \)

Figure 2. Test results for alloimmunised pregnant women tested from 2003 up to 2010.
No false-positive or false-negative results were reported for the allele-specific assays for c and E used in our protocol. Similar conclusions were published by Finning et al., who used the same set of primers and reached 100% accuracy in 46 samples from pregnant women with anti-E alloantibodies at a more advanced gestational age (mean 23 weeks) and 44 samples from pregnant women with anti-c alloantibodies (mean 26 weeks), with an initial three inconclusive results in the latter. Adhering to their protocols, Gutensohn et al. confirmed their findings in 87 and 100 samples from nonimmunised c-negative and E-negative pregnant women, respectively.

Using conventional allele-specific primers on a real-time PCR platform, detection of the fetal K allele can be hampered by non-specific amplification of the maternal k allele and only a few laboratories therefore offer this test diagnostically. To increase the specificity of the assay we used a PNA probe, preventing mispriming of the K-allele-specific primer. Only in two of the 60 samples tested for K did non-specific amplification prevent a conclusive result. Using conventional allele-specific primers on a real-time PCR platform, detection of the fetal K allele can be hampered by non-specific amplification of the maternal k allele and only a few laboratories therefore offer this test diagnostically. Therefore, accuracy statements should only be made based on these cases.

As other methylation markers are emerging, their use as a universal fetal marker may be implemented in diagnostic protocols soon.

A limitation of our study is the fact that test results could be compared with cord blood serology or genotyping results of amniotic fluid cells in only 59% of women. Therefore, accuracy statements should only be made based on these cases. As our study is an evaluation of data obtained in a clinical diagnostic setting, cord blood serology results were collected in a retrospective manner. It was left to the physician’s clinical judgement at the time of birth whether or not to have cord blood serology performed. We presume that infants for whom cord blood serology was not performed did not have any clinical signs of anaemia. This is supported by the finding that no false-negative results were found in those women in whom cord blood serology was performed. Although we cannot fully exclude false-positive results in those women for whom cord blood serology was not performed, no false-positive results were observed in those women for whom cord blood serology was performed. Although we cannot fully exclude false-positive results in those women for whom cord blood serology was not performed, no false-positive results were observed in those women for whom cord blood serology was performed. Although we cannot fully exclude false-positive results in those women for whom cord blood serology was not performed, no false-positive results were observed in those women for whom cord blood serology was performed.

**Conclusion**

Noninvasive fetal blood group genotyping is a clinical reality. There is no longer a need for invasive procedures to determine the fetal D, c, E or K antigen status.
In alloimmunised pregnant women, noninvasive fetal blood group genotyping provides an easy and safe method to determine whether a fetus is at risk of haemolytic disease, preventing extensive laboratory and clinical monitoring in antigen-negative cases.

Disclosure of interests
None.

Contribution to authorship
All authors were involved in the design of the study. PS collected data and wrote the manuscript. CvdS, GP-C and MdH discussed data and critically revised the manuscript for intellectual content. All authors approved the final version of the manuscript.

Details of ethics approval
According to the Dutch legal system, ethics approval was not needed for this study, as it was not subject to the Medical Research Involving Human Subjects Act (WMO) (see website for the Central Commission on Research Involving Human Subjects at www.ccmo-online.nl).

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Supporting information
The following supplementary materials are available for this article:

Table S1. Primer and probe sequences for fetal D, c, E and K typing.

Table S2. Scoring model for fetal RHD exon 5, RHD exon 7, c, E and K polymerase chain reaction.

Table S3. Antibody-dependent cell-mediated cytotoxicity test results and antibody titres before fetal blood group genotyping in maternal plasma (year 2009).

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

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