DOI: 10.1111/j.1471-0528.2011.03028.x
www.bjog.org

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

PG Scheffer,[a,b] CE van der Schoot,[b] GCML Page-Christiaens,[a] M de Haas[b,c]

[a] Division of Perinatology and Gynaecology, University Medical Centre Utrecht, Utrecht, the Netherlands [b] Department of Experimental Immunohaematology, Sanquin Research Amsterdam and Landsteiner Laboratory, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands [c] Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, Amsterdam, the Netherlands
Correspondence: M de Haas, Department of Immunohaematology Diagnostics, Sanquin Diagnostic Services, PO Box 9190, 1006 AD Amsterdam, the Netherlands. Email m.dehaas@sanquin.nl

Accepted 14 April 2011. Published Online 14 June 2011.

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.

Please cite this paper as: Scheffer P, van der Schoot C, Page-Christiaens G, de Haas M. Noninvasive fetal blood group genotyping of rhesus D, c, E and of K in alloimmunised pregnant women: evaluation of a 7-year clinical experience. BJOG 2011;118:1340–1348.

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.[1,2] 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.[3,4] Antibodies against the C and E antigens of the Rh system or against antigens of other blood group systems rarely lead to clinical manifestations.[5]

In alloimmunised pregnant women, knowledge of the fetal antigen status is beneficial to tailor pregnancy management.[1] 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[6] and could potentially enhance maternal sensitisation.[7] The discovery of cell-free fetal DNA in the plasma of pregnant women at the end of the twentieth century presented a noninvasive, and therefore safe, method to determine the fetal blood group genotype.[8,9] Since then, numerous groups have reported on fetal *RHD* genotyping in D-negative mothers with close to 100% accuracy.[10,11]

Although a small number of laboratories across Europe now offer this test to alloimmunised pregnant women diagnostically,[12] most studies have been performed with samples from nonimmunised D-negative pregnant women, to evaluate the use of this test to restrict antenatal anti-D immunoglobulin prophylaxis.[13–16] Moreover, most data have been obtained in a research setting rather than in a clinical setting and lacked a control for the presence of fetal DNA in case negative results were obtained.[17] Few studies have reported on noninvasive genotyping of fetal *c*, *E* and *K*.[18–20]

As a national reference laboratory, we have been offering noninvasive fetal blood group genotyping of rhesus D, c, E and of K in maternal plasma for alloimmunised pregnant women since the beginning of 2003 using a stringent diagnostic algorithm with the inclusion of fetal DNA identifiers to exclude false-negative results. The aim of the present study was to evaluate the diagnostic performance of these noninvasive fetal blood group genotyping tests, performed in a clinical setting over a 7-year period.

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%.[21] 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 $1200 \times g$ for 10 minutes within 48 hours of sampling. The plasma fraction was again centrifuged at $2400 \times g$ for 20 minutes and the supernatant was collected and stored at −20°C until further processing.[22] 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[23] and *RHD* exon 7[22] 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 nonfunctional *RHD* pseudogene or an *RHD-CE-D*^{s} gene is present, both genes commonly found in people from African descent.[24,25] *RHD* exon 7 PCR is positive in almost all *RHD* genes, but not in *RHD-CE-D*^{s}. The use of both of these targets prevents false-positive results in fetuses carrying only an *RHD* pseudogene or *RHD-CE-D*^{s} gene, and, at the same time, allows for fetal *RHD* typing in D-negative mothers carrying these variant *RHD* genes.[26] For detection of the *c* and *E* alleles of the *RHCE* gene, allele-specific primers from Finning et al.[18] 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

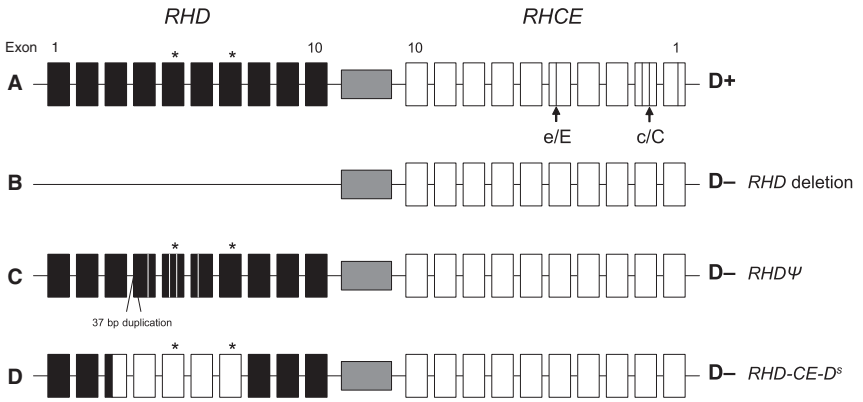

Figure 1. Diagrammatic representation of the *RHD* and *RHCE* genes in four haplotypes. (A) Normal *RHD* gene leading to a D-positive (D+) phenotype. (B) Complete deletion/absence of the *RHD* gene leading to a D-negative (D–) phenotype. (C) Mutations (white bands) in exons 4, 5 and 6 of the *RHDΨ* leading to a D– phenotype. (D) *RHCE*-derived exons (white boxes) in the *RHD-CE-D*^{s} 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; *RHDΨ*, *RHD* pseudogene.

probe to the *k* allele prevents non-specific amplification, ensuring allele-specificity of the reaction. To minimise the non-specific amplification of maternal 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 was performed for fetal E typing (39%), followed by typing for D (29%), c (17%) and K (15%). Most 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* allele. Maternal *RHD* analysis confirmed the presence of a nonfunctional *RHD* pseudogene. In three further women (cases 7–9), a discrepancy between the level of amplification of fetal *RHD* exon 5 and *RHD* exon 7 was observed. Serological and molecular RhD typing of the father revealed the presence of an *RHD* variant gene (DIVa, DAU5 and DNU, respectively), that apparently was inherited by the fetus (leading to, respectively, a negative *RHD* exon 7, a negative *RHD* exon 5, and a weaker *RHD* exon 7 PCR result).

Table 1. Number of fetal blood group genotyping tests performed from 2003 up to 2010

Test	Year							
	2003	2004	2005	2006	2007	2008	2009	Total
D	14	10	20	33	20	39	32	168
c	–	–	–	–	9	21	19	49
E	–	–	–	–	6	36	43	85
K	–	–	–	3	16	24	17	60
Total	14	10	20	36	51	120	111	362

In 51 pregnancies, both *RHD* exon 5 and exon 7 PCR were negative, pointing to a D-negative fetus. In two further pregnancies with a negative *RHD* exon 5 PCR result (Table 2; cases 10 and 11), an abnormally high level of amplification of *RHD* exon 7 exposed a maternal *RHD* pseudogene, which was confirmed after maternal *RHD* analysis. Of these 53 pregnancies, the presence of fetal DNA could be confirmed in 48 and a D-negative fetus was reported. In the other five, the presence of fetal DNA could not be confirmed and the test result was reported as inconclusive.

In two more cases of fetal *RHD* typing (Table 2; cases 12 and 13), no test result could be issued because of a high level of amplification of both *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 2. Results for maternal and paternal *RHD* analysis performed because of atypical fetal *RHD* exon 5 and/or *RHD* exon 7 polymerase chain reaction results

Case	*RHD* PCR plasma		Maternal *RHD* analysis (genotype)	Paternal *RHD* analysis (genotype)	Conclusion fetal RhD status
	Exon 5 Ct	Exon 7 Ct			
1	37	32	*RHDΨ/d*	N/A	D positive
2	39	29	*RHDΨ/d*	N/A	D positive
3	35	30	*RHDΨ/d*	*RHD/DAU*	D positive
4	38	31	*RHDΨ/d*	N/A	D positive
5	38	32	*RHDΨ/d*	*RHD/RHD*	D positive
6	37	30	*RHDΨ/RHD-CE-D^{s}*	*RHD/DIII type 5*	D positive
7	38	Und	*d/d*	*DIVa/d*	D positive
8	Und	36	*d/d*	*RHD/DAU5*	D positive
9	35	39	*d/d*	*RHD/DNU*	D positive
10	Und	31	*RHDΨ/d*	*RHDΨ/DAU*	D negative
11	Und	31	*RHDΨ/RHD-CE-D^{s}*	N/A	D negative
12	30	30	*RHD(343delC)/d*	N/A	inconclusive
13	31	31	*RHD(IVS1+1G>A)/d*	*d/d*	inconclusive

Ct, cycle threshold value; *d*, deletion/complete absence of *RHD* gene; N/A, not available; *RHD*, normal *RHD* gene; *RHDΨ*, *RHD* pseudogene; Und, undetermined (no amplification).
RHDΨ, *RHD-CE-D^{s}*, *RHD(343delC)* and *RHD(IVS1+1G>A)*: *RHD* variant genes leading to a D-negative phenotype.
DAU, DIII type 5, DIVa, DAU5 and DNU: *RHD* variant genes leading to a D-positive phenotype.

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.

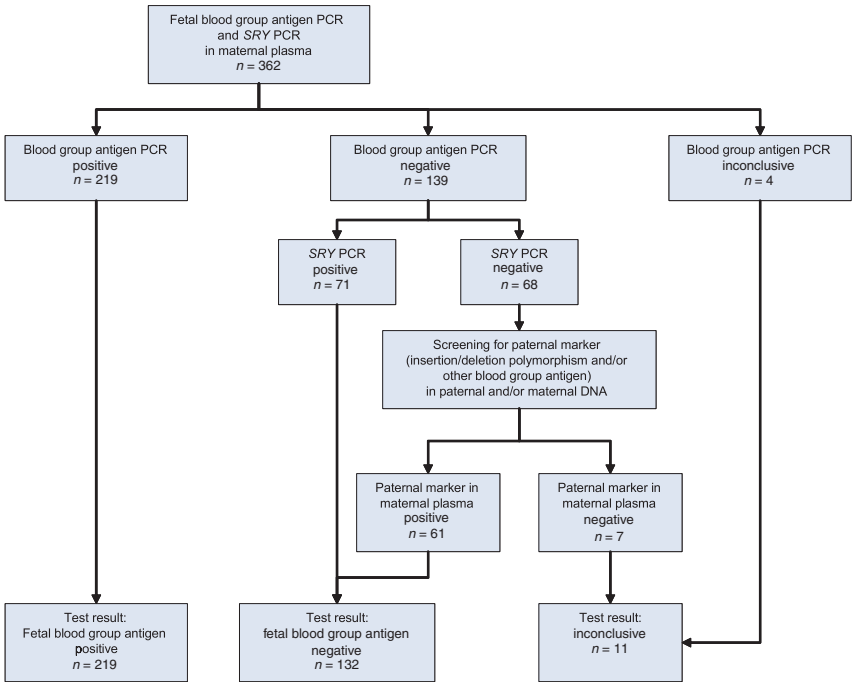

Figure 2. Test results for alloimmunised pregnant women tested from 2003 up to 2010.

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).[30] 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*^{s} 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*^{s}), 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*^{s}, 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]

Table 3. Maternal plasma polymerase chain reaction results vs cord blood serology or genotyping results of amniotic fluid cells (n = 212)

Assay	Newborn phenotype or fetal genotype*	*n*	Maternal plasma PCR result		
			Positive	Negative	Inconclusive
D	D positive	97	96	0	1
	D negative	42	0	37	5
c	c positive	14	14	0	0
	c negative	5	0	5	0
E	E positive	16	16	0	0
	E negative	5	0	5	0
K	K positive	18	17	0	1
	K negative	15	0	13	2

*As determined by cord blood serology or by genotyping of amniotic fluid cells.

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.[18], 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.[20] 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[18] and only a few laboratories therefore offer this test diagnostically.[12] 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. In a publication by Finning et al.[18] in which the authors introduced locked nucleic acids in the *K*-specific primer to increase specificity, three inconclusive results and one false-negative result were reported out of 70 samples tested for K. Because locked nucleic acids reduce the sensitivity of the assay, the authors recommended retesting at 28 weeks of samples with a K-negative result tested before 28 weeks of gestation. This is relatively late given the fact that K alloimmunisation can lead to severe fetal anaemia already early in pregnancy.[3] With our approach, the fetal K status can be reliably predicted before 18 weeks of gestation.

To ensure a low-as-possible maternal DNA concentration in the sample, blood samples drawn for fetal K typing are processed within 8 hours of sampling. Recently, a new type of blood collection tube, Cell-free DNA BCT (Streck Inc., La Vista, NE, USA), was shown to minimise post-sampling maternal cell-free DNA background for up to 14 days after sampling.[38] Use of these tubes might allow for a longer period of time before processing, obviating the need for courier transport.

Because a fetal antigen-negative phenotype is not detected directly but only inferred by a negative result for the antigen-specific PCR, we regard the confirmation of the presence of fetal DNA in such a case to be of the utmost importance. We were able to confirm the presence of fetal DNA by other paternally inherited DNA sequences (i.e. *SRY*, an insertion/deletion polymorphism or other blood group antigen) in 95% (132 of 139) of women with a negative PCR result for a specific blood group antigen. Although this percentage may be clinically acceptable, the ideal fetal marker would be universally applicable (in 100% of women) and independent of paternally inherited sequences. The *RASSF1A* gene, methylated differently between mother and child, has been proposed by Chan et al.[39], but in our hands the use of this methylation marker has not yet been proven specific and sensitive enough for routine application (unpublished results). Recently however, an Australian group published a description of its successful use in confirming the presence of fetal DNA in 16 of 16 samples negative for *RHD* and *SRY* in a study with 140 samples from D-negative pregnant women.[40] As other methylation markers are emerging,[41] 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 antigen-positive-predicted women in which it was (65% for all assays combined).

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).

Funding
None.

Acknowledgements
The development and validation of the *RHD* polymerase chain reaction protocols have been performed within the European Commission for Special Non-invasive Advances in Fetal and Neonatal Evaluation (SAFE) Network of Excellence (LSHB-CT-2004-503243). We greatly acknowledge Aicha Ait Soussan (Sanquin Research Amsterdam, Amsterdam) and Bernadette Bossers (Sanquin Diagnostic Services, Amsterdam) for technical support. We thank Diana van den Akker, Peter Ligthart and Lianne Schuitemaker (Sanquin Diagnostics Amsterdam) and Jennie Verdoes (Leiden University Medical Centre, Leiden) for help with retrieving cord blood serology results.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author. ■

References

1 Moise KJJ. Management of rhesus alloimmunization in pregnancy. *Obstet Gynecol* 2008;112:164–76.
2 Koelewijn JM, de Haas M, Vrijkotte TGM, van der Schoot CE, Bonsel GJ. Risk factors for RhD immunisation despite antenatal and postnatal anti-D prophylaxis. *BJOG* 2009;116:1307–14.
3 van Wamelen DJ, Klumper FJ, de Haas M, Meerman RH, van Kamp IL, Oepkes D. Obstetric history and antibody titer in estimating severity of Kell alloimmunization in pregnancy. *Obstet Gynecol* 2007;109:1093–8.
4 Koelewijn JM, Vrijkotte TGM, van der Schoot CE, Bonsel GJ, de Haas M. Effect of screening for red cell antibodies, other than anti-D, to detect hemolytic disease of the fetus and newborn: a population study in the Netherlands. *Transfusion* 2008;48:941–52.
5 Moise KJ. Fetal anemia due to non-Rhesus-D red-cell alloimmunization. *Semin Fetal Neonatal Med* 2008;13:207–14.
6 Mujezinovic F, Alfirevic Z. Procedure-related complications of amniocentesis and chorionic villous sampling: a systematic review. *Obstet Gynecol* 2007;110:687–94.
7 Tabor A, Bang J, Norgaard-Pedersen B. Feto-maternal haemorrhage associated with genetic amniocentesis: results of a randomized trial. *Br J Obstet Gynaecol* 1987;94:528–34.
8 Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485–7.
9 Faas BH, Beuling EA, Christiaens GC, dem Borne AE, van der Schoot CE. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* 1998;352:1196.
10 Geifman-Holtzman O, Grotegut CA, Gaughan JP. Diagnostic accuracy of noninvasive fetal Rh genotyping from maternal blood – a meta-analysis. *Am J Obstet Gynecol* 2006;195:1163–73.
11 Daniels G, Finning K, Martin P, Massey E. Noninvasive prenatal diagnosis of fetal blood group phenotypes: current practice and future prospects. *Prenat Diagn* 2009;29:101–7.
12 Daniels G, van der Schoot CE, Gassner C, Olsson ML. Report of the third international workshop on molecular blood group genotyping. *Vox Sang* 2009;96:337–43.
13 van der Schoot CE, Soussan AA, Koelewijn J, Bonsel G, Page-Christiaens GCML, de Haas M. Non-invasive antenatal RHD typing. *Transfus Clin Biol* 2006;13:53–7.
14 Finning K, Martin P, Summers J, Massey E, Poole G, Daniels G. 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. *BMJ* 2008;336:816–8.
15 Minon JM, Gerard C, Senterre JM, Schaaps JP, Foidart JM. Routine fetal RHD genotyping with maternal plasma: a four-year experience in Belgium. *Transfusion* 2008;48:373–81.
16 Muller SP, Bartels I, Stein W, Emons G, Gutensohn K, Kohler M, et al. The determination of the fetal D status from maternal plasma for decision making on Rh prophylaxis is feasible. *Transfusion* 2008;48:2292–301.
17 Freeman K, Szczepura A, Osipenko L. Non-invasive fetal RHD genotyping tests: a systematic review of the quality of reporting of diagnostic accuracy in published studies. *Eur J Obstet Gynecol Reprod Biol* 2009;142:91–8.

18 Finning K, Martin P, Summers J, Daniels G. Fetal genotyping for the K (Kell) and Rh C, c, and E blood groups on cell-free fetal DNA in maternal plasma. *Transfusion* 2007;47:2126–33.

19 Geifman-Holtzman O, Grotegut CA, Gaughan JP, Holtzman EJ, Floro C, Hernandez E. Noninvasive fetal RhCE genotyping from maternal blood. *BJOG* 2009;116:144–51.

20 Gutensohn K, Muller SP, Thomann K, Stein W, Suren A, Kortge-Jung S, et al. Diagnostic accuracy of noninvasive polymerase chain reaction testing for the determination of fetal rhesus C, c and E status in early pregnancy. *BJOG* 2010;117:722–9.

21 Oepkes D, van Kamp IL, Simon MJ, Mesman J, Overbeeke MA, Kanhai HH. Clinical value of an antibody-dependent cell-mediated cytotoxicity assay in the management of Rh D alloimmunization. *Am J Obstet Gynecol* 2001;184:1015–20.

22 Rijnders RJP, Christiaens GCML, Bossers B, van der Smagt JJ, van der Schoot CE, de Haas M. Clinical applications of cell-free fetal DNA from maternal plasma. *Obstet Gynecol* 2004;103:157–64.

23 Finning KM, Martin PG, Soothill PW, Avent ND. Prediction of fetal D status from maternal plasma: introduction of a new noninvasive fetal RHD genotyping service. *Transfusion* 2002;42:1079–85.

24 Singleton BK, Green CA, Avent ND, Martin PG, Smart E, Daka A, et al. The presence of an RHD pseudogene containing a 37 base pair duplication and a nonsense mutation in africans with the Rh D-negative blood group phenotype. *Blood* 2000;95:12–8.

25 Faas BH, Beckers EA, Wildoer P, Ligthart PC, Overbeeke MA, Zondervan HA, et al. Molecular background of VS and weak C expression in blacks. *Transfusion* 1997;37:38–44.

26 Grootkerk-Tax MGHM, Soussan AA, de Haas M, Maaskant-van Wijk PA, van der Schoot CE. Evaluation of prenatal RHD typing strategies on cell-free fetal DNA from maternal plasma. *Transfusion* 2006;46:2142–8.

27 Lo YM, Tein MS, Lau TK, Haines CJ, Leung TN, Poon PM, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768–75.

28 Pongers-Willemse MJ, Verhagen OJ, Tibbe GJ, Wijkhuijs AJ, de Haas V, Roovers E, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. *Leukemia* 1998;12:2006–14.

29 Scheffer PG, van der Schoot CE, Page-Christiaens GCML, Bossers B, van Erp F, de Haas M. Reliability of fetal sex determination using maternal plasma. *Obstet Gynecol* 2010;115:117–26.

30 Avent ND, Reid ME. The Rh blood group system: a review. *Blood* 2000;95:375–87.

31 Rouillac-Le Sciellour C, Puillandre P, Gillot R, Baulard C, Metral S, Le Van Kim C, et al. Large-scale pre-diagnosis study of fetal RHD genotyping by PCR on plasma DNA from RhD-negative pregnant women. *Mol Diagn* 2004;8:23–31.

32 Chinen P, Nardozza L, Martinhago C, Camano L, Daher S, Pares D, et al. Noninvasive determination of fetal Rh blood group, D antigen status by cell-free DNA analysis in maternal plasma: experience in a Brazilian population. *Am J Perinatol* 2010;27:759–62.

33 Brojer E, Zupanska B, Guz K, Orzinska A, Kalinska A. Noninvasive determination of fetal RHD status by examination of cell-free DNA in maternal plasma. *Transfusion* 2005;45:1473–80.

34 Clausen FB, Krog GR, Rieneck K, Nielsen LK, Lundquist R, Finning K, et al. Reliable test for prenatal prediction of fetal RhD type using maternal plasma from RhD negative women. *Prenat Diagn* 2005;25:1040–4.

35 Gautier E, Benachi A, Giovangrandi Y, Ernault P, Olivi M, Gaillon T, et al. Fetal RhD genotyping by maternal serum analysis: a two-year experience. *Am J Obstet Gynecol* 2005;192:666–9.

36 Clausen F, Krog G, Rieneck K, Rasmark E, Dziegiel M. Evaluation of two real-time multiplex PCR screening assays detecting fetal RHD in plasma from RhD negative women to ascertain the requirement for antenatal RhD prophylaxis. *Fetal Diagn Ther* 2011;29:155–63.

37 Tynan J, Angkachatchai V, Ehrich M, Paladino T, van den Boom D, Oeth P. Multiplexed analysis of circulating cell-free fetal nucleic acids for noninvasive prenatal diagnostic RHD testing. *Am J Obstet Gynecol* 2011;204:251.

38 Fernando MR, Chen K, Norton S, Krzyzanowski G, Bourne D, Hunsley B, et al. A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenat Diagn* 2010;30:418–24.

39 Chan KCA, Ding C, Gerovassili A, Yeung SW, Chiu RWK, Leung TN, et al. Hypermethylated RASSF1A in maternal plasma: a universal fetal DNA marker that improves the reliability of noninvasive prenatal diagnosis. *Clin Chem* 2006;52:2211–8.

40 Hyland CA, Gardener GJ, Davies H, Ahvenainen M, Flower RL, Irwin D, et al. Evaluation of non-invasive prenatal RHD genotyping of the fetus. *Med J Aust* 2009;191:21–5.

41 Nygren A, Dean J, Jensen T, Kruse S, Kwong W, van den Boom D, et al. Quantification of Fetal DNA by Use of Methylation-Based DNA Discrimination. *Clin Chem* 2010;56:1627–35.

