

PRENATAL DIAGNOSIS OF FETAL RhD STATUS BY MOLECULAR ANALYSIS OF MATERNAL PLASMA

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ABSTRACT

Background The ability to determine fetal RhD status noninvasively is useful in the treatment of RhD-sensitized pregnant women whose partners are heterozygous for the *RhD* gene. The recent demonstration of fetal DNA in maternal plasma raises the possibility that fetal RhD genotyping may be possible with the use of maternal plasma.

Methods We studied 57 RhD-negative pregnant women and their singleton fetuses. DNA extracted from maternal plasma was analyzed for the *RhD* gene with a fluorescence-based polymerase-chain-reaction (PCR) test sensitive enough to detect the *RhD* gene in a single cell. Fetal RhD status was determined directly by serologic analysis of cord blood or PCR analysis of amniotic fluid.

Results Among the 57 RhD-negative women, 12 were in their first trimester of pregnancy, 30 were in their second trimester, and 15 were in their third trimester. Thirty-nine fetuses were RhD-positive, and 18 were RhD-negative. In the samples obtained from women in their second or third trimester of pregnancy, the results of RhD PCR analysis of maternal plasma DNA were completely concordant with the results of serologic analysis. Among the maternal plasma samples collected in the first trimester, 2 contained no *RhD* DNA, but the fetuses were RhD-positive; the results in the other 10 samples were concordant (7 were RhD-positive, and 3 RhD-negative).

Conclusions Noninvasive fetal RhD genotyping can be performed rapidly and reliably with the use of maternal plasma beginning in the second trimester of pregnancy. (N Engl J Med 1998;339:1734-8.)

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THE Rh blood-group system is involved in hemolytic disease of the newborn, transfusion reactions, and autoimmune hemolytic anemia.¹ Despite the widespread use of Rh immune globulin prophylaxis in RhD-negative pregnant women, Rh isoimmunization still occurs.² In cases in which the father is heterozygous for the *RhD* gene, and the mother is RhD-negative, there is a 50 percent chance that the child will be RhD-positive. Prenatal determination of RhD status in these cases is clinically useful because no further testing or therapeutic procedures will be necessary if the fetus is RhD-negative. If the fetus is RhD-positive, fur-

ther studies will be necessary to determine the level of fetal hemolysis (e.g., by fetal-blood sampling).

The human *RhD* gene³ has been cloned, and it is absent in RhD-negative subjects.⁴ Fetal RhD status has been determined in samples of amniotic fluid and chorionic villi with the use of techniques based on the polymerase chain reaction (PCR).⁵ However, because of the invasive means by which such samples are obtained, these approaches increase the risk of further sensitizing the mother. To circumvent this risk, several groups have investigated the possibility of determining fetal RhD status through the use of fetal cells isolated from maternal blood.⁶⁻⁹ The main problem with this approach is that the procedures needed to isolate sufficient numbers of fetal cells from maternal blood are time consuming, technically demanding, and expensive.^{7,8} An alternative approach based on the detection of *RhD* messenger RNA in fetal nucleated red cells has also been described,¹⁰ but the small number of subjects analyzed precludes any firm conclusion as to the reliability of this method.

In a recent study, we identified fetal DNA in maternal plasma and serum.¹¹ Therefore, in the current study, we assessed the feasibility of fetal RhD genotyping using fetal DNA extracted from plasma samples from RhD-negative pregnant women.

METHODS

Subjects

We collected 10-ml blood samples from 30 blood donors who were positive on serologic testing for RhD and 30 blood donors who were negative at the Department of Hematology, John Radcliffe Hospital, Oxford, United Kingdom. We used these samples to establish the accuracy of the RhD PCR system.

To assess the value of the system for prenatal diagnosis, we collected 10-ml blood samples from 57 women with singleton pregnancies who were patients at the Nuffield Department of Obstetrics and Gynecology, John Radcliffe Hospital. Twelve women were in the first trimester of pregnancy (7 to 14 weeks), 30 were in the second trimester (15 to 23 weeks), and 15 were in the third trimester (37 to 41 weeks). Ten were primigravidas. The blood samples were collected from the women who were in the first trimester of pregnancy during a routine prenatal checkup. Blood

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samples from the women in their second trimester were collected just before routine amniocentesis; 10 ml of amniotic fluid was also collected for fetal RhD genotyping. The blood samples were collected from the women in their third trimester just before delivery. For the women who were studied during the first and third trimesters, a sample of cord blood was collected after delivery for the determination of fetal RhD status by serologic methods. The project was approved by the Central Oxfordshire Research Ethics Committee, and all the women gave informed consent.

Preparation of Samples

The blood samples were collected in tubes containing EDTA and centrifuged at 3000×g, and the plasma was then transferred into plain polypropylene tubes, with care taken to ensure that the buffy coat was not disturbed. The buffy coat was then removed and stored at -20°C until further processing. The plasma samples were recentrifuged at 3000×g, and the supernatants were stored at -20°C until further processing.

DNA Extraction

DNA was extracted from samples of plasma (800 µl), buffy coat, and amniotic fluid (200 µl each) with a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the "blood and body fluid protocol" recommended by the manufacturer.¹² An elution volume of 50 µl was used for the final washing of the DNA from the column.

Real-Time Fluorogenic PCR Analysis

Real-time fluorogenic PCR analysis was performed with a Perkin-Elmer Sequence Detector (model 7700, Perkin-Elmer Applied Biosystems, Foster City, Calif.), which is a combined thermal cycler and fluorescence detector with the ability to monitor the progress of individual PCR reactions optically.¹³ The RhD fluorogenic PCR system consisted of the amplification primers RD-A (5'CCTCTCACTGTTGCCTGCATT3') and RD-B (5'AG-TGCCTGCGGAACATT3') and a dual-labeled fluorescent probe, RD-T (5'(FAM)TACGTGAGAAACGCTCATGACAGCAAAG-TCT(TAMRA)3'; FAM [6 carboxyfluorescein] and TAMRA [6 carboxytetramethylrhodamine] were the fluorescent reporter dye and quencher dye, respectively).¹³ The primers and probe were targeted toward the 3' untranslated region (exon 10) of the *RhD* gene.³ The β -globin PCR system consisted of the amplification primers and probe as previously described.¹⁴ The fluorescent probes contained a 3'-blocking phosphate group to prevent extension of the probe during the PCR. Combinations of primers and probes were designed with Primer Express software (Perkin-Elmer). Sequence data for the *RhD* gene were obtained from the GenBank data base (accession number, X63097).

The fluorogenic PCR reactions were set up according to the manufacturer's instructions in a reaction volume of 50 µl with all components except the fluorescent probes and amplification primers obtained from a TaqMan PCR Core Reagent Kit (Perkin-Elmer). The RhD and β -globin fluorescent probes were custom-synthesized by Perkin-Elmer and were used at concentrations of 25 nM and 100 nM, respectively. The PCR primers were synthesized by Life Technologies (Gaithersburg, Md.) and were used at a concentration of 300 nM. A total of 5 µl of the extracted plasma or amniotic fluid DNA was used for amplification; for buffy-coat DNA, 10 ng was used. DNA amplifications were carried out in 96-well reaction plates that were designed to capture optical data (Perkin-Elmer).

Thermal cycling was initiated with a two-minute period of incubation at 50°C to allow time for the enzyme uracil *N*-glycosylase, which destroys any contaminating PCR amplicons, to act. This step was followed by initial denaturation for 10 minutes at 95°C and then by 40 cycles of denaturation at 95°C for 15 seconds and reannealing and extension for 1 minute at 60°C.

Amplification data collected by the Sequence Detector and stored in a Macintosh computer (Apple, Cupertino, Calif) were analyzed with Sequence Detection System software (Perkin-Elmer).

The threshold of detection was set at 10 SD above the mean baseline fluorescence calculated from cycles 1 to 15.¹³ An amplification reaction in which the intensity of fluorescence increased above the threshold during the course of thermal cycling was defined as a positive reaction.

Anticontamination Measures

Strict precautions against contamination of the PCR assay were used.¹⁵ Aerosol-resistant pipette tips were used to handle all liquids. Separate areas were used to set up amplification reactions, add DNA template, and carry out amplification reactions. The use of the Sequence Detector offered an extra level of protection in that its optical-detection system obviated the need to reopen the reaction tubes after the completion of the amplification reactions, thus minimizing the possibility of carryover contamination. In addition, the PCR assay included a further anticontamination measure in the form of preamplification treatment with uracil *N*-glycosylase, which destroyed uracil-containing PCR products.¹⁶ Multiple water blanks were included as negative controls in every analysis.

RESULTS

The RhD PCR system was used to genotype buffy-coat DNA extracted from the 30 RhD-positive blood donors and the 30 RhD-negative blood donors. There was complete concordance between the results of RhD PCR genotyping and the serologic results.

To determine the sensitivity of fluorogenic RhD PCR analysis, genomic DNA from an RhD-positive subject was diluted serially both in water and in 1 µg of genomic DNA from an RhD-negative subject. The smaller the amount of DNA, the more amplification cycles were needed to produce detectable amounts of fluorescent reporter molecules (Fig. 1). Positive signals were detected with as little DNA as the approximate amount (7.8 pg) contained in a single RhD-positive cell.

All 57 of the pregnant women were RhD-negative on serologic testing. Analysis of DNA extracted from buffy-coat samples from the 45 women who were in the second or third trimester of pregnancy revealed no *RhD* DNA, a finding in agreement with the serologic results. Among the 57 fetuses, 39 were RhD-positive and 18 were RhD-negative on serologic analysis of cord blood or PCR testing of amniotic fluid.

The results of the RhD PCR assay of plasma samples from the 57 women are shown in Table 1. Representative amplification data are shown in Figure 2. Among the women who were in the second or third trimester of pregnancy, there was complete concordance between results of the fetal RhD genotyping with use of the RhD PCR assay of maternal plasma samples and the results obtained from genotyping of amniotic fluid or serologic testing of cord blood. Plasma samples from two women in the first trimester of pregnancy who were carrying RhD-positive fetuses, with gestational ages of eight and nine weeks, yielded false negative results. The results in the other 10 women in their first trimester of pregnancy were concordant: 7 were RhD-positive on PCR

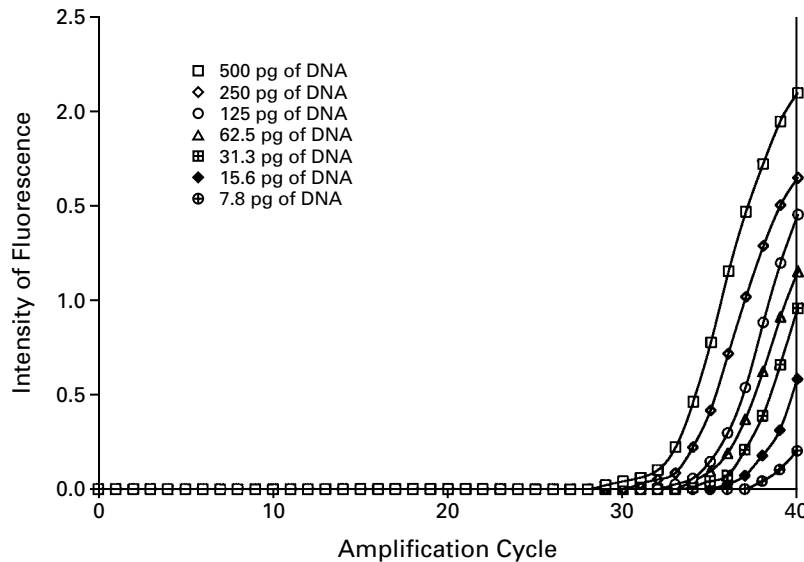


Figure 1. Sensitivity of the PCR Analysis for the Detection of *RhD* DNA.

Genomic DNA from an RhD-positive subject was serially diluted and subjected to real-time fluorogenic RhD PCR analysis. The intensity of fluorescence was monitored optically during each amplification cycle.¹³ With progressively fewer target molecules, more cycles of amplification were required to achieve a detectable level of fluorescence. The final dilution (7.8 pg) corresponded to the approximate DNA content of a single cell.

testing and had RhD-positive fetuses, and 3 were RhD-negative on PCR testing and had RhD-negative fetuses. Forty-seven of the 57 subjects had had previous pregnancies.

As a control for the amplifiability of DNA extracted from maternal plasma, the samples were also subjected to the β -globin PCR assay. The signal was positive in all 57 samples of maternal plasma DNA.

TABLE 1. RESULTS OF RhD GENOTYPING OF FETUSES OF RhD-NEGATIVE WOMEN WITH THE USE OF THE RhD PCR ASSAY.*

TRIMESTER OF PREGNANCY	RhD-POSITIVE FETUS†	RhD-NEGATIVE FETUS†
	no. of positive fetuses on PCR testing/total no. of fetuses (%)	
First	7/9 (78)	0/3
Second	22/22 (100)	0/8
Third	8/8 (100)	0/7

*The RhD PCR assay used plasma samples from the women.

†The RhD status was determined by serologic analysis of cord-blood samples in the case of samples obtained during the first or third trimester and by PCR testing of amniotic fluid in the case of samples obtained during the second trimester.

DISCUSSION

Our study demonstrates the feasibility of fetal RhD genotyping with the use of DNA extracted from maternal plasma. This type of analysis should be very useful for the treatment of sensitized RhD-negative women whose partners are heterozygous for the *RhD* gene. If testing shows that the fetus is RhD-negative, the parents can be reassured that the fetus is not at risk. On the other hand, if testing shows that the fetus is RhD-positive, treatment can be planned. The advantage of this test, which analyzes maternal plasma, is that neither the mother nor the fetus is exposed to the risks normally associated with amniocentesis or chorionic-villus sampling.¹⁷ An additional important advantage of this approach is the avoidance of further immunologic sensitization as a result of fetomaternal hemorrhage after invasive procedures.^{18,19}

Our data suggest that the results of the RhD PCR test are reliable beginning in the second trimester. The availability of such early, reliable results gives clinicians sufficient time to plan for further tests or treatment such as fetal-blood sampling and fetal transfusion,^{20,21} which are usually performed beginning in the middle of the second trimester. The results for two first-trimester samples were false negative, presumably because of the low concentration of fetal DNA in maternal plasma at that time.¹⁴

This test may also have an application in the routine testing of nonsensitized RhD-negative pregnant

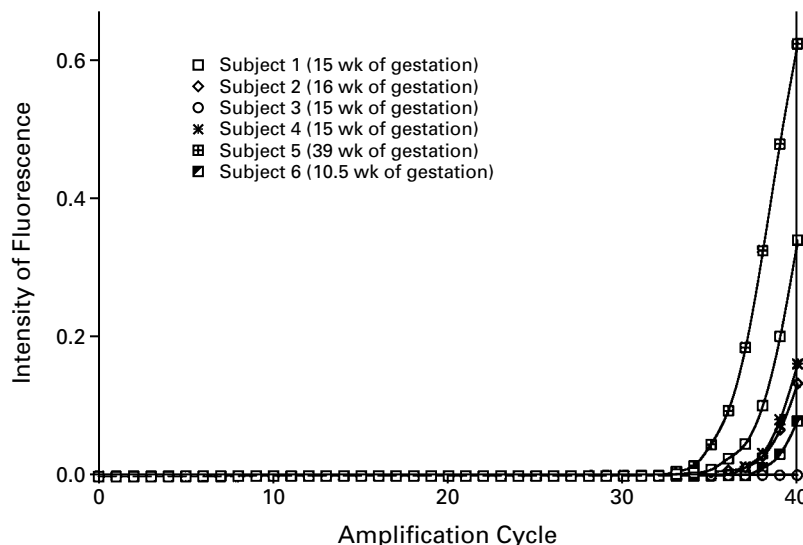


Figure 2. Detection of Fetal *RhD* DNA in Maternal Plasma.

DNA extracted from plasma samples from six pregnant women was analyzed with the RhD PCR system. Subjects 1, 2, 4, 5, and 6 were carrying RhD-positive fetuses and had positive amplification signals, corresponding to the presence of fetal DNA in maternal plasma. Subject 3 was carrying an RhD-negative fetus, and there was no amplification signal.

women. If the fetus is found to be RhD-negative, then unnecessary use of RhD immune globulin can be avoided.²²

From the data obtained so far, analysis of fetal DNA in maternal plasma does not appear to be affected by the persistence of fetal cells from previous pregnancies.²³ For example, we found no false positive results in plasma from women who had been pregnant before and who were carrying RhD-negative fetuses in the current pregnancy. This finding is consistent with our previous data obtained using Y-chromosome-specific PCR testing: there were no false positive results in women who had previously been pregnant with a male fetus.¹⁴

Because of the high concentration of fetal DNA in maternal plasma,¹⁴ the results of fetal genotyping of DNA extracted from maternal plasma are more reliable than those obtained by fetal genetic analysis of the cellular fraction of maternal blood. It also does not rely on the isolation of fetal cells, which requires the use of specialized, time-consuming, and technically demanding techniques such as cell sorting²⁴ and micromanipulation.²⁵ The high sensitivity of our PCR system is most likely the result of the use of an efficient protocol for the extraction of DNA and a fluorescence-based DNA system of amplification detection. Our current protocol for the extraction of DNA allows us to use eight times as much plasma DNA per amplification as was used in our previous study.¹¹

The method that we used has a number of advantages. First, it is based on an optical system of detection that obviates the need for any postamplification manipulation or analysis of samples. Second, the system is efficient, because the amplification and product-detection steps are combined. This allows 96 samples to be analyzed within a period of two hours. Even when one factors in the time needed to extract DNA from plasma, this method of fetal genotyping can easily be performed in one day. The brevity of this method should facilitate efficient clinical decision making and decrease the time that sensitized RhD-negative women spend waiting to learn the RhD status of their fetuses.

The Rh family of polypeptides is encoded by two related genes: the *RhCE* gene and the *RhD* gene.^{3,26} Because of the genetic complexity of the Rh system, several primer sets have been described for use in RhD genotyping.^{5,6,27} The extent of agreement between the results of genotyping and serologic results is high, although the results can be discordant, possibly because of the existence of uncommon polymorphisms.²⁷

Our findings indicate that the results of genotyping of fetal DNA extracted from maternal plasma are accurate and can potentially be used for the diagnosis of many disorders involving single genes. This approach may also be used to identify disorders such as cystic fibrosis and β -thalassemia in families in which the father and mother carry different mutations.²⁸

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Drs. Lo and Wainscoat have applied for a patent for the RhD test procedure described in this paper.

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