Rapid Rh D Genotyping by Polymerase Chain Reaction–Based Amplification of DNA


Rh (rhesus) D is the dominant antigen of the Rh blood group system. Recent advances in characterization of the nucleotide sequence of the cDNA(s) encoding the Rh D polypeptide allow the determination of the Rh D genotype at the DNA level. This can be of help in cases in which red blood cells are not available for phenotyping, eg, when it concerns a fetus. We have tested three independent DNA typing methods based on the polymerase chain reaction (PCR) for their suitability to determine the Rh D genotype. DNA derived from peripheral blood mononuclear cells from 234 Rh-phenotyped healthy donors (178 Rh D positive and 56 Rh D negative) was used in the PCR. The Rh D genotypes, as determined with a method based on the allele-specific amplification of the 3′ noncoding region of the Rh D gene described by Bennett et al (N Engl J Med 328:607, 1993), were not concordant with the serologically established phenotypes in all cases. We have encountered 5 discrepant results, ie, 3 false-positive and 2 false-negative (a father and child). Rh D genotyping with the second method was performed by PCR amplification of exon 7 of the D gene with allele-specific primers. In all donors phenotyped as D positive tested so far (n = 178), the results of molecular genotyping with this method were concordant with the serologic results, whereas a false-positive result was obtained in one of the D-negative donors (also false-positive in the first method). Complete agreement was found between genotypes determined in the third method, based on a 600-bp deletion in intron 4 of the Rh D gene described by Arce et al (Blood 82:651, 1993), and serologically determined phenotypes. The Rh blood group system is complex, and unknown polymorphisms at the DNA level are expected to exist. Therefore, although genotypes determined by the method of Arce et al were in agreement with serotypes, it cannot yet be regarded as the golden standard. More experience with this or other methods is still needed.

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THE Rh BLOOD GROUP SYSTEM is of clinical interest because Rh antibodies are involved in the immune-mediated destruction of red blood cells (RBCs), eg, after transfusion with Rh incompatible blood or in hemolytic disease of the newborn (HDN). Most commonly, HDN is caused by maternal antibodies directed against the Rh D antigen, despite common usage of Rh immune globulin prophylaxis. The maternal antibodies are formed in response to alloimmunization of the mother by fetal RBCs after delivery or sometimes late in the first pregnancy, if there is fetal-maternal incompatibility. In subsequent pregnancies, it is important to investigate the Rh D status of the fetus, especially when the father is heterozygous for the Rh D antigen. Prenatal determination of the fetal Rh D genotype will thus help in the antenatal management of fetuses at risk. Unnecessary invasive diagnostic methods and expensive therapeutic strategies will be prevented in case the fetus is D negative.

Recently, it has been established that the Rh locus on chromosome 1 p34.3-p36.1 comprises at least two distinct, but highly homologous genes, a D gene and a CcEe gene.5,6 The Rh D-negative phenotype is caused by a deletion of the Rh D gene rather than by an allele of this gene. Complementary DNA derived from the Rh D gene, encoding the Rh D polypeptide, has been characterized.6,8 It has been proposed that full-length messenger RNA transcript derived from the Rh CcEe gene encodes the polypeptide carrying the C/E antigens, whereas, by alternative splicing, shorter polypeptides are produced that carry the C/c antigens.5,9 Several characteristic nucleotide differences between the Rh D cDNA and the non-D cDNAs have been observed.4,6,9-11

Colin et al12 were the first to report the applicability of Southern blotting of genomic DNA to determine the Rh D genotype. However, because this assay is time-consuming and requires a relatively large number of cells, its usefulness in the routine laboratory, especially in prenatal testing, is limited. Recently, Bennett et al13 were able to determine the Rh D genotype of 15 fetuses using DNA derived from amniocytes in the polymerase chain reaction (PCR). This assay is more suitable for routine application. However, we have encountered discrepancies between the results of serologic typing and of genotyping by this assay.13 This was not an unexpected finding, because this assay is based on sequence differences between the D and CcEe genes in the 3′ noncoding regions. This finding prompted us to evaluate the reliability of three different PCR-based Rh D typing methods. Genomic DNA derived from 234 individuals with a known Rh D phenotype were used for this purpose.

MATERIALS AND METHODS

Serology

Rh D phenotyping of the individuals under investigation was performed with standard methods using polyclonal as well as monoclonal antibodies, as described.1

DNA Preparation

The mononuclear cell fraction was isolated by density gradient centrifugation and used to prepare DNA according to the method described by Ciulla et al.14

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2975
Each of the PCR oligonucleotide primers, and PCR Ampl(jiccuior1

Model 480 (Norwalk, CT) with L

oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA).

polymerase (Promega, Madison, WI)
yields a product of

Thus, with these allele-specific primers (ASPA), a 96-bp product

and visualized by
donors. The PCR products were analyzed by polyacrylamide gel

PCR Strategies I, II, and III

Method I. Method I was performed as described (see Table 1 for the oligonucleotide primer sequences A1, A2, A3, and A4). For a schematic PCR strategy, see Fig 1A. 136 bp of exon 7 common to the Rh CcEe and Rh D genes, is amplified with the primer set A1 and A2, thus serving as an internal control for the PCR. The antisense primer A4 of the second primer set recognizes a sequence in the 3' noncoding region of the Rh D gene and therefore yields a product of 186 bp only from DNA from Rh D-positive donors. The PCR products were analyzed by polyacrylamide gel (8%) electrophoresis, subsequently stained with ethidium bromide, and visualized by UV illumination.

Method II. The first primer set A5 and A6 (see Table 1 for the sequences) was used as consensus primers to amplify a 141-bp segment from exon 5 of both the Rh D and the Rh CcEe gene. In contrast, primers A7 and A8 are specific for exon 7 of the Rh D gene only. Thus, with these allele-specific primers (ASPA), a 96-bp product will only be obtained with DNA from Rh D-positive subjects (for a schematic representation, see Fig 2A). Analysis of the PCR products was performed by electrophoresis in an 8% polyacrylamide gel and subsequent ethidium bromide staining and UV illumination was used to visualize the amplimers.

Method III. Method III is based on the method originally described by Arce et al with different oligonucleotide primers, A9 (for a sequence located in exon 4) and A6 (for a sequence located

in exon 5) for the sequences, see Table 1). A schematic illustration is shown in Fig 3A. In this technique, the CcEe gene is distinguished from the D gene, because in the D gene there is a deletion in intron 4 between exon 4 and 5, which results in a DNA fragment that is 600 bp smaller than that obtained from the CcEe gene. With primers A9 and A6, a PCR product of approximately 1,200 bp is derived from the CcEe gene in all donors, whereas the smaller PCR product of approximately 600 bp, derived from the D gene, is lacking when the donor is D negative. PCR products were size-separated on 2% agarose gels and visualized by UV illumination after ethidium bromide staining.

PCR Conditions

Method I. The first cycle of the PCR was performed at 95°C for 5 minutes to denature the DNA; followed by 35 cycles of 1 minute at 95°C (denaturation), 1.5 minutes at 49°C (primer-annealing), and 2.5 minutes at 72°C (extension); and finally 1 cycle of 9 minutes at 72°C to complete the extension.

Methods II and III. After an initial cycle of denaturation at 95°C for 5 minutes, 35 cycles were performed consisting of 1 minute at

Table 1. Sequence of the Oligonucleotide Primers Selected for the PCR

<table>
<thead>
<tr>
<th>Oligonucleotide Sequences</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1. 5' TGTGTTGTAACCGAGT 3' (sense) 941-956</td>
<td></td>
</tr>
<tr>
<td>A2. 5' ACATGCGTCTCCGCGT 3' (antisense) 1073-1062</td>
<td></td>
</tr>
<tr>
<td>A3. 5' TAAAGCAGGATCCCAA 3' (sense) 1252-1268</td>
<td></td>
</tr>
<tr>
<td>A4. 5' GCTGCGCGGCACGGTATC 3' (antisense) 1437-1422</td>
<td></td>
</tr>
<tr>
<td>A5. 5' GCCCTTCTTGGTGATG 3' (sense) 637-654</td>
<td></td>
</tr>
<tr>
<td>A6. 5' TGACCCTCAGATGGCTGT 3' (antisense) 768-751</td>
<td></td>
</tr>
<tr>
<td>A7. 5' AGCTCCATCATGSCTACA 3' (sense) 973-992</td>
<td></td>
</tr>
<tr>
<td>A8. 5' ATGGTGGTACCTCCTGGT 3' (antisense) 1523-1506</td>
<td></td>
</tr>
<tr>
<td>A9. 5' ATGAATCCAGTTTCTTCT 3' (sense) 657-624</td>
<td></td>
</tr>
<tr>
<td>A10. 5' tagaattcACAGACTACCACATGAAC 3' (sense) 487-504</td>
<td></td>
</tr>
<tr>
<td>A11. 5' atagaCTTTGCGACGACAGGCCAC 3' (antisense) 568-547</td>
<td></td>
</tr>
</tbody>
</table>

The primers are numbered according to the coding sequence described by Mouro et al. and Simsek et al. Primer A4 is deduced from the sequence of the 3' noncoding region of the D gene. The Rh D-specific nucleotides in oligonucleotide primers A7 and A8 are underlined. In primers A10 and A11, the small written nucleotides are not part of the coding sequence, but are added as a restriction site that can be used for cloning purposes. These nucleotides are not involved in the numbering of the primer nucleotides.

PCR Amplification

The PCR was performed in a Perkin-Elmer Cetus Thermal Cycler Model 480 (Norwalk, CT) with 1 μg of genomic DNA, 20 pmol of each of the PCR oligonucleotide primers, and 2 U of Taq DNA polymerase (Promega, Madison, WI) in a buffer containing 50 mmol/ L KCl, 10 mmol/L Tris-HCl (pH 9.0), 0.1% (wt/vol) Triton X-100, and 1.5 mmol/L MgCl2, in a total volume of 50 μL. All PCR primers (see Table 1 for the sequences) were purchased from Applied Biosystems (Palo Alto, CA). The primers A8 and A9 were purified with oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA).

Fig 1. (A) Schematic representation of the organization of the Rh D and Rh CcEe genes at the Rh locus of chromosome 1p34.3-p36.1. The Rh D and the Rh CcEe genes are distinct but highly homologous genes comprising 10 exons (represented as boxes, not to scale). Oligonucleotide primers A1 and A2 (consensus primers) are used to amplify a 136-bp fragment of exon 7 of both the Rh D and the Rh CcEe genes. A3 and A4 (Rh D-specific primer) yield a 186-bp PCR product, derived from the Rh D gene, in Rh D-positive subjects only. (B) DNA of individuals phenotyped for the Rh D antigen were subjected to PCR amplification with primers A1/A2 and A3/A4 in one PCR tube. After subsequent electrophoresis through polyacrylamide gels, the PCR products were stained with ethidium bromide and visualized by UV illumination. Lanes 1 through 3 contain the PCR products of Rh D-positive individuals; lanes 4 and 5 contain the PCR samples of Rh D-negative individuals.
PCR-BASED RhD TYPING

Allele-Specific Restriction Enzyme Analysis (ASRA) With Dde I in Exon 4

Two nucleotide differences in the Rh D gene as compared with the Rh CcEe gene (C → A at position 505 and G → T at position 509, resulting in Leu^{106} → Met and Arg^{509} → Met amino acid changes, respectively) were used for a PCR-ASRA. The target site (CTNAG) for the restriction enzyme Dde I in the Rh CcEe gene (CTGAG) is absent in the Rh D gene (ATGAT). A segment of 94 bp in exon 4 of the Rh D gene as well as that of the Rh CcEe gene was amplified (the same PCR condition as used for methods II and III) with consensus primers A10 and A11 (see Table 1 for the sequences). Genomic DNA from the discrepant cases was used for this purpose. The PCR products were digested with Dde I according to conditions recommended by the manufacturer (Bethesda Research Laboratories, Gaithersberg, MD), electrophoresed on 10% polyacrylamide gels, and stained with ethidium bromide. The product of 94 bp is expected to be cut completely into fragments of 67 bp and 27 bp in the case of Rh D-negative individuals, whereas only 50% of the 94-bp product derived from Rh D-positive individuals will be digested because of the absence of the Dde I site in the Rh D gene (94 bp, 67 bp, and 27 bp).

Southern Blot Analysis

Southern blot analysis was performed according to standard methods. Genomic DNA was digested with the restriction endonuclease

95°C of denaturation, 1.5 minutes at 55°C of primer annealing, and 2.5 minutes at 72°C of extension, followed by a final cycle of 9 minutes at 72°C.

Note that in all three PCR typing methods an internal control was included to check the efficiency of the Taq DNA polymerase used. In methods I and II, a second primer set that yields a PCR product regardless of the Rh D genotype was used. In method III, only one primer set was applied. This primer set is sufficient for both checking the efficiency of the PCR and for typing for the Rh D gene, for the reasons stated above.

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Rh D Genotyping

tive (father and child) were genotyped as only the control PCR product, with a size of 141 bp (Fig 3).

The individuals studied with method I are summarized in Table 2. Figure 1B shows a representative example of the characteristic bands visualized on polyacrylamide gels. The samples containing only the 136-bp band were scored as Rh D negative (lanes 1 through 3). The genotypes of the individuals studied with method I are summarized in Table 2. Two of the 178 subjects phenotyped as Rh D positive (father and child) were genotyped as D negative. Three of the 56 subjects phenotyped as Rh D negative were genotyped as D positive.

Rh D Genotyping Using Method II and the Correlation of the Results With Those of Serotyping

As can be seen in Table 1, primer A7 as well as primer A8 contain 6 Rh D-specific nucleotides. Most importantly, the 3' end of both primers contains Rh D-specific nucleotides. As expected, the Rh D-negative samples contained only the control PCR product, with a size of 141 bp (Fig 2B, lanes 1, 4, and 5), whereas the Rh D-positive samples contained also a Rh D-specific PCR product with a size of 96 bp in addition (Fig 2B, lanes 2, 3, and 6). However, one Rh D-negative individual was typed in this PCR assay as Rh D positive (Table 2). This false-positive case was also observed in method I.

Rh D Genotyping Using Method III and the Correlation of These Results With Those of Serotyping

As can be seen in Fig 3B, the Rh D-negative PCR samples have only the 1,200-bp band (lanes 1 through 3), whereas the Rh D-positive samples have the 1,200-bp as well as the 600-bp band (lanes 4 and 5). In all individuals studied so far, no discrepancies between serotyping and DNA typing (method III) were encountered (Table 2).

Discrepant Cases

PCR-ASRA. The discrepancies between serology and the DNA assays are summarized in Table 3. PCR-ASRA (Dde I) with the discrepant cases was performed to evaluate whether the C to A and G to T nucleotide mutations in the more upstream located exon 4 are normally present in these Rh D-positive individuals. This study showed the presence of the expected fragments with a size of 67 bp and 27 bp (data not shown) in Rh D-negative individuals (indicated with a minus in Table 3) and fragments of 94 bp, 67 bp, and 27 bp (data not shown) in Rh D-positive individuals (indicated with a plus in Table 3).

Southern blot analysis. To further evaluate the discrepant results, genomic DNA from the discrepant cases no. 1, 2, 3, and 5 were digested with BamHI restriction enzyme and subjected to Southern blot analysis with the full-length Rh cDNA probe (Fig 4). Unfortunately, for case no. 4 there was not enough DNA available to perform Southern blot analysis and we could not obtain any more material. DNAs from D-negative (dCcee, lane 1; dCcee, lane 3) and D-positive (DCcee, lane 5) donors were used as control.

For cases no. 1 and 2 (father and child), the Southern blot showed exactly the same pattern as for normal Rh D-positive donors (results not shown).

For cases no. 3 and 5, the results from the Southern blot analysis are shown in Fig 4, lanes 4 and 2, respectively. In case no. 3, the D-specific band of 16 kb, corresponding to the 3' region of the D gene, was present. However, the 4.0-kb D-specific band (exons 4, 5, and 6) was missing, whereas an additional fragment of approximately 4.6 kb appeared. In agreement with the PCR results of methods I and III, in case no. 5 the D-specific 16-kb band was present, whereas the 4.0-kb D-specific band was missing; however, an additional band of approximately 12 kb was seen.

DISCUSSION

We have compared three different Rh D genotyping methods using genomic DNA from 234 donors. The results of only one of the three methods studied (method III) were in full agreement with those of serotyping in individuals with a normal Rh D phenotype.

DNA typing for Rh D in method I is based on the difference in the 3' noncoding regions of the Rh D and the Rh CcEe gene. Also, in method III a difference in a noncoding region, ie, an intronic sequence (flanked by exons 4 and 5) of the Rh D- and the Rh CcEe gene, is used to differentiate Rh D-positive from Rh D-negative individuals. In our opinion, more reliable distinction for Rh D positivity and Rh D negativity at the molecular level would be expected based on differences in the coding regions. Therefore, we took advantage of the major nucleotide differences in exon 7 of the Rh D and Rh CcEe genes. Based on this knowledge, the

Table 2. Serology Versus Genotyping

<table>
<thead>
<tr>
<th>Method I</th>
<th>Method II</th>
<th>Method III</th>
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</thead>
<tbody>
<tr>
<td>D pos</td>
<td>D neg</td>
<td>D pos</td>
</tr>
<tr>
<td>Ser D pos</td>
<td>176</td>
<td>2</td>
</tr>
<tr>
<td>Ser D neg</td>
<td>3</td>
<td>53</td>
</tr>
</tbody>
</table>

BamHI, size-separated by electrophoresis on a 1% agarose gel, and transferred to a nitrocellulose membrane. Blots were hybridized with a 32P-labeled Rh cDNA probe (kindly provided by Dr D. Anstee, International Blood Group Reference Laboratory, Bristol, UK) and visualized by autoradiography.

RESULTS

Rh D Genotyping Using Method I and the Correlation of the Results With Those of Serotyping

We determined the Rh D phenotypes of 234 individuals. Of these 234 individuals, 178 were typed as Rh D positive and 56 as Rh D negative (Table 2). Figure 1B shows a representative example of the characteristic bands visualized on polyacrylamide gels. The samples containing only the 136-bp band were scored as Rh D negative (lanes 4 and 5), whereas those containing in addition the band of 186 bp were scored as Rh D positive (lanes 1 through 3). The genotypes of the individuals studied with method I are summarized in Table 2. Two of the 178 subjects phenotyped as Rh D positive (father and child) were genotyped as D negative. Three of the 56 subjects phenotyped as Rh D negative were genotyped as D positive.

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Table 3. The Discrepant Case Encountered in the DNA Assays

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Serology</th>
<th>Method I</th>
<th>Method II</th>
<th>Method III</th>
<th>ASRA Exon 4</th>
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<td>+</td>
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<td>dcCee</td>
<td>+</td>
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</table>

Abbreviations: +, Rh D positive; --, Rh D negative; *, weak positive.
**PCR-BASED RhD TYPING**

![Southern blot analysis of human genomic DNA. DNA derived from individuals with Dccee (lane 1), DcCee (lane 3), and DCCcE (lane 5) phenotypes and from the discrepant cases no. 5 and 3 (lanes 2 and 4, respectively) was digested with the restriction endonuclease BamHI, electrophoresed, blotted onto nitrocellulose blot, and hybridized with the complete Rh cDNA probe. λ/HindIII fragments were used as size markers.](image)

Fig 4. Southern blot analysis of human genomic DNA. DNA derived from individuals with Dccee (lane 1), DcCee (lane 3), and DccCee (lane 5) phenotypes and from the discrepant cases no. 5 and 3 (lanes 2 and 4, respectively) was digested with the restriction endonuclease BamHI, electrophoresed, blotted onto nitrocellulose blot, and hybridized with the complete Rh cDNA probe. λ/HindIII fragments were used as size markers.

From the results of the PCR-based methods using DNA from cases no. 1 and 2 (a father and child, who were both typed D negative in method I and D positive in methods II and III and serology), we may conclude that the 3' noncoding region of the Rh D gene in these two individuals is altered or deleted. However, the patterns in Southern blot analysis were not different from patterns obtained with DNA from a normal Rh D-positive donor. From these results we can conclude that there is no deletion in the D gene in these individuals, but that probably the 3' region of the D gene is altered so that primer A3 or A4 can no longer anneal. The false-positivity in cases no. 4 and 5 may be caused by a partial deletion of the Rh D gene in the concerning region, i.e., the Rh D gene in these individuals is not entirely deleted, but still contains its 3' region. Unfortunately, for case no. 4 there was not enough material to perform a Southern blot analysis. Indeed, Southern blot analysis of case no. 5 showed the presence of the 16-kb band corresponding to the 3' noncoding region of the D gene, whereas the 4.0-kb band was missing. But, in addition, an extra restriction fragment of approximately 12 kb was found. Because we were not able to obtain enough material from the parents, we could not confirm the origin of this band. It could be that the 16-kb band was inherited from one parent and that the 12-kb band was inherited from the other parent. This would mean that both parents have an abnormal Rh D gene. In case no. 3, the subject was typed D negative by both serology and two DNA-methods (methods III and IV [exon 4 ASRA]), but D positive by two other DNA methods (methods I and II). Southern blot analysis showed an abnormal band of 4.6 kb, whereas the band of 4.0 kb present in case of D positivity was missing. An identical restriction fragment length pattern had been found earlier by Mouro et al when they described the rearrangements involved in the Rh D\(^{11}\) variant (see below). Thus, in this case we are probably dealing with the Rh D\(^{11}\) type, which was not noticed in serology.

Mouro et al described two different rearrangements of the Rh D gene to be associated with the Rh D\(^{11}\) phenotypes. In the type I D\(^{11}\) variant, exons 4, 5, and 6 of the Rh D gene were found to be deleted because of an unequal crossing over within the Rh D gene. In contrast, in the second D\(^{11}\) variant, exons 4, 5, and 6 of the Rh D gene are derived from the Rh CcEe gene, caused by intergenic double crossing over or gene conversion between the Rh D and the Rh CcEe gene. Genomic DNA derived from type I D\(^{11}\) as well as from type II D\(^{11}\) individuals was also used in the above methods of Rh D genotyping. As expected, these individuals were typed as Rh D positive in methods I and II. However, in method III, all D\(^{11}\) samples showed only the 1,200-bp band, which is specific for the Rh CcEe gene.

The concordance of the results in method III and the ASRA with Dde I in exon 4 (only performed in the discrepant cases) with those of serology and the discordance of the results in method I and method II in some cases all together suggest that the major Rh D epitopes are located upstream from exon 7.

Our main aim was to construct a reliable molecular genotyping technique for Rh D for prenatal typing fetuses at risk. Accurate prediction of the Rh genotypes of fetuses is crucial when the risks and costs of prenatal interventions, including fetal blood sampling and high-dose intravenous Ig administration to the mother, are considered. Until recently, prenatal determination of the fetal Rh D status could only be performed by fetal blood sampling. Southern blot analysis of genomic DNA for the determination of the Rh D genotypes is an impractical technique.

In contrast to the simplicity of the molecular basis of the platelet alloantigens, there are many differences within the coding as well as in the noncoding regions of the Rh D- and Rh CcEe genes. Moreover, it is likely that these differences will vary among individuals. The Rh gene frequencies as well as the frequencies of the different Rh haplotypes vary among individuals from different racial backgrounds. Therefore, it is also likely that there will be differences in the Rh D genes. Although we and others have
shown that nucleotide differences that result in amino acid substitutions between the Rh D gene and the non-D gene are indeed linked to the Rhesus phenotype. More investigations are needed to correlate the Rh genes and the Rh phenotypes because the association between the characteristics of the Rh D polypeptide and their immunogenicity still remains to be determined.

In conclusion, the three different approaches presented in this report to genotype for Rh D were useful but not sufficient in all cases. Additional methods, such as PCR-ASRA, as well as simultaneous analysis of parental DNA, may prove necessary, especially in case RBCs are not available for phenotyping. For some cases, a possible discrepancy between the results of serotyping and genotyping in the parents can be detected in this way, thus decreasing the risk of a false-negative result for the fetus. More research is necessary to understand the nature of these discrepancies.

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Rapid Rh D genotyping by polymerase chain reaction-based amplification of DNA

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