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


The Rh blood group system is of clinical interest because Rh antibodies are involved in the immunemediated 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. 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. It has been proposed that full-length messenger RNA transcript derived from the Rh CcEe gene encodes the polypeptide carrying the CcEe antigens, whereas, by alternative splicing, shorter polypeptides are produced that carry the Cce antigens. Several characteristic nucleotide differences between the Rh D cDNA and the non-D cDNAs have been observed.

Colin et al 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 al 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. 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.

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.

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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' TGTGTGTGAACCGAGT 3' (sense)</td>
<td>941-956</td>
</tr>
<tr>
<td>A2. 5' ACATGCCATTGCGCG 3' (antisense)</td>
<td>1073-1062</td>
</tr>
<tr>
<td>A3. 5' TAAGCCTAAAGCATTCACA 3' (sense)</td>
<td>1252-1268</td>
</tr>
<tr>
<td>A4. 5' ATGGTGAGATCTCTCT 3' (sense)</td>
<td>1437-1422</td>
</tr>
<tr>
<td>A5. 5' GCCCTCTCTTGTTGATG 3' (sense)</td>
<td>637-654</td>
</tr>
<tr>
<td>A6. 5' TGACCTGAGATGCTGT 3' (antisense)</td>
<td>768-751</td>
</tr>
<tr>
<td>A7. 5' AGCTCATCAGGCTAACA 3' (sense)</td>
<td>973-892</td>
</tr>
<tr>
<td>A8. 5' ATTGCCGCGGCAAGGCAT 3' (antisense)</td>
<td>1068-1048</td>
</tr>
<tr>
<td>A9. 5' CACATCCAGTTGCTG 3' (sense)</td>
<td>697-624</td>
</tr>
<tr>
<td>A10. 5' tagaattgcagactacccatcgac 3' (sense)</td>
<td>487-504</td>
</tr>
<tr>
<td>A11. 5' atacaGGTGGCCAAGGCGACACAC 3' (antisense)</td>
<td>568-547</td>
</tr>
</tbody>
</table>

The primers are numbered according to the coding sequence described by Moura et al10 and Simsek et al.11 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 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% (v/vol) Triton X-100, and 1.5 mM MgCl2, in 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).

PCR Strategies I, II, and III

Method I. Method I was performed as described12 (see Table 1 for the oligonucleotide primer sequences A1, A2, A3, and A4). For a schematic PCR strategy, see Fig 1A. A segment of 126 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 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) is 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 bands.

Method III. Method III is based on the method originally described by Arce et al12 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

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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\sup{109} → Met and Arg\sup{270} → 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, Gaithersburg, 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

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Fig 2. (A) A schematic illustration of the PCR strategy used to amplify a Rh D-specific region and a region that is present in both genes, ie, an internal control. A PCR product of 141 bp, derived from exon 5 of both the Rh D and the Rh CcEe genes ("internal control"), is obtained with oligonucleotide primers A5 and A6. In contrast, A7 and A8 are Rh D ASPA. Only in Rh D-positive subjects is a PCR-ASP product of 96 bp generated. (B) The PCR products are separated on polyacrylamide gels and stained with ethidium bromide. Lanes 1, 4, and 5 contain the PCR products of Rh D-negative individuals. Lanes 2, 3, and 6 contain the PCR products of Rh D-positive individuals.

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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Fig 3. (A) Schematic representation (not to scale) of the Rh locus and the PCR strategy for the amplification of intron 4 between exons 4 and 5. Oligonucleotide primer A9 in exon 4 and A6 in exon 5 flanking the intron were used on genomic DNA of Rh D phenotyped individuals in the PCR. When DNA of Rh D-positive individuals is used, 1,200-bp and 600-bp PCR products are generated, whereas, in Rh D-negative individuals, only a 1,200-bp product is obtained because of the lack of the 600-bp deletion. (B) Ethidium bromide staining after 2% agarose gel electrophoresis of the PCR products in Rh D-positive and Rh D-positive phenotypes. Lanes 1 through 3 contain PCR samples of Rh D-negative individuals and lanes 4 and 5 contain the PCR samples of Rh D-positive individuals.
Rh D Genotyping

The results of the control PCR product, with a size of 141 bp (Fig 1B, lanes 2, 3, and 5), were digested with BamHI restriction enzyme and subjected to Southern blot analysis with the full-length Rh D-specific PCR product (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. DNA samples from D-negative donors (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.

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 typed as Rh D positive (father and child) were genotyped as D negative. Three of the 56 subjects typed 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 → A and G → 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 D-specific PCR product (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 donors (DCcee, lane 1; dCcee, lanes 3 and 5) and D-positive (DCcee, lane 5) donors were used as control.

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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
primers A7 and A8 were constructed and used in the ASPA for the determination of Rh D genotypes. The discrepant case no. 3 (Table 3), which was already encountered in assay I, remained false positive in this assay.

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 D positivity was missing. An identical restriction fragment length pattern had been found earlier by Mourou et al. when they described the rearrangements involved in the Rh D11 variant (see below). Thus, in this case we are probably dealing with the Rh D11 type, which was not noticed in serology.

Mourou et al. described two different rearrangements of the Rh D gene to be associated with Rh D11 phenotypes. In the type I D11 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 D11 variant, exons 4, 5, and 6 of the Rh D gene are derived from the Rh CeEe gene, caused by intergenic double crossing over or gene conversion between the Rh D and the Rh CeEe gene. Genomic DNA derived from type I D11 as well as from type II D11 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 D11 samples showed only the 1,200-bp band, which is specific for the Rh CeEe 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 CeEe 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.\textsuperscript{8,11} More investigations are needed to correlate the Rh genes and the Rh phenotypes because the association between the characteristic amino acids 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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