# *RHD/CE* typing by polymerase chain reaction using sequence-specific primers

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**BACKGROUND:** Current DNA-based Rh system typing strategies may detect the two *RH* genes and their prevalent alleles, but they are known to fail sometimes, when rare *RH* alleles (e.g., D category phenotypes) are encountered. It is almost impossible to find a single DNA-based method that can accommodate the great heterogeneity within the human Rh system.

**STUDY DESIGN AND METHODS:** An easy-to-perform DNA-based method for the detection of the two *RH* genes and their alleles, including variant *RHD* alleles, was developed. By the use of one *RHD/C-*, seven *RHD-*, and four *RHCE*-specific polymerase chain reactions, all triggered to work at identical thermocycling conditions, the DNA of 77 blood donors carrying weak D and that of 200 random donors with common D phenotype was investigated. In addition, 77 selected samples of ccDee and rare Rh system phenotypes were examined.

**RESULTS:** Among 77 samples of weak D, one Rh33 and six $D^{VI}$ categories were detected, one of which showed new *RHD*-specific nucleotide patterns. In DFR and CCee samples, novel variant *RHD* alleles were found. *RHD* DNA types of 200 random donors were found to be concordant with their D phenotype. For *RHE* and *RHe* genotyping, a full correlation with serologic phenotypes was found. Our method for genotyping *RHC* and *RHc* failed in some cases, because of an already published *RHc* allelic variation, which we have called *RHc(cyt$^{48}$)*. An estimate of the frequency of this *RHc(cyt$^{48}$)* allele in a white population was made.

**CONCLUSION:** The presented exon-scanning *RHD/CE* polymerase chain reaction using sequence-specific primers complements current DNA-based Rh system typing strategies and is superior in the detection of variant *RHD* alleles.

The antigens of the Rh blood group system are carried on two proteins encoded by two genes, denoted *RHD* and *RHCE*. Each gene consists of 10 exons, and, although their coding sequences are very closely related, the immunologic heterogeneity within the resulting proteins is remarkable.[1-4] Besides the most common *RHD* DNA sequence, some unexpressed *RHD* (*RHD$^{nex}$*) and several allelic *RHD variants* (*RHD$^{var}$*) have been described. They differ from the consensus *RHD* DNA sequence by deletions (in some *RHD$^{nex}$*), point mutations, and replacements with homologous *RHCE* sequences (*RHD$^{var}$*).[5,6] Most of these mutations result in serologically definable D categories.[7-10] The *RHCE* gene also bears its own heterogeneity, which is responsible for C, c, E, and e. *RHC* and *RHc* differ by five nucleotides in exon 2, resulting in three amino acid substitutions, and *RHE* and *RHe* differ by one single nucleotide in exon 5, which results in a Pro226Ala substitution.[11-13] This heterogeneity within the Rh protein family in conjunction with the antigens' immunogenicity is thought to be the main reason for the production of alloantibodies to antigens acquired by blood transfusion or by pregnancy. Alloantibodies can be directed against D or D category antigens, C, c, E, and e.[14,15]

Rh system antibodies and their antigenic counterparts are of great clinical importance. It is desirable to perform

**ABBREVIATIONS:** PCR(s) = polymerase chain reaction(s); PCR-SSP = PCR using sequence-specific primer(s).

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Supported by grant P.239 from the Klinikum der Universität Ulm Forschungsförderung and by a grant from the DRK-Blutspendedienst Baden-Württemberg, Baden-Baden, Germany.

Received for publication January 14, 1997; revision received April 18, 1997, and accepted April 24, 1997.

**TRANSFUSION** 1997;37:1020–1026.

typing of the Rh system antigens without the use of red cells. This will be of particular importance for a reliable DNA typing approach in prenatal testing and for anti-D prophylaxis in pregnancy. It may also be used to check the DNA type for $RHD^{var}$ if a D variant type suspected as a result of serologic testing. Molecular biology methods offer these advantages. Several groups have published DNA-based typing systems for the Rh system applying a variety of techniques.[16-23]

Our aim was to develop an easy-to-perform, systematic, and more accurate DNA-based typing system for the Rh system without the need for time-consuming postamplification procedures, such as restriction endonuclease cleavage, or the use of (radio)labeled DNA probes. DNA typing for *RHCE* and *RHD* should be possible in one step and be more accurate with respect to $RHD^{var}$. Although a previously published heteroduplex generator method (exon 2 nt 179-294 and exon 4 nt 604 to exon 5 nt 772) met the same needs, we asked for testing of the entire *RHD* gene in a rapid way.[23] Therefore, we developed 12 polymerase chain reactions using sequence-specific primers (PCR-SSP) to specifically detect the *RH* genes and their alleles (*RH* PCR-SSP). PCR-SSP has proven to be a powerful tool in detecting particular nucleotides in known DNA sequences, but both variability and homology between the two *RH* genes complicate such attempts.[24-26]

One PCR that was specific for *RHD/C* in exon 2 and seven PCRs that were specific for *RHD* in exons 3 to 7 and 9 and 10, allowed us to screen for $RHD^{var}$ in a group of individuals with weak D phenotypes. Four other PCRs were employed to detect *RHC, RHc, RHE,* and *RHe.*

## MATERIALS AND METHODS

### Typing by serologic methods

The Rh phenotypes were determined by standard serologic methods with commercially available test systems according to the manufacturer's instructions (DiaMed-ID Micro Typing System, DiaMed AG, Cressier, Switzerland; Gamma Biologicals, Houston, TX; Biotest AG, Dreireich, Germany; Baxter Diagnostics AG, Düdingen, Switzerland; Manfred R. Hofmann Serologische Reagenzien, Bad Homburg, Germany; Ortho Diagnostic Systems, Neckargemünd, Germany). D variants were confirmed by a panel of monoclonal antibodies (D-Screen, Diagast, Lille, France) as described previously.[27]

### Isolation of genomic DNA

All individuals investigated were white and from Austria or Germany. Blood was collected in EDTA and centrifuged at 2000 × *g* for 20 minutes. The buffy coat (1 mL) was separated

**TABLE 1. Primers used for *RH* PCR-SSP***

| Exon | Specificity | Detected base | Name of primers | DNA sequence of primers (5'-3') | Resulting specificity | PCR number/ PCR product (bp) |
|---|---|---|---|---|---|---|
| 2 | *RHD/C* | 201 | D-2-201 | GCT TGG GCT TCC TCA CCT CG | *RHD/C* | 1/148 |
| 2 | *RHD/C* | 307 | D-2-307 | CAG TGT GAT GAC CAC CTT CCC AGA | | |
| 3 | *RHD* | 383 | D-3-383 | TTG TCG GTG CTG ATC TCA GTG GA | *RHD* | 2/113 |
| 3 | $RH^{all}$† | 451 | D-3-451 | ACT GAT GAC CAT CCT CAG GTT GCC | | |
| 4 | $RH^{all}$ | 527 | D-4-527 | ACA TGA TGC ACA TCT ACG TGT TCG C | *RHD* | 3/122 |
| 4 | *RHD* | 602 | D-4-602 | CAG ACA AAC TGG GTA TCG TTG CTG | | |
| 5 | *RHD/e* | 676 | D-5-676 | ATG TTC TGG CCA AGT GTC AAC TCT G | *RHD* | 4/157 |
| 5 | *RHD* | 787 | D-5-787 | ctg ctc acC TTG CTG ATC TTC CC | | |
| 6 | $RH^{all}$ | 826 | D-6-826 | TTA TGT GCA CAG TGC GGT GTT GG | *RHD* | 5/132 |
| 6 | *RHD* | 916 | D-6-916 | CAG GTA CTT GGC TCC CCC GAC | | |
| 7 | $RH^{all}$ | 967 | D-7-967 | GTT GTA ACC GAG TGC TGG GGA TTC | *RHD* | 6/122 |
| 7 | *RHD* | 1048 | D-7-1048 | TGC CGG CTC CGA CGG TAT C | | |
| 9 | $RH^{all}$ | 1168 | D-9-1168 | tat gca ttt aaa cag GTT TGC TCC TAA ATC | *RHD* | 7/83 |
| 9 | *RHD* | 1193 | D-9-1193 | AGA AAA CTT GGT CAT CAA AAT ATT TAG CCT | | |
| 10 | $RH^{all}$ | 1255 | D-10-1255 | TCC TCA TTT GGC TGT TGG ATT TTA AG | *RHD* | 8/147 |
| 10 | *RHD* | 1358 | D-10-1358 | CAG TGC CTG CGC GAA CAT TG | | |
| 1 | $RH^{all}$ | –24 | C-1-(–24) | GAT GCC TGG TGC TGG TGG AAC | $RHC/c(cyt^{48})$ | 9/112 |
| 1 | $RHC/c(cyt^{48})$ | 48 | C-1-48 | GCT GCT TCC AGT GTT AGG GCG | | |
| 2 | $RHc/c(cyt^{48})$ | 201 | C-2-201 | GGC TTG GGC TTC CTC ACC TCA | $RHc/c(cyt^{48})$ | 10/149 |
| 2 | $RHc/c(cyt^{48})$ | 307 | C-2-307 | AG TGT GAT GAC CAC CTT CCC AGG | | |
| 5 | *RHE* | 676 | E-5-676 | GAT GTT CTG GCC AAG TGT CAA CTC TC | *RHE* | 11/158 |
| 5 | *RHE/e* | 787 | E-5-787 | ct gct cac CAT GCT GAT CTT CCT | | |
| 5 | *RHD/e* | 676 | D-5-676 | ATG TTC TGG CCA AGT GTC AAC TCT G | *RHe* | 12/158 |
| 5 | *RHE/e* | 787 | E-5-787 | ct gct cac CAT GCT GAT CTT CCT | | |
| | *HGH*‡ | 5580 | 5580 hgh | TGC CTT CCC AAC CAT TCC CTT A | *HGH* | 1-12/434 |
| | *HGH* | 5967 | 5967 hgh | CCA CTC ACG GAT TTC TGT TGT GTT TC | *HGH* | |

* For all primers, the respective exons, their specificity for *RHD* and *RHCE* alleles, and their position from the 3' end on the *RHD* and *RHCE* coding sequences are given. Lower-case letters are sequences occurring in introns, upper-case letters represent those in exons.
† $RH^{all}$ denotes primers that are specific for either *RHD, RHC, RHc, RHc(cyt$^{48}$), RHE,* or *RHe.*
‡ As controls for amplification, primers for the human growth hormone (HGH) are used in all PCRs.

from the red cells. The DNA was isolated using a modified salting-out method described by Miller et al.[28] After the addition of 10 mL of lysis buffer I to the buffy coat, we continued according to the method of Miller et al. DNA was also prepared from small samples (e.g., 50 µL-1 mL whole blood) using a salting-out method that was reduced in scale or a kit (QIAamp, Qiagen, Hagen, Germany).

## Allele-specific PCR amplification

The DNA sequences used were those published for *RHCE* and *RHD*.[1-4] Additional information used included data on the differences between the alleles of *RHCE* given by Mouro et al.[11] and data on exon-intron borders given by Chérif-Zahar et al.[12] PCR primer selection was performed with a computer program (MacVector, version 4.5.3; Kodak, New Haven, CT); synthesis was done by a company that synthesizes oligonucleotides (Microsynth, Balgach, Switzerland).

Each *RHD* exon-specific reaction consisted of at least one *RHD*-specific primer (except for PCR for *RHD* exon 2; Table 1) and its common *RH*-specific counterpart (except for PCRs for *RHD* exons 2 and 5; Table 1). For exon 1 and 8, no gene-specific nucleotide differences between *RHD* and *RHCE* are known, and hence no *RHD*-specific PCRs could be established. Because coding sequences of *RHC* and *RHD* are identical in exon 2, *RHC* detection is omitted in a D-positive individual. Therefore, *RHC* was typed at cytosine 48 in exon 1, although this position is not indicative only for *RHC*, but also for an infrequent *RHc* variant, which will be referred to as *RHc(cyt$^{48}$)*.[29,30] *RHc* was identified with two primers that are specific for *RHc* at positions 201 and 307. Discrimination of *RHE* and *RHe* was done in two PCRs. Both reactions included the same primer that was specific for *RHCE* at nucleotide position 787 and one primer that was specific for *RHE* at nucleotide position 676 or one primer that was specific for *RHe* at nucleotide position 676.

Oligonucleotide primers used in the *RH* typing system and their combinations, amplification product lengths, and specificities are given in Table 1. Nucleotide substitutions between the various *RHD* and *RHCE* alleles and the nucleotides detected with our *RH* PCR-SSP are shown in Fig. 1.

In each PCR, control human growth hormone oligonucleotides were used to amplify a 434-bp PCR fragment from the human growth hormone locus position 5559 to 5992,[31] which served as a positive amplification control. In the case of a specific *RHD/CE* amplification, the 434-bp control fragment may not occur in the PCR, because of competition. The concentration of the detection primers was 0.2 µ*M*, with the exception of PCRs 7 and 11, in which 0.35 and 0.1 µ*M*, respectively, were used. Concentration of the control primers was 0.05 µ*M* (0.08 µ*M* in PCRs 9, 10, and 12).

Amplification was carried out in a final volume of 10 µL, containing 50 m*M* KCl, 10 m*M* Tris/HCl (pH 8.3), 0.01-percent gelatin, 5.0-percent glycerol, 100 µg per mL of cresol red, 200 µ*M* of each dNTP, 100 ng of genomic DNA (quantitated by ultraviolet light) or 2 µL of QIAamp spin column eluate, and 0.4 units of *Thermus aquaticus* polymerase (Perkin-Elmer Cetus, Norwalk, CT). Concentrations of $MgCl_2$ varied among the PCRs and were 0.9 m*M* (PCR 10), 1.1 m*M* (PCR 5), 1.2 m*M* (PCRs 1 and 4), 1.35 m*M* (PCR 12), and 1.5 m*M* (PCRs 2, 3, 6 to 9, and 11). To facilitate *RH* DNA typing, all 12 PCRs were triggered to work under the same thermocycling conditions on a DNA thermal cycler (PCR System 9600, Perkin-Elmer Cetus). The conditions were an initial denaturation step of 120 seconds at 94°C, 10 incubation cycles for 10 seconds at 94°C and 60 seconds at 65°C; and 20 incubation cycles for 30 seconds at 94°C, 60 seconds at 61°C, and 30 seconds at 72°C. PCR fragments were sepa-

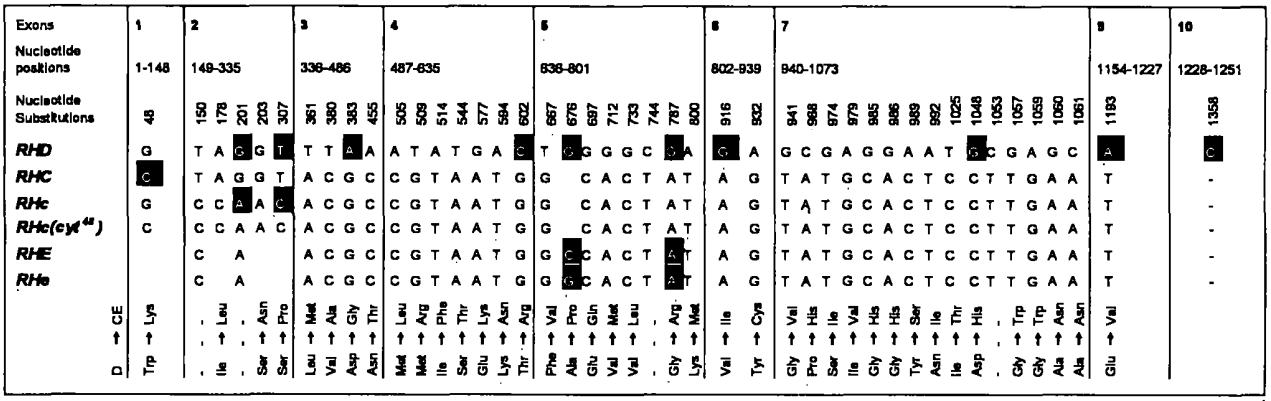
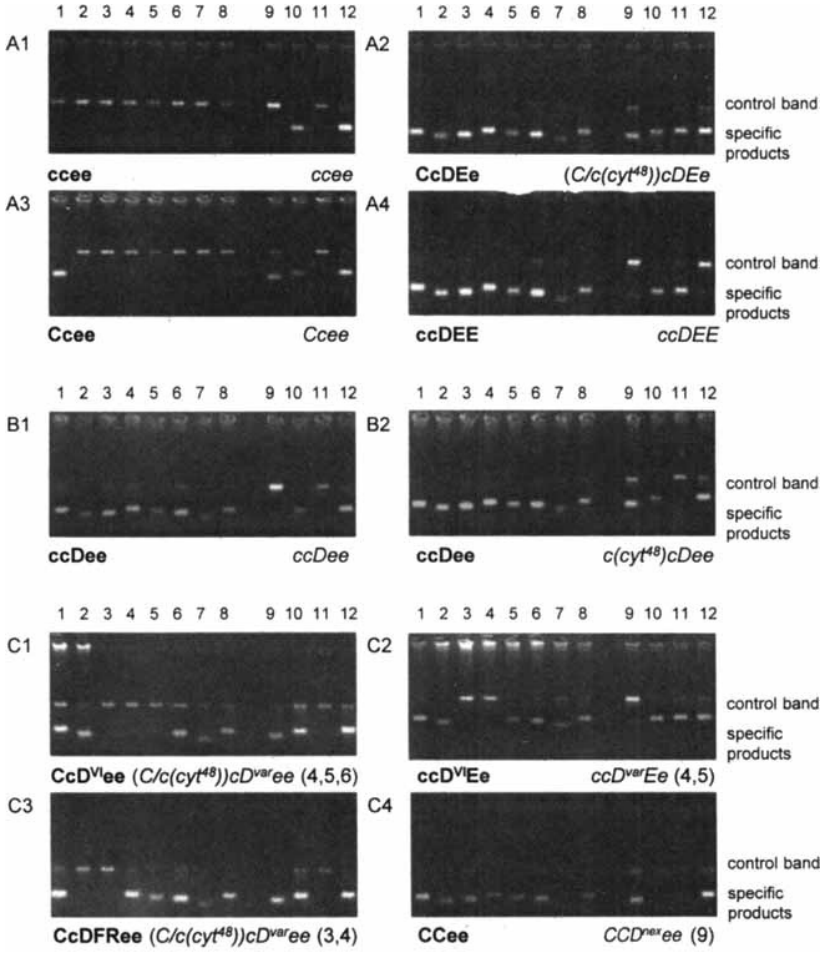



**Fig. 1. DNA sequence data for the exons of the published *RHD* and *RHCE* alleles. The exons are given along with their portion of the coding sequence of *RHD* (+1 refers to the A of the initiation codon ATG). The positions of nucleotide substitutions occurring in various *RHD* and *RHCE* alleles are indicated. The resulting amino acid substitutions are shown. White letters on black background represent nucleotide residues detected by the *RH* PCR-SSP. The DNA sequence of the *RHCE* gene is unrelated to the *RHD* gene in the region of position 1358 and is denoted by dashes.**




| Reaction | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Specificity for | *D/C* | *D* | *D* | *D* | *D* | *D* | *D* | *D* | *C/c(cyt48)* | *C/c(cyt48)* | *E* | *e* |
| in Exon | 2 | 3 | 4 | 5 | 6 | 7 | 9 | 10 | 1 | 2 | 5 | 5 |
| at nucleotide(s) | 201 307 | 383 | 602 | 676 787 | 916 | 1048 | 1193 | 1255 | 48 | 201 307 | 787 | 787 |
| Product (bp) | 148 | 113 | 122 | 157 | 132 | 122 | 83 | 147 | 112 | 149 | 158 | 158 |

**Fig. 2. *RH* PCR-SSP results of 10 representative *RHD/CE* types. Panels A1 to C4 show agarose gel electrophoresis of 12 *RH* PCR-SSP performed with DNA from persons with various known Rh phenotypes. The PCR numbers are given at the top of each panel group. The phenotype (bold face) and the detected DNA type (italics) are shown at the bottom of each panel. The *RH* PCR-SSP characteristics are shown in the interpretation scheme at the bottom of the figure. A1 through A4 represent common Rh phenotypes (ccee, CcDEe, Ccee, ccDEE). B1 and B2 show samples of ccDee phenotypes. Reaction 9 of B2 is positive, which indicates the presence of *c(cyt48)cDee*. In C1 through C4, exons of missing *RHD*-specific nucleotides are given in brackets. C1, C2, and C3 are two samples of the $D^{VI}$ phenotype and one sample of the DFR phenotype, respectively, and each fails to present some of the different *RHD*-specific nucleotides. C4 is a sample with CCee phenotype with an *RHD* allele, whose product cannot be detected serologically (data not shown). DNA of this $RHD^{nex}$ allele fails to contain the exon 9-specific amplification product.**

rated by size in a 2-percent agarose gel containing 0.5 µg per mL of ethidium bromide, visualized with ultraviolet light, and photodocumented.

### Statistical analysis

CIs of frequencies were calculated according to the binomial distribution. The haplotype frequency of *RHc(cyt48)De* was based on published data.[27]

## RESULTS

We tested DNA from 200 consecutive blood donors with common Rh phenotypes, 77 blood donors with weak D phenotype, and 78 donors with rare and very rare phenotypes with an *RH* gene- and allele-specific PCR-SSP. *RH* PCR-SSP types obtained by our method were compared to serologically defined Rh phenotypes. Representative results of our *RH* PCR-SSP typings are shown in Fig. 2. The results of the genetic typing and the correlation to the serologic phenotype are given in Table 2.

### Typing for *RHD*

In all 200 random donors, the expected *RHD* exon-specific nucleotides were present in D-positive samples and absent in D-negative samples. Concordant results were also obtained in the weak D and rare Rh phenotype samples, with one exception. The exception was found in a sample of CCee phenotype, which presented an unknown *RHD*-specific amplification pattern indicating the absence of *RHD*-specific adenine 1193 in exon 9 (Fig. 2, panel C4).

### Typing for $RHD^{var}$ in weak D

Among 77 samples of consecutive blood donors representing weak D phenotypes, all *RHD*-specific nucleotides were present in 70 samples, but some of the *RHD*-specific PCRs failed to yield amplified DNA in 7 samples. In all cases with an absence of *RHD*-specific nucleotides, D variant proteins could be defined serologically (1

**TABLE 2. Results of *RH* PCR-SSP**

| Individuals investigated | Phenotype | DNA type* | Absent *RHD*-specific nucleotides and position | Number | Percentage† |
|---|---|---|---|---|---|
| Common phenotypes (n = 200) | ccee | *ccee* | None | 48 | 24.0 |
| | Ccee | *Ccee* | None | 2 | 1.0 |
| | CcDee | *(C/c[cyt$^{48}$])cDee* | None | 68 | 34.0 |
| | CCDee | *CCDee* | None | 32 | 16.0 |
| | ccDEe | *ccDEe* | None | 20 | 10.0 |
| | ccDEe | *(C/c[cyt$^{48}$])cDEe*‡ | None | 1 | 0.5 |
| | ccDee | *(C/c[cyt$^{48}$])cDee*‡ | None | 1 | 0.5 |
| | CcDEe | *(C/c[cyt$^{48}$])cDEe* | None | 24 | 12.0 |
| | ccDEE | *ccDEE* | None | 4 | 2.0 |
| Rare phenotypes (n = 3) | CCee | *CCee* | None | 1 | |
| | CCee | *CCD$^{ex}$ee*§ | A 1193 | 1 | |
| | CcDFRee | *(C/c[cyt$^{48}$])cD$^{var}$ee* | A 383, C 602 | 1 | |
| Weak D and D$^{cat}$ phenotypes (n = 77) | ccD$^{weak}$Ee | *ccDee* | None | 21 | 27.3 |
| | CcD$^{weak}$ee | *(C/c[cyt$^{48}$])cDee* | None | 39 | 50.6 |
| | CcD$^{weak}$Ee | *(C/c[cyt$^{48}$])cDEe* | None | 1 | 1.3 |
| | CCD$^{weak}$ee | *CCDee* | None | 9 | 11.7 |
| | ccD$^{VI}$Ee | *ccD$^{var}$Ee* | C 602, G 676 and/or G 787 | 4 | 5.2 |
| | CcD$^{VI}$ee | *(C/c[cyt$^{48}$])cD$^{var}$ee* | C 602, G 676 and/or G 787, G 916 | 2 | 2.6 |
| | ccD$^{var}$ee(Rh33) | *ccD$^{var}$ee* | Only exon 5 *RHD*-specific bases are present: G 676 and/or G 787 | 1 | 1.3 |
| Phenotypes tested for determination of *RHc(cyt$^{48}$)* frequency (n = 74) | ccDee | *ccDee* | None | 19 | |
| | ccDee | *(C/c[cyt$^{48}$])cDee*‡ | None | 40 | |
| | ccDEE | *ccDEE* | None | 12 | |
| | ccEE | *ccEE* | None | 3 | |

* (C/c[cyt$^{48}$]) indicates the presence of cytosine 48, which eliminates discrimination of *RHC/c(cyt$^{48}$)* in these cases. *D$^{var}$* indicates the absence of *RHD*-specific nucleotides. In these cases, absent *RHD*-specific nucleotides and their position on the coding sequence of *RHD* are shown.
† Percentages are given where appropriate.
‡ Samples for which detection for nucleotide cytosine 48 (in exon 1) is not specific for *RHC* because of the likely presence of *RHc(cyt$^{48}$)*.
§ The presence of *RHCe/RHCe* with an unexpressed *RHD*.

Rh33, 6 D$^{VI}$). In typing of the Rh33 sample, the only positive reactions were those for *RHD* exon 5, *RHc*, and *RHe*. In four D$^{VI}$ samples, the *RHD*-specific nucleotide cytosine 602 (exon 4), one or both of the nucleotides guanosine 676 and guanosine 787 (both exon 5) were absent (Fig. 2, panel C2). The presence of *RHD*-specific guanosine 916 in exon 6, which we observed in these four D$^{VI}$ samples, has not been described previously among D$^{VI}$ phenotypes.[7,8] In two additional D$^{VI}$ samples only, we found the same pattern plus the lack of guanosine 916 (exon 6) as has repeatedly been reported for D$^{VI}$ (Fig. 2, panel C1). One known DFR sample was investigated by our method; it was negative in two PCRs that were specific for *RHD* at nucleotides adenine 388 (exon 3) and cytosine 602 (exon 4) (Fig. 2, panel C3). The absence of *RHD*-specific nucleotide adenine 388 (characteristic of exon 3) is also a new finding not explained by previous RHDFR characterization.[8] Our finding likely indicated a split in DFR phenotypes.

## Typing for *RHCc*

As already mentioned, typing for *RHC* at nucleotide 48 is inappropriate for discrimination between *RHC* and *RHc*. This can be explained by an infrequent *RHc* variant, denoted *RHc(cyt$^{48}$)*, that shares cytosine 48 in exon 1 with *RHC*.[29,30] Nevertheless, the use of PCR number 9 *RHC/c(cyt$^{48}$)* together with PCR number 10 *RHc/c(cyt$^{48}$)* allowed correct identification of the *RHC/c* type in all typings with a negative reaction in PCR number 9 or 10. These DNA typings could be correctly identified as *RHc/RHc* or *RHC/RHC*, respectively. In D-negative individuals, PCR 1 (specific for *RHD* and *RHC* in exon 2) also allowed the correct identification of *RHC/c* type (Fig. 2, panel A3). For each D-positive sample with positive reactions in PCRs 9 and 10, there was shared c in the phenotype as expected, but we were unable to predict the second *RHCc* allele presenting either an *RHC* or *RHc(cyt$^{48}$)* band (Fig. 2, panel A2).

## Calculation of *RHc(cyt$^{48}$)* frequency

To clarify whether *RHc(cyt$^{48}$)* is associated with a specific haplotype, we investigated additional 59 ccDee, 12 ccDEE, and 3 ccEE blood samples. Therefore, including the results obtained by the typing of 200 random donors, a total of 60 ccDee, 16 ccDEE, 3 ccEE, and 48 ccee samples were analyzed. Forty-one of the ccDee samples, but none of those of other phenotypes, showed cytosine 48 in exon 1 as evidence of an *RHc(cyt$^{48}$)* allele. The resulting 95-percent CIs for *RHc(cyt$^{48}$)* occurring in this phenotype were ccDee, 0.55 to 0.89; ccDEE, ≤0.17; and ccee, ≤0.06. The resulting relative haplotype frequencies were c(cyt$^{48}$)De/cDe, 0.68 (CI, 0.54-0.88); c(cyt$^{48}$)DE/cDE, 0 (CI, ≤0.09); and c(cyt$^{48}$)de/cde, 0 (CI, ≤0.03).

### Typing for *RHcEe*

Typing for *RHc*, *RHE*, and *RHe* revealed complete concordance between serologic and genetic testing in all the cases investigated in this study. In addition, two phenotypes of rare occurrence were tested. Three ccEE samples and two individuals with the CCee phenotype were correctly identified by our *RH* PCR-SSP.

## DISCUSSION

We developed an *RH* PCR-SSP, which offered a rapid method of genetic characterization of the *RHD* and *RHCE* allele heterogeneity. Our method is also useful for routine *RH* DNA typing. It can be performed easily, cost-effectively, and in less than 2.5 hours after DNA preparation. Furthermore, the technique will facilitate the genetic characterization of known D category phenotypes and the detection of new D variants by molecular screening. Because the information about *RH*-specific sequences is rapidly increasing, it was advantageous to devise a modular *RH* PCR-SSP, which can be complemented by additional PCRs.

With our *RH* PCR-SSP, we were able to genetically identify more samples in congruence with their serologic phenotype than with previously published *RHD* typing methods. Because our method allows a total of eight reactions at once, it allows genetic testing for several known and some novel $RHD^{cat}$ samples and does not run any risk of typing them as *D*-negative samples.

Our exon-scanning method is capable of properly identifying several *RHD/CE* hybrids and $RHD^{cat}$ samples such as *RHDFR*, $RHD^{IVa,b}$, $RHD^{Va}$, $RHD^{VI}$, and *RH*33.[32] We confirmed the exon pattern reported for $RHD^{VI}$ and *RHDFR*.[7,8] It is interesting that novel allelic variants were found among these samples of partial *RHD*, which expanded the genetic heterogeneity of $RHD^{VI}$ and *RHDFR*. $D^{II}$, DNU, and $D^{VII}$ are caused by single-point mutations,[6,33] which cannot be detected by the present method. Therefore, a reaction pattern indistinguishable from common *RHD* is expected for these $RHD^{var}$ typings and was confirmed for $D^{VII}$ (data not shown). These partial D samples (and, potentially, new allelic variants) may be identified by 1) single-strand conformation polymorphism or heteroduplex analysis methods covering the entire gene for *RHD* or 2) an appropriate modular extension of our exon-scanning PCR-SSP. Preliminary results with an $RHD^{VII}$-specific module proved the presence of the Leu(110)Procoding nucleotide substitution in 28 $D^{VII}$ samples (Flegel et al. Manuscript in preparation).

All common Rh phenotypes (n = 200) were properly characterized for D, E, and e. For 53 percent (106/200) of all common Rh phenotypes, the C/c type could be determined precisely. However, we observed two interesting limitations of our method. First, typing for cytosine at nucleotide 48 was correctly, but not exclusively, correlated to C, as already reported by Wolter's group.[29,30] Taking into account serologic data, we were able to calculate the frequency of $RHc(cyt^{48})$ in a white population. Currently, *RHC* and $RHc(cyt^{48})$ are not distinguished by our method. Recent reports of a restriction fragment length polymorphism in intron 2,[22] also specific for *RHC*, may offer the possibility of devising an exclusively *RHC*-specific PCR. Second, a novel nonfunctional *RHD* allele ($RHD^{nex}$) was detected in a CCee sample, which was lacking the *RHD*-specific adenine 1193 in exon 9 (Fig. 2, panel C4). This is suggestive of a $CCee(r'r')$ genotype described by Hyland and colleagues.[5] However, an $RHD^{nex}$ allele, lacking the *RHD*-specific exon 9 (adenine 1193) only, has not previously been observed.

We conclude that our *RH* PCR-SSP is practical for both routine typing of *RHD* DNA types and molecular screening for novel $RHD^{var}$. The *RH* PCR-SSP results may point to the rearranged parts of the *RHD* gene, which can then be fully characterized by DNA sequencing. In this study, we investigated a limited number of random-donor samples and a few samples from people with rare Rh phenotypes. We were surprised by the great heterogeneity of *RHD*, which we could detect in this relatively small panel of samples. The readily detected heterogeneity is evidence for the considerable value of our exon-scanning *RH* PCR-SSP as a scientific tool in the further characterization of the *RHD/CE* polymorphism.

#### ACKNOWLEDGMENTS

The authors thank Verena Mayr, Evelyn Draxl, and Claudia Gerstenbräun for excellent technical assistance.

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