Transfusion Medicine, 1998, **8,** 281–302

REVIEW ARTICLE

Rh phenotype prediction by DNA typing and its application to practice

W. A. Flegel,* F. F. Wagner,* T. H. Müller† and C. Gassner‡ **Abteilung Transfusionsmedizin, Universitätsklinikum Ulm and DRK-Blutspendedienst Baden-Württemberg, Institut Ulm, Ulm, Germany, †DRK-Blutspendedienst Niedersachsen-Oldenburg, Institut Oldenburg, Oldenburg, Germany, and ‡Zentralinstitut für Bluttransfusion und Immunologische Abteilung Innsbruck, Innsbruck, Austria*

Received 27 April 1998; accepted for publication 20 August 1998

SUMMARY. The complexity of the *RHD* and *RHCE* genes, which is the greatest of all blood group systems, confounds analysis at the molecular level. *RH* DNA typing was introduced in 1993 and has been applied to prenatal testing. PCR-SSP analysis covering multiple polymorphisms was recently introduced for the screening and initial characterization of partial D. Our objective is to summarize the accrued knowledge relevant to the approaches to Rh phenotype prediction by DNA typing, their possible applications beyond research laboratories and their limitations. The procedures, results and problems encountered are highly detailed. It is recommended that DNA typing comprises an analysis of more than one polymorphism. We discuss future directions and propose a piecemeal approach to improve reliability and cost-efficiency of blood group genotyping that may eventually replace the prevalent serology-based techniques even for many routine tasks. Transfusion medicine is in the unique position of being able to utilize the most extensive phenotype databases available to check and develop genotyping strategies.

Key words: blood group, DNA typing, genomic analysis, genotyping, human diversity, partial D, PCR-RFLP, PCR-SSO, PCR-SSP, phenotype prediction, red blood cell, Rh, *RHCE*, RhD, *RHD*, Rhesus, RT-PCR, transfusion.

The genes of almost all blood group systems have been cloned and the molecular bases of their major antigens elucidated. Hence, DNA typing has become possible for many blood group antigens that are mostly defined by single amino acid polymorphisms and expressed by proteins of the red blood cell surface (Anstee, 1995; Avent, 1997; Huang, 1997b). Suitable PCR techniques for genotyping of *ABO* (Gassner *et al*., 1996), *Kell* (Hessner *et al*., 1996), *Duffy* (Mallinson *et al*., 1995), *Kidd* (Olives *et al*., 1997), *MN* and *Ss* (Eshleman *et al*., 1995) have been described. In a similar fashion, DNA typing for alleles of both *RH* genes can be performed.

The first application of Rh phenotype prediction by DNA typing was published in 1993 (Bennett *et al*., 1993; Lo *et al*., 1993). A rather high rate of false positive results (Simsek *et al*., 1994) and false negative results (Bennett & Cartron, 1994; Simsek *et al*., 1994) was immediately recognized and the authors proposed that 'the use of two independent primer sets should reduce the risk of incorrect genotyping.' (Bennett & Cartron, 1994; Simsek *et al*., 1994). Many polymorphisms have been utilized for RhD phenotype prediction since then (previously reviewed by Wolter *et al*., 1993; Hyland *et al*., 1995; van den Veyver & Moise, 1996; Aubin *et al*., 1997). No consensus has yet been achieved as to which polymorphism may be most reliable for testing of any given population.

RH and particularly *RHD* DNA typing is much confounded by the presence of two highly homologous genes, *RHCE* and *RHD*, and the complex polymorphisms between both genes. In contrast to most other blood group antigens, the RhD antigen does not derive from amino acid polymorphisms, but from the presence of a separate protein absent in the RhD-negative phenotypes. Furthermore, there are proteins, such as R_0^{Har} and D^{VI}, that express RhD-immunoreactivity but do not share any common *RHD*-specific nucleotide sequences. To establish reliable and workable solutions for *RH* DNA typing

Correspondence to: Willy A. Flegel, Priv.-Doz. Dr med., Abteilung Transfusionsmedizin, Universitätsklinikum Ulm, and DRK-Blutspendedienst Baden-Württemberg, Institut Ulm, Helmholtzstrasse 10, D-89081 Ulm, Germany. Tel: +49 731 150 600; fax: +49 731 150 602; e-mail: waf@ucsd.edu

is probably the most challenging task among all blood group genotyping applications.

The discrimination of *RHD* homozygotes from *RHD* heterozygotes is hampered (Cossu *et al*., 1996) because the most prevalent *RHD*-negative allele cannot yet be detected specifically. For the time being, we prefer therefore the term DNA typing rather than genotyping, when an RhD phenotype prediction by molecular techniques is attempted.

We have summarized the molecular genetics of the *RH* genes according to the current understanding. A list of *RHD* alleles, catalogued by molecular rather than serological criteria, and of polymorphisms between *RHD* and *RHCE*, useful for DNA typing, is provided. The development of suitable DNA techniques is discussed. We present a survey of published *RH* DNA typing methods. The propagation of DNA typing methods for blood groups, like Rh, from the benches of some specialized laboratories to a widely established routine tool for transfusion medicine is under way. Efficient and affordable solutions should be widely adapted.

CLINICAL SIGNIFICANCE

The first application of *RH* DNA typing was prenatal testing for fetal RhD status to indicate the requirement for anti-D prophylaxis and clinical management of pregnant women with anti-D (Bennett *et al*., 1993; Lo *et al*., 1993). DNA typing may require less fetal tissue and can be performed before the Rh proteins are expressed, rendering the fetal DNA typing less invasive than serological phenotyping. The DNA typing can be done with any fetal tissue, such as amniotic fluid ('amniocytes'), trophoblastic cells ('chorionic villi') or cord blood ('fetal blood cells'). Essentially noninvasive methods for fetal testing by retrieving fetal (trophoblastic) cells shed into the endocervical canal (Adinolfi *et al*., 1995) or fetal cells circulating in the mother's peripheral blood (Lo *et al*., 1994a,b) appear possible. For the latter application, selection of the appropriate fetal cell type is crucial, because fetal lymphocytes may persist for many years in the mother's circulation (Bianchi *et al*., 1996) preventing a meaningful test interpretation in multiparous women. Recent approaches stressed the importance of test sensitivity (Hengstschlager *et al*., 1997; Tonn *et al*., 1997) and focused on fetal nucleated red blood cells, which may be enriched (Gänshirt-Ahlert *et al*., 1993; Busch *et al*., 1994; Geifman-Holtzman *et al*., 1996b; Sekizawa *et al*., 1996), tested as single cells (Ferguson-Smith *et al*., 1994; van den Veyver *et al*., 1995; Sekizawa *et al*., 1996; Reubinoff *et al*., 1996) or assayed for *RHD* mRNA (Hamlington *et al*., 1997). Prenatal genotyping has already been applied to many other blood group proteins like RhCE (Le Van Kim *et al*., 1994; Spence *et al*., 1995b; Dildy *et al*., 1996; Geifman-Holtzman *et al*., 1996a), Kell (Lee *et al*., 1996; Spence *et al*., 1997), MN and Ss (Eshleman *et al*., 1995).

Other applications of *RH* DNA typing are in paternity testing and forensic medicine (Ikemoto *et al*., 1996). Like all genetic testing, *RH* DNA typing would be feasible with any source of human tissue carrying nucleated cells or remnants thereof, including blood, serum, plasma, skin, hair, hair follicle, bone, semen or urine.

Emerging evidence is pointing to the possibility that DNA-based phenotype prediction is superior to serotyping, when serological typing cannot be accomplished with its usual ease. After massive transfusion of blood components, the serological typing is frequently hampered by the admixture of allogeneic red blood cells obscuring the recipient's antigens. Despite the transfer of donor leucocytes, DNA typing was feasible for *HLA* (Wenk & Chiafari, 1997), *MN* and *Ss* (Eshleman *et al*., 1995). It seems promising that suitable methods will be developed for other blood groups so that a reliable typing can be provided even after massive transfusions. In partial D with reduced antigen density, serological discrimination is often hampered by limiting antibody sensitivity. DNA-based phenotype prediction was shown to be superior to serological discrimination of such RhD phenotypes, as exemplified by the different D^{VI} types (Wagner *et al*., 1998a).

The relevance of *RH* DNA typing for clinical management depends critically on the reliability of the Rh phenotype prediction. Allele variants, especially *RHD*/*CE* hybrid alleles, and random nucleotide substitutions in the gene account for discrepancies between the results of DNA-based phenotype prediction and of serology-based phenotyping. *RH* genotyping may eventually replace current serological Rhesus testing, if two major obstacles can be overcome. A more comprehensive understanding of the *RH* locus and its variant organization in different populations needs to be achieved (Carritt *et al*., 1994) and, more importantly, the cost-efficiency needs to be vastly improved.

SCIENTIFIC SIGNIFICANCE

A unique combination of features applying to the *RH* genes and Rh proteins qualifies them as an attractive model system. They represent two highly homologous genes that are located closely adjacent on a chromosome. Their polymorphism is shaped by a recent duplication and later deletion events. A multitude of gene conversion and recombination events are evident from the limited characterization of the extant alleles, which has been conducted to date. With a prudent combination of serological and molecular screens the knowledge will soon increase considerably. Hence, the *RH* polymorphism

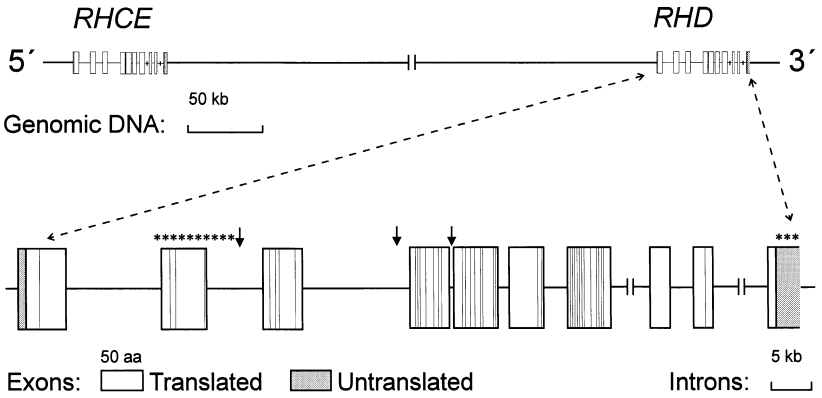


Fig. 1. Chromosomal organization of the *RHCE* and *RHD* genes according to the current understanding. The locus (top) spans less than 450 kb on the short arm of chromosome 1 between the positions 1p34·3 and 1p36·1 (Cherif-Zahar *et al*., 1991; MacGeoch *et al*., 1992). The physical distance between both genes is unknown. The *RHD* gene is located 3′ to the *RHCE* gene. The orientation of the genes in regard to each other and to the centromere is still unresolved. The genomic structure was analysed for the *RHCE* gene (Cherif-Zahar *et al*., 1994). The *RHD* gene is believed to have a very similar structure (bottom). There are 37 amino acid positions varying between the *ce* allele of *RHCE* and the prevalent *RHD* allele (thin lines in the 10 exons). The 3′ untranslated region (UTR) of *RHD* exon 10 (hatched) covers more than 1500 bp (Le Van Kim *et al*., 1992); a shorter stretch is known for *RHCE* (Cherif-Zahar *et al*., 1990), which deviated considerably (asterisks above 3′ UTR). Many of these exon polymorphisms have been utilized to detect the presence of the *RHD* allele (see Table 1). There is a 4247-bp stretch of near nucleotide identity between the *C* allele of *RHCE* and the *RHD* alleles spanning from intron 1 to intron 2 (asterisks around exon 2). The positions of nucleotide deletions occurring in introns of the *RHCE* gene are indicated by arrows: All three deletions (109 bp in intron 2, 288 bp in intron 3 and 651 bp in the very short intron 4) have been utilized for DNA typing to detect the presence of the *RHD* allele (see Table 4).

presents itself as a model system for the group of homologous adjacent genes, which comprise a large number of members and are frequent throughout the genomes of all species. With the exception of the *HLA* genes' locus the *RH* genes already represent the best characterized members of that group. It is a potential advantage that in contrast to HLA the Rh proteins do not seem to be exposed to stringent selective pressure and may therefore represent more faithfully the processes involved in near-random molecular evolution.

RH allele polymorphism will also be of interest to population biology because it has become feasible to determine in a massive way the frequencies of molecularly defined rare alleles in natural populations. Poisson-like allele distributions were predicted by mathematical models (Joyce & Tavare, 1995), which have so far not been checked in any real population.

The observation of alleles with multiple nucleotide substitutions in the *RHD* gene that were characteristic for the *RHCE* gene, but interspersed by *RHD*-specific DNA segments, cannot be explained by a single gene conversion event. This observation pointed to more complicated mechanisms shaping the polymorphism of proteins. The occurrence of various Rh proteins seems to represent a natural experiment for protein membrane integration. The mechanisms involved can be more completely explained once the function of the Rh protein has been discerned and the relationship of function and protein morphology has been checked.

RELEVANCE OF THE MOLECULAR GENETICS OF THE *RH* LOCUS

All antigens of the Rh blood group system are carried by two proteins encoded by two highly homologous genes, *RHD* and *RHCE*, with adjacent chromosomal positions. Recent reviews (Cartron, 1994; Rouger & Muller, 1996; Huang, 1997b; Scott *et al*., 1997; Sonneborn & Voak, 1997) summarize the accrued knowledge. The genes were derived from an older duplication process that was followed far more recently by a deletion encompassing the whole *RHD* gene in the prevalent RhD-negative haplotype. The allelic variability known from serological studies was confirmed by the current molecular work-up, which revealed many more alleles than anticipated by serology. A multitude of genetic mechanisms seems to have contributed to this allelic variability including multiple exchanges of DNA stretches between or recombination events affecting both genes, and numerous nucleotide substitutions occurring in both genes (Carritt *et al*., 1997).

The complex organization of the *RH* locus (Fig. 1) and the variable distribution of *RH* haplotypes among populations confound molecular genetics analysis and

Table 1. Rh antigen prediction by sequence-specific detection of polymorphic nucleotide positions in various exons and introns of the *RHD* and *RHCE* genes

Antigen	cDNA position(s)	Polymorphic nucleotide(s) assayed	Gene segment	Specific for antigen	Problems and notes
D (RH1)	383	A	exon 3	yes	–
	602	C	exon 4	yes	–
	n.a.	deletion	intron 4	yes	–
	676/787	G/G	exon 5	yes	–
	916	G	exon 6	yes	–
	1048	G	exon 7	yes	–
	1193	A	exon 9	yes	–
	1358	C	3′ UTR	yes	–
C (RH2)	48	C	exon 1	no	C^w, *c*(cyt48) positive
	n.a.	insertion	intron 2	yes	–
E (RH3)	676/787	C/A	exon 5	yes	–
c (RH4)	201/307	A/C	exon 2	yes	–
e (RH5)	676/787	G/A	exon 5	yes	–
C^w (RH8)	122	G	exon 1	yes	–
G (RH12)	201/307	G/T	exon 2	yes	D and C positive
D^{VII} (RH40)	329	C	exon 2	yes	–

The specificity is indicated for the prevalent alleles at the *RH* locus in Caucasians. Rare alleles may limit the specificity as discussed in the text (see Tables 2 and 3). At least one nucleotide position in each exon and intron with known polymorphisms is tested. There are no polymorphisms in exon 8 or in the coding sequence of exon 10 (see Fig. 1) and no suitable polymorphism for SSP in intron 3. D^{VII} represents *RHD*(L110P); C^w – *RHC*(Q41R); *c*(cyt48) – the *c*(W16C) allele of *RHCE* which is frequent in the cDe haplotype and serologically indistinguishable from the prevalent *c* alleles of *RHCE* (Gassner *et al*., 1997b); 3′ UTR – 3′ untranslated region of exon 10; n.a. – not applicable because of intronic location of the polymorphism. Primer sequences may be found in Gassner *et al*. (1997b) and for intron 4 in Avent *et al*. (1997c). For intron 2, C^w and D^{VII} the method is given in Gassner *et al*. (1997a), although no PCR-SSP primer sequences have yet been published.

its exploitation for DNA typing. Whilst understanding of the arrangement of the *RH* genes is far from complete, many polymorphisms between both genes along with allele polymorphisms have been established. Apart from exon sequences, polymorphisms in intron 2 (Poulter *et al*., 1996), 3 (Wagner *et al*., 1998a), 4 (Arce *et al*., 1993) and 8 (Kemp *et al*., 1996; Carritt *et al*., 1997) were utilized to predict the phenotype for many Rh antigens. Examples for 'diagnostic sites' suitable for PCR using sequence-specific priming are given in Table 1.

Partial D phenotypes

Anti-D immunization in RhD-positive individuals (Argall *et al*., 1953) was exploited to classify some RhD proteins into 'D categories' (Tippett & Sanger, 1962). The term 'partial D' (Salmon *et al*., 1984) refers to RhD proteins lacking epitopes that are often defined by monoclonal anti-D. Several partial D antigens permit anti-D immunization in their carriers. Rather good data on anti-D immunization is available for the more frequent D^{VI} and D^{VII} phenotypes. Only further studies will reveal which other partial D are frequently, rarely or never permissible for anti-D immunization. Aberrant *RHD* coding sequences have been shown for all investigated partial D. The molecular causes comprise (i) exchange of large gene segments between *RHD* and *RHCE* resulting in RhD/RhCE hybrid proteins; (ii) single or multiple nucleotide exchanges between *RHD* and *RHCE* not involving larger gene segments; and (iii) mis-sense mutations. Molecular alterations in partial D generally affect at least one extrafacial amino acid as predicted by the model for RhD membrane integration (Wagner *et al*., 1998b).

Several of these *RHD* alleles with aberrant *RHD* coding sequences are clinically important. Their discrimination from the prevalent *RHD* allele in transfusion recipients would be advantageous to guide RhD-negative transfusion therapy. The best characterized partial D to be set apart from RhD-positive in routine serological testing are the different D^{VI} types (Wagner *et al*., 1998a). However, as more data for the partial D population frequencies (Flegel & Wagner, 1996; Roubinet *et al*.,

1996) and the propensity for immunization by RhD-positive transfusion and pregnancy are gathered, the specific detection of other partial D may become desirable.

Weak D antigen expression

In contrast to the previously accepted dogma (Issitt & Telen, 1996; Roubinet *et al*., 1996; Rouillac *et al*., 1996; Aubin *et al*., 1997; Beckers *et al*., 1997; Fukumori *et al*., 1997; Huang, 1997b), the vast majority of *RHD* alleles found with weak D antigen expression encode aberrant RhD with amino acid substitutions (Flegel *et al*., 1998). We noted that in all weak D samples characterized at the molecular level the substituted residues were located in transmembranous and intracellular RhD protein segments (Wagner *et al*., 1998b).

Towards a molecular-based Rh nomenclature

For the purpose of an *RH*-allele-based nomenclature, we would favour the term 'aberrant *RHD*' allele referring to all *RHD* alleles coding for one or more amino acid substitutions compared to the prevalent, standard *RHD* allele. This DNA-based definition would broaden the serology-based definition of partial D and encompass a wider range of *RHD* alleles and the proteins/antigens encoded by these alleles. The name reflects the sporadic occurrence of these alleles and, hence, of their 'aberrant RhD' phenotypes, including but not limited to partial D. The proposed nomenclature has the advantage of covering almost all known *RHD* alleles (Table 2) including all known clinically relevant partial D and molecular weak D types (Wagner *et al*., 1998b). The nomenclature is unequivocally defined and does not depend on the availability of suitable anti-D. With regard to *RHCE*, for example, the *Rh33* allele and its protein would likewise be called aberrant *RHCE* and aberrant RhCE. Null alleles of the *RHD* gene, which are associated with ineffective RhD protein expression, like *RHD*(Q41X) (Avent *et al*., 1997c), may be referred to as nonfunctional *RHD*.

RhD-negative phenotype and nonfunctional alleles

The characterization of molecular structure(s) underlying the *RHD*-negative haplotype is incomplete. Lack of the whole *RHD* gene leads to the prevalence of the RhD-negative phenotype (Colin *et al*., 1991; Arce *et al*., 1993). Only the knowledge of the *RHD*-negative molecular structure(s) in various populations will permit '*RH* genotyping' for RhD phenotype prediction in the future.

It is noteworthy that nonfunctional alleles generally occur rather frequently in most genes, as originally shown by electrophoresis studies of enzymes in *Drosophila melanogaster* (Langley *et al*., 1981; Ohnishi *et al*., 1982). Similar results were obtained for the population frequency of nonfunctional alleles in the human H transferase gene (*FUT1*) (Wagner & Flegel, 1997). The majority of nonfunctional alleles are due to mis-sense, frame shift and non-sense mutations in the coding sequence (Cooper & Krawczak, 1993); promoter defects, loss of start codon and alterations of splice sites may also occur. Nonfunctional alleles are of principal practical importance for the specificity of any genotyping strategy aiming for a phenotype prediction (Flegel, 1997).

Thus, it was not surprising frequently to encounter RhD-negative samples harbouring *RHD*-specific DNA stretches (Table 3). This list of nonfunctional *RHD* alleles is expected to grow considerably. Their more frequent occurrence among RhD-negative samples in non-Caucasian populations (Daniels *et al*., 1997; Fukumori *et al*., 1997; Okuda *et al*., 1997) is explained in part by the lower prevalence of RhD-negative phenotypes in these populations. These alleles confound DNA typing, because they are refractory to some, most or all current DNA typing approaches.

The Rh_{null} phenotype of the regulator variant (Cherif-Zahar *et al*., 1996; Hyland *et al*., 1998) may carry an intact and functional *RHD* allele and, hence, cannot be specifically detected by any molecular analysis of the *RH* gene locus. Fortunately, this phenotype is even less frequent than the very rare Bombay phenotype in Caucasians (Wagner *et al*., 1995).

GENERAL TECHNICAL ASPECTS FOR DNA TYPING TECHNIQUES

DNA typing by PCR requires sample preparation and nucleic acid extraction; amplification of the desired 'diagnostic' DNA fragments; and the specific detection of the amplicons. Robust methods, starting from the sample preparation to the final interpretation of the results, are mandatory for any genotyping strategy. The development of convenient procedures and reliable instrumentation is anticipated to allow efficient and safe handling. The total number of pipetting and transfer steps should be minimized to avoid contamination and sample mix-ups. Automatic pipetting robots are available for contamination-free and reliable pipetting of small volumes of reagents.

DNA extraction and DNA amplification by PCR

Extraction of DNA from the biological sample deserves special attention to ensure adequate purity (including efficient removal of inhibitors such as haemoglobin) and adequate yield (even with relatively low numbers of amniotic cells) for the ensuing PCR. Adsorption-based extraction methods (e.g. spin-columns) have been

Table 2. A molecular based nomenclature for aberrant *RHD* alleles and a *RHCE* allele carrying *RHD* specific DNA stretches*

Allele	Location	Nucleotide change	Phenotype: Trivial name† Partial D	Haplotype(s)	Additional antigen(s)	Anti-D immunization‡	References
Missense mutations							
RHD(L110P)	exon 2	T→C at 329	D^{VII}	CDe	RH40 (Tar)	weak, infrequent	(Rouillac *et al.*, 1995b; Flegel *et al.*, 1996)
RHD(R229 L)	exon 5	G→A at 686	DHR	cDE	–	not reported	(Jones *et al.*, 1997)
RHD(T283I)	exon 6	C→T at 848	DHMi	c(D)E	–	strong, one sample	(Jones, 1995; Liu *et al.*, 1996; Wagner *et al.*, 1998b)
RHD(G353R)	exon 7	G→A at 1059	DNU	CDe	–	not reported	(Avent *et al.*, 1997a)
RHD/RHCE alleles with single or multiple short conversions							
RHD(S103P)	exon 2	T→C at 307	D (G negative)	cDE	RH12 (G) neg.	anti-G, one sample	(Faas *et al.*, 1996)
RHD(A354D)	exon 7	C→A at 1063	D^{II}	CDe	–	strong, rare	(Avent *et al.*, 1997a)
RHD(N152T,T201R,*F*223 V)	exon 3, 4 and 5	conversions	D^{IIIa}	cDe	RH20 (VS)	strong, few samples	(Huang *et al.*, 1997; Huang, 1997b)
RHD(L62F,N152T,D350H)	exon 3 and 7	conversions	D^{IVa} (D^{IV} type I)	cDe, CDe, cDE	RH30 (Go^a)	strong, few samples	(Rouillac *et al.*, 1995a; Huang, 1997b)
RHD/RHCE hybrid alleles (single larger conversions)							
RHD-CE(2)-D	exon 2	hybrid	D^{IIIb}	cDe	RH12 (G) neg.; RH20 (VS)	strong	(Rouillac *et al.*, 1995c)
RHD-CE(3)-D	exon 3	hybrid	D^{IIIc}	CDe	–	strong, several samples	(Beckers *et al.*, 1996a)
RHD-CE(3–5)-D§	exon 3–5	hybrid	DHMii	cDE	–	not reported	(Jones, 1995; Liu *et al.*, 1996)
RHD-Ce(3–6)-D	exon 3–6	hybrid	D^{VI} type III	C(D)e	RH52 (BARC)	strong, one sample¶	(Wagner *et al.*, 1998a)
RHD-CE(4)-D	exon 4 partial	hybrid	DFR	CDe > cDE	RH50 (FPTT)	weak, one sample	(Lomas *et al.*, 1994; Rouillac *et al.*, 1995a)
RHD-cE(4–5)-D	exon 4–5	hybrid	D^{VI} type I	c(D)E	–	strong, frequent	(Maaskant-van Wijk *et al.*, 1997a; Avent *et al.*, 1997b; Huang, 1997a)
RHD-CE(4–6)-D	exon 4–6	hybrid	D^{VI} type II	C(D)e	RH52 (BARC)	strong, frequent	(Mouro *et al.*, 1994)
RHD-CE(5)-D	exon 5	hybrid	D^{Va}	cDe, CDe, cDE	RH23 (D^w)	strong, few samples	(Rouillac *et al.*, 1995a)
RHD-CE(5–7)-D	exon 5–7	hybrid	DBT	CDe	RH32	strong, few samples	(Beckers *et al.*, 1996b; Wallace *et al.*, 1997)
RHD-CE(6–9)-D	exon 6–9	hybrid	D^{IV} type III	C(D)e	–	not reported	(Wagner *et al.*, 1998b)
RHD-CE(7–9)-D	exon 7 part to 9	hybrid	D^{IVb} (D^{IV} type II)	CDe	–	strong, rare	(Rouillac *et al.*, 1995a)
RHCE/RHD hybrid alleles							
RHCE-D(5)-CE	exon 5	hybrid	Rh33, R_0^{Har}	c(D)(e)	RH33, RH50 (FPTT)	weak, few samples	(Beckers *et al.*, 1996c; Beckers *et al.*, 1996d)

*Allele nomenclature in accordance with published recommendations (Beaudet *et al.*, 1996; Beutler *et al.*, 1996). Most known aberrant *RHD* alleles are shown excluding D – –, molecular weak D types (Flegel *et al.*, 1998) and RhD-negative phenotypes (see Table 3). A characterization of more than 15 alleles, all represented by mis-sense mutations, for molecular weak D types is forthcoming (Wagner *et al.*, 1998b). †Reviewed by Tippett *et al.* (1996). Partial D was defined (Salmon *et al.*, 1984), among other features, by its expressing of the antigen D in conjunction with the established lack of one or more D epitopes or allo-anti-D immunization or both. The phenotypes of D categories constitute a subgroup of partial D. The listing of D categories (D^{II} to D^{VII}) is complete, since no further partial D will be designated a 'D category': D^I and D^{Vc} are obsolete (Tippett & Sanger, 1977; Lomas *et al.*, 1989); D^{Vb} characterization is pending. As exemplified by D^{IV} and D^{VI}, the D category phenotypes can be subdivided by molecular characterization. ‡Summarized by Jones *et al.* (1995) and in the cited source reports. §Unexpectedly, *RHD* intron 4 was reported to be present (Jones, 1995). A full characterization is pending. ¶In a blood sample kindly provided by Zhu Zi-yan, Shanghai, China (Flegel & Wagner, 1998).

Table 3. RhD-negative phenotypes harboring *RHD*-specific DNA sequences*

Allele	Location	Nucleotide change	Probable haplotype	Populations	References
RHD(Q41X)	exon 1	C→T at 121	Cde	White	(Avent *et al*., 1997c)
RHD-CE(2–9)-D	exon 2 or 3–9	possible hybrid allele	Cde	White† & African	(Hyland *et al*., 1994; Andrews *et al*., 1998b) (Huang, 1996)
RHD-CE(3–7)-D	exon 3–7	hybrid allele	Cde^{s}	African & Asian	(Blunt *et al*., 1994; Carritt *et al*., 1994; Faas *et al*., 1997a)
RHD(488del4)	exon 4	deletion of 4 bp from 488: frameshift	Cde	White	(Hyland *et al*., 1994; Andrews *et al*., 1998a,b)
RHD-CE(4–7)-D	exon 4–7	hybrid allele	cdE	White†	(Faas *et al*., 1996,1997a; Avent *et al*., 1997c;
RHD(exon 5 variant)	exon 5	not communicated‡	cde	not communicated	(Carritt *et al*., 1994)
RHD(G314 V)	exon 7	G→T at 941	Cde	Japanese	(Okuda *et al*., 1997)
RHD(exon 9 variant)	exon 9	unknown	Cde	White	(Gassner *et al*., 1997b)

*Although no data have been gathered, frequent anti-D immunizations would be expected, if carriers were transfused RhD positive. †Australian blood donors of white descent (C. A. Hyland and B. H. W. Faas, written and personal communications). ‡The presence of a stop codon in exon 5 was reported without indicating the affected nucleotide position(s); no full report has been published since.

developed and are faster and easier than conventional phenol-chloroform and salting-out procedures, but should be carefully controlled for batch-to-batch variations. Fully automated validated commercial systems to extract nucleic acids from cells will help to facilitate and standardize this crucial step, which should be checked in quality control programmes.

Substantial advances in the quality of commercial thermocyclers during the past decade have shifted the realization of adequate reaction conditions for the PCR from a delicate technical problem to a highly reproducible routine procedure. Hot-start PCR techniques efficiently suppress mispriming. In a modular set-up several PCR-SSPs are performed in separate test tubes and analysed in parallel. In a multiplex set-up two or more PCR-SSPs are run in one test tube. Integrating several different primer pairs into a multiplex PCR in a single tube offers the important advantage of increasing the efficiency of genotyping by reducing the number of procedures and the required amounts of both DNA and reagents. PCR methods for successfully accommodating 11 or even more primer pairs in a single tube have been described (Lin *et al*., 1996). In DNA samples that contain only small amounts of contaminants interfering with PCR the yield of amplicons from a few primer pairs may still be sufficient; DNA samples of lesser purity and quality may be more representative of the routine situation and prone to yield less reliable results. Thus, multiplex PCR has to be most thoroughly optimized and standardized to accommodate adequate amplification from DNA samples of variable quality.

Amplicon detection

RHD and *RHCE* allele-specific amplicons synthesized by PCR are easily detected by electrophoresis in agarose gels followed by ethidium bromide staining. Sensitivity of this step is appropriate for the investigation of many samples and can be further improved by using either acrylamide gels or different dyes for staining, such as SYBR green I or silver. When testing a large number of samples, gel electrophoresis can easily limit the sample throughput. ELISA detection of amplicons is feasible by digoxigenin- or fluorescein-labelled primers used in combination with a biotinylated primer (Legler *et al*., 1996; Müller *et al*., 1997a). Incorporation of biotin into the PCR products allows capture of the amplicons on an avidin-coated microtitre plate (Fischer *et al*., 1995); digoxigenin and fluorescein antibodies then offer convenient means for detection of amplicons in hundreds of samples and for automation (Müller *et al*., 1997a). Simultaneous generation of several different amplicons by multiplex PCR requires not only adequate detection sensitivity but also high separation efficiency, especially for amplicons of similar size. Excellent resolution of

Table 4. Results of some published PCR set-ups applied to *RH* DNA typing

References	PCR		Mother (M) fetus (F) tested	Genes/ alleles	Sites (*n*)	Polymorphisms tested		Samples tested (*n*)	Aberrant allele		Technical problem§	
						Location						
	set-up	methods*				Intron	Exon		Detected† (*n*)	Mistyped‡ (*n*)	Mistyped (*n*)	No amplicon (*n*)
Lo *et al.* (1993)	single	SSP	F	*RHD*	1	–	10¶	71	0	0	2	0
Arce *et al.* (1993)	single	LP	no	*RHD*	1	4	–	8	0	0	0	0
Bennett *et al.* (1993)	single	SSP	F	*RHD*	1	–	10	15	0	0	0	0
Wolter *et al.* (1993)	single	SSP	no	*RHD*	1	–	7	3	0	0	0	0
Rossiter *et al.* (1994)	single	LP	F	*RHD*	1	4	–	28	0	0	0	0
Yankowitz *et al.* (1995)	single	LP	no	*RHD*	1	4	–	765	0	9	0	1
van den Veyver *et al.* (1995)	single	SSP	F	*RHD*	1	–	7	100	0	0	2	1
Lighten *et al.* (1995)	single	SSP	F	*RHD*	1	–	10	135	0	0	2	1
Adinolfi *et al.* (1995)	single	SSP	F	*RHD*	1	–	10	12	0	0	2	0
Simsek *et al.* (1994, 1995)	modular	LP/SSP	F	*RHD*	3	4	7 and 10	234	5	0	0	0
Spence *et al.* (1995a)	multiplex	LP/SSP	F	*RHD*	2	4	10	50	0	0	0	0
Pope *et al.* (1995)	multiplex	LP/SSP	F	*RHD*	2	4	10	63	1	0	0	0
Poulter *et al.* (1996)	single	RFLP	no	*RHD*	1	2	–	105	0	0	0	0
van den Veyver *et al.* (1996)	single	SSP	F	*RHD*	1	–	7	108	0	0	0	0
Sekizawa *et al.* (1996)	single	SSP	M	*RHD*	1	–	7	10	0	0	0	4
Dildy *et al.* (1996)	single	SSP	F	*RHD*	1	–	10	347	0	0	1	0
Tonn *et al.* (1997)	single	SSP	no	*RHD*	1	–	10	78	0	0	0	0
Maas *et al.* (1997)	modular	SSP/LCR	no	*RHD*	3	–	2, 5 and 7	207	0	0	0	0
Gassner *et al.* (1997b)**	modular	SSP	no	*RHD*	8	–	2–7, 9 and 10	354	2	0	0	0
Avent *et al.* (1997c)	multiplex	SSP	F	*RHD*	2	4	10	357	13	7	0	0
Maaskant-van Wijk *et al.* (1997b)	multiplex	SSP	no	*RHD*	6	–	3, 4, 5, 6, 7 and 9	126	4	0	0	0
Müller *et al.* (1997b)††	multiplex	SSP	F	*RHD*	9	–	2–7, 9 and 10	110	0	0	0	0
Wagner *et al.* (1998b)	single	LP	no	*RHD*	1	3	–	27	0	0	0	0
Total sample number assayed for *RHD*								3313	25 (0·75%)	16 (0·5%)		
Le Van Kim *et al.* (1994)	multiplex	SSP	F	*c and E*	2	–	2 and 5	14	0	0	0	0
Faas *et al.* (1995)	modular	SSP	no	*E and e*	1	–	5	158	0	0	0	0
Poulter *et al.* (1996)	modular	SSP	no	*C and c*	1	2	–	105	0	0	0	0
Yankowitz *et al.* (1997)	modular	RFLP	no	*C, c and E*	3	–	1, 2 and 5	655	0	23/17/8	0	0

Table 4. Results of some published PCR set-ups applied to *RH* DNA typing

References	PCR set-up	PCR methods*	Mother (M) fetus (F) tested	Genes/ alleles	Polymorphisms tested: Sites (*n*)	Location: Intron	Location: Exon	Samples tested (*n*)	Aberrant allele: Samples Detected† (*n*)	Aberrant allele: Mistyped‡ (*n*)	Technical problem§: Mistyped (*n*)	Technical problem§: No. amplicon (*n*)
Gassner *et al.* (1997b)**	modular	SSP	no	*C, c, E and e*	4	–	1, 2 and 5	354	0	42/0/0/0	0	0
Tanaka *et al.* (1997)	modular	SSP	no	*C, c, E and e*	5	–	1, 2 and 5	513	0	2/0/0	0	0
Müller *et al.* (1997b)††	multiplex	SSP	F	*C, C^w, c, E and e*	6	2	1, 2 and 5	110	0	0	0	0

*SSP – sequence-specific primer; LP – length polymorphism; RFLP – restriction fragment LP; LCR – ligase chain reaction. †Discrepant results not explained by known partial D. ‡The high rate of mistypings in some studies was often caused by highly selected samples. §All technical problems were caused by contamination or amplification failure in fetal tissue testing. ¶Because there is no polymorphic site in the coding sequence of exon 10, the 3′ untranslated region (UTR) of exon 10 is assayed. **Modular extensions of the PCR set-up (Gassner *et al.*, 1997a) have been described since (see Fig. 2) and utilized in the multiplex assay of Müller *et al.* (1997b). ††Fifteen sites representing 8 *RHD*, 5 *RHCE*, 1 D^{VII} and 1 C^w specific sites were utilized for amplifications in four multiplex PCR and analysed in one semiautomated fluorescence reading (for an outline of the method see Fig. 4).

the amplicon separation is routinely achieved by the techniques established for DNA sequencing.

APPLICATION OF PCR-SSP TO *RH* DNA TYPING

PCR using sequence-specific priming (PCR-SSP), also known as allele-specific primer amplification (ASPA), allows the specific detection of nucleotides at predetermined sequence positions. PCR-SSP can be devised to suit modular and multiplex PCR set-ups, may be adapted for detection of most nucleotide polymorphisms and for standardized thermocycling conditions, and is rapid. Thus, PCR-SSP is preferred for *RH* DNA typing by most research groups and in more recent PCR set-ups (Table 4).

Single primer pairs

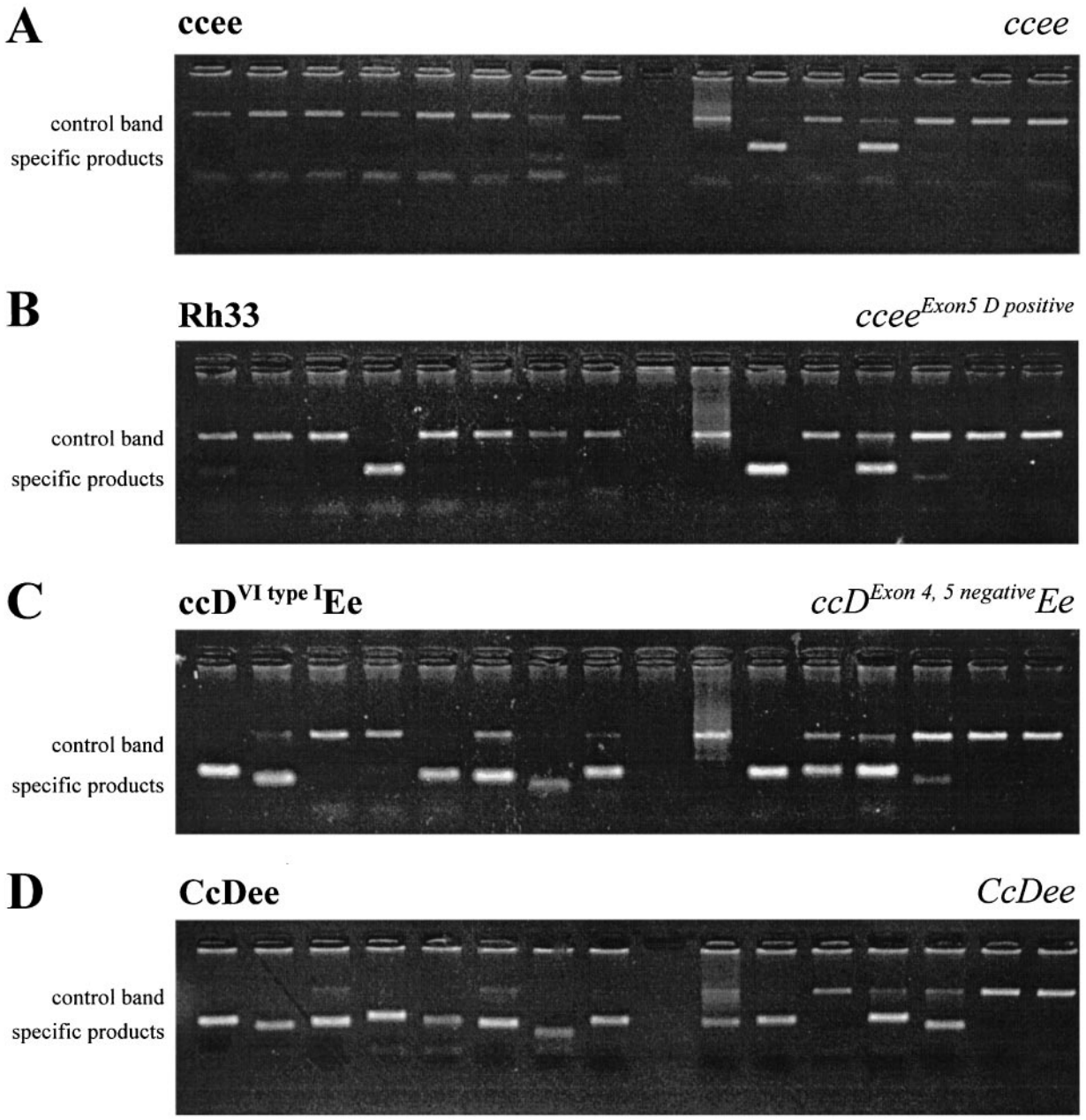
As noted previously, PCR set-ups with a single primer pair only are not considered reliable and should not generally be applied for diagnostic purposes. They may be very useful to distinguish and characterize rare alleles. Limited studies with new primer pairs for single sites are conducted to establish specificity and robustness, before a primer pair is added to established modular or multiplex systems.

Modular systems

With PCR set-ups comprising two or more primer pairs the rate of false positive and false negative results can be diminished. However, samples of unknown *RHD* structure may still be retrieved, whose clinical relevance is not immediately apparent.

We developed a modular *RHD* PCR-SSP system consisting of seven PCR reactions specific for *RHD* exons 3–7, 9 and 10 plus one PCR reaction detecting exon 2 of *RHD* or the *C* allele of *RHCE* (Table 1 and Fig. 2). To maximize the information obtained about the protein sequence, only nucleotide substitutions determining amino acid polymorphisms were chosen for detection. The screening of all *RHD* exons differing from *RHCE* allows the identification of any *RHD–CE–D* hybrid allele that involves at least one full exon, because the lack of any *RHD*-specific exon would always be detected by one or more negative *RHD*-specific reaction. This system proved very efficient for the rapid identification and preliminary characterization of new forms of partial D due to *RHD–CE–D* hybrid proteins (Gassner *et al.*, 1997b; Wagner *et al.*, 1998a). Additional PCR-SSP reactions may be added to detect partial D, like D^{VII}, caused by mis-sense mutations (Fig. 2).

The *RHD* typing system is complemented by six PCR reactions detecting the Rh antigens C^w, C, c, E and e



Reaction	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Specificity	*D/C*	*D*	*D*	*D*	*D*	*D*	*D*	*D*	*C*	*c**	*E*	*e*	*C**	C^{W}	D^{VII}
Exon	2	3	4	5	6	7	9	10	-	2	5	5	1	1	2
Nucleotides	201 307	383	602	676 787	916	1048	1193	1255		201 307	787	787	48	122	329
Product (bp)	148	113	122	157	132	122	83	147	123	149	158	158	112	184	167

(Table 1 and Fig. 2) (Gassner *et al*., 1997b). Typing for the *C* allele of *RHCE* was generally considered difficult and unreliable (Faas *et al*., 1997b): the identity of *RHD* alleles and the *C* allele of *RHCE* in exon 2 prevents the specific detection of the *C* allele in *RHD*-positive samples by these nucleotide positions. Typing for the *C* allele at position 48 in exon 1 was also unreliable (Faas *et al*., 1997b; Gassner *et al*., 1997b; Faas, 1998). This was recently explained by our finding that C48 is shared by a *c* allele occurring in most cDe (Gassner *et al*., 1997b) and a few cde haplotypes (own unpublished observation; Tanaka *et al*., 1997). In the meantime we added a *C*-allele-specific PCR-SSP (Gassner *et al*., 1997a) based on a *C*-allele-specific intron 2 polymorphism (Poulter *et al*., 1996) to our modular PCR set-up. A *C*-allele-specific DNA typing performed with this reaction was concordant with the C antigen serology without exception in more than 500 samples investigated (unpublished observations) rendering this system reliable for *C*-allele-specific DNA typing. Although data from the African populations are lacking, a similar excellent concordance is predicted, if the high false-positive rate in Africans can be explained by the prevalance of the cDe haplotype in that population.

The sequence of the *c* alleles of *RHCE* is identical either to the sequence of the *C* alleles of *RHCE* or to *RHD* alleles in all exons but exon 2. Hence, typing for a *c* allele of *RHCE* is performed utilizing two polymorphic nucleotide positions in exon 2 (Table 1). The only difference between the *E* and *e* alleles of *RHCE* is a C (*E* alleles)/G (*e* alleles) polymorphism at position 676 located in exon 5. The *RHD* alleles share the G at position 676. A PCR-SSP detecting this polymorphic nucleotide only may hence coamplify all *RHD*-positive haplotypes. For this reason, the PCR-SSP reactions for the *E*/*e* polymorphism utilizes two sequence-specific primers: one primer detects an *RHCE*-specific nucleotide at position 787 and suppresses the coamplification of all *RHD* alleles, and the other discriminates the *E*/*e* polymorphism at position 676 by being specific for either the *E* or the *e* alleles of *RHCE* (Table 1). Finally, a reaction detecting position 122 in exon 2 specific for the C^w allele was recently added to our modular typing system (Table 1 and Fig. 2).

In summary, a total of 15 PCR-SSP reactions detect 17 of about 41 amino acid polymorphisms crucial for most antigen specificities of the Rh blood group system. This enabled us to type samples with an unprecedented accuracy. Since all PCR-SSP reactions are performed with identical thermocycling conditions, the number of PCR reactions does not affect the assay time. If the detection of alleles, defined by many other mis-sense mutations and coding for Rh antigens, like D^{II}, DNU, DHR, DHMi, C^x, RH26 and VS, is considered important, additional modules may be added easily. On the other hand, *RHD*-specific reactions may be omitted if they were shown redundant for a given accuracy in any distinct population. Most importantly for a rational testing strategy, *RHD* PCR-SSP modules specific for nonfunctional *RHD* alleles may be added as soon as their occurrence and molecular cause is identified (Table 3). Once a modular system for *RH* DNA typing is established, it may be easily transferred to applications using fluorescence and otherwise labelled primers.

Modular systems with fluorescence-labelled primers

Fluorescence detection in a modular PCR-SSP for *RHD*/*CE* DNA typing has been shown to enhance the assay sensitivity (Tonn *et al*., 1997). Promising technologies have been introduced to detect newly formed amplicons whilst the PCR amplification is still in progress (Heid *et al*., 1996; Morris *et al*., 1996; Kalinina *et al*., 1997; Woo *et al*., 1998). A relevant DNA stretch is amplified by PCR using sequence-specific priming, for example. A short oligonucleotide ('TaqMan probe') carrying a fluorescence dye as reporter at its 5′ end and a quencher dye at its 3′ end can hybridize specifically to the amplicon. The ensuing PCR releases the reporter dye from the probe due to the 5′ nuclease activity of the polymerase. The released fluorescence dye no longer being quenched is indicative of the amount of amplicons produced. In arrays of 96 samples, the change of fluorescence in closed PCR tubes can be detected simultaneously and offers an extraordinarily efficient and quantitative read out whilst the PCR thermocycling is still under way. This

Fig. 2. Representative results of a modular *RH* PCR-SSP system. Panels A to D show agarose gel electrophoresis of 15 *RH* PCR-SSP reactions. The Rh phenotypes (bold type) and the detected DNA type (italics) are indicated above each panel. The *RH* PCR-SSP reactions are specified in the boxed interpretation scheme below the panels; the reactions 10 and 13 indicated by c* and C* are detecting the *c*(W16C) allele of *RHCE* in addition to the *c* and *C* alleles of *RHCE* that are prevalent among Caucasians. In the ccddee phenotype shown in panel A no reactions specific for *RHD* are detected. The reaction patterns in panel B, PCR-SSP positive for *RHD* exon 5, and in panel C, PCR-SSP negative for *RHD* exons 4 and 5, indicate the presence of the *Rh33* and D^{VI} *type I* alleles. The CcDee phenotype in panel D shows specific products in all PCR-SSP reactions geared to detect *RHD* exons. Specific products were also found in the *RHCE* PCR-SSP reaction of all four samples in concordance with their RhCE phenotypes. The 434-bp internal control amplicon, which was devised to be larger than any *RH*-specific amplicon, may be suppressed because of competition, if a specific product is amplified.

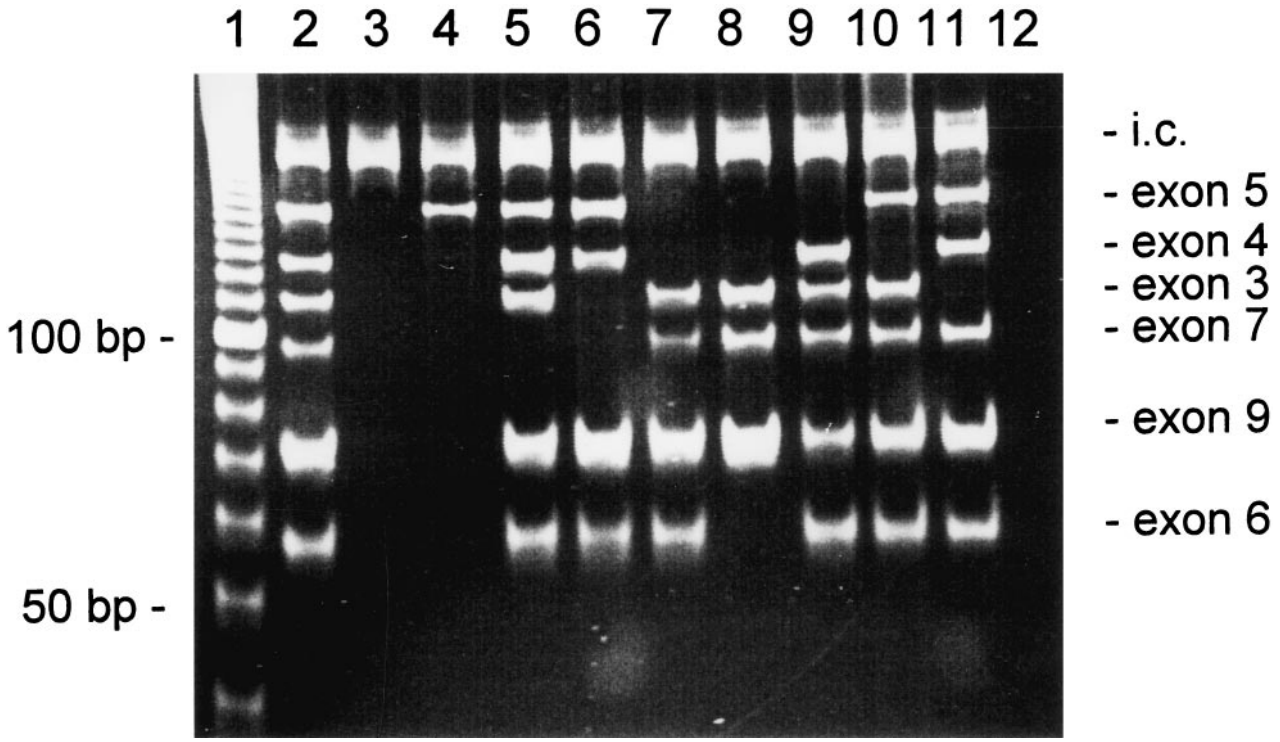


Fig. 3. Representative results of a multiplex *RHD* PCR-SSP system. Specific products of different lengths are amplified for *RHD* exons 3, 4, 5, 6, 7 and 9 and separated by polyacrylamide gel electrophoresis (PAGE). The regular RhD-positive phenotype, lane 2, carries all *RHD*-specific sites; the ccddee phenotype, lane 3, no *RHD*-specific site. R_0^{Har} and partial D are represented by exon patterns that may be diagnostic: lane 4, R_0^{Har}; lane 5, D^{IVb}; lane 6, D^{IVa}; lane 7, D^{VI} *type I*; lane 8, D^{VI} *type II*; lane 9, D^{Va}; lane 10, *DFR*; lane 11, D^{IIIc}. Lane 1: DNA size markers (10-bp ladder); lane 12: water control; i.c.: 200-bp internal control amplicon. The gel image was kindly provided by Petra A. Maaskant-van Wijk.

technology reduces dramatically the risk of contamination by amplicons generated during the PCR.

Multiplex systems

In multiplex systems, several specific PCR reactions are performed in a single tube. This allows detection of more than one polymorphism in a single DNA sample and in one PCR reaction. The development of advanced multiplex systems is a laborious task. The different PCR reactions must be orchestrated to work with equal efficiencies under the same thermocycling conditions and without loss of specific bands due to competition.

The first multiplex systems proposed for *RHD* DNA typing involved combinations of the intron 4 length polymorphism with the exon 10 SSP reaction (Spence *et al*., 1995a; Pope *et al*., 1995) and included specific PCR products of up to 600 bp length. To improve the stability of the intron 4 system, Avent *et al*. (1997c) engineered two PCR-SSP reactions whose sequence-specific primers are located in the *RHCE* intron 4 sequence and span the *RHD* deletion point. These two reactions were combined with an exon-10-based *RHD* PCR-SSP resulting in a true SSP multiplex system detecting *RHD* intron 4 and exon 10. This system was useful for screening a large number of samples for aberrant *RHD* alleles and led to the identification of the *RHD*(Q41X) allele. The authors' data indicated that false positive results due to sporadic mutations may be frequent among Cde and cdE haplotypes and may remain a problem even for multiplex systems.

Maaskant-van Wijk *et al*. (1997b) recently presented a multiplex system that incorporates reactions for *RHD* exons 3, 4, 5, 6, 7 and 9, all the informative *RHD* exons, along with a β-actin control, in a single tube. Specific products range from 157 to 57 bp and were visualized in a polyacrylamide gel (Fig. 3, gel image courtesy of P. A. Maaskant-van Wijk). This elaborate system allowed the correct identification of all known *RHD–CE–D* hybrid alleles with the only exception of D^{IIIb}. The inclusion of so many reactions into a single tube is complicated, and internal mismatch bases had to be introduced into several primers to increase specificity. Furthermore, strict quality control is needed, because with a DNA concentration of less than 10 ng, specific bands may be lost while the control band is still amplified. Full concordance for all

systems was observed in 40 RhD-positive and 13 RhD-negative Caucasians. However, among 46 non-Caucasian RhD-positive individuals, two partial D (D^{IVa} and D^{Va}) were identified, suggesting that these partial D are frequent among non-Caucasians. The authors proposed the application of this single tube system for prenatal testing and for population screening, which would facilitate the immediate identification and classification of most partial D.

Multiplex systems with fluorescence labelled primers

We have developed a strategy for *RHD*/*CE* DNA typing in large numbers of samples (Müller *et al*., 1997b), which is outlined in Fig. 4. Fifteen primer pairs specific for *RHD* or *RHCE* (Gassner *et al*., 1997b) were used in four multiplex PCR reactions to amplify *RHD*/*CE*-allele-specific segments. One primer of each pair is labelled with either 6-carboxy-fluorescein, tetrachloro-6-carboxy-fluorescein or hexachloro-6-carboxy-fluorescein. The PCR products of all four tubes with the same DNA sample are pooled after completion of the PCR reactions and the primers are removed by adsorption to columns. The pooled amplicons are finally separated by capillary electrophoresis and fluorescence detection of the labels using an automated nucleotide sequencer (ABI Prism Genetic Analyser 310; Applied Biosystems, Foster City, CA, USA). Electrophoretic separation of up to 15 different products from the multiplex PCR in a 47-cm-long capillary (0·05 mm internal diameter) filled with the POP-6 polymer is completed within 25 min after injection of the sample. A highly reproducible measurement of the size of the amplicons is ensured by comparison of the retention times of the amplicons to those of internal standards of defined size. Combining these size measurements with analysis of the fluorescence of the different dyes reliably identifies the products generated by multiplex PCR. Up to 96 samples, which may contain the mixed products of the four multiplex PCR, are automatically injected and analysed sequentially. Validation experiments demonstrate the high reliability (concordant results with serotyping for samples from 100 donors) and good sensitivity (adequate for the analysis of samples from amniotic fluid) of this approach. This method of multiplex PCR with fluorescent primers together with automated identification of the pooled amplification products allowed semiautomated *RHD*/*CE* genotyping based on a multitude of 'diagnostic' DNA polymorphisms.

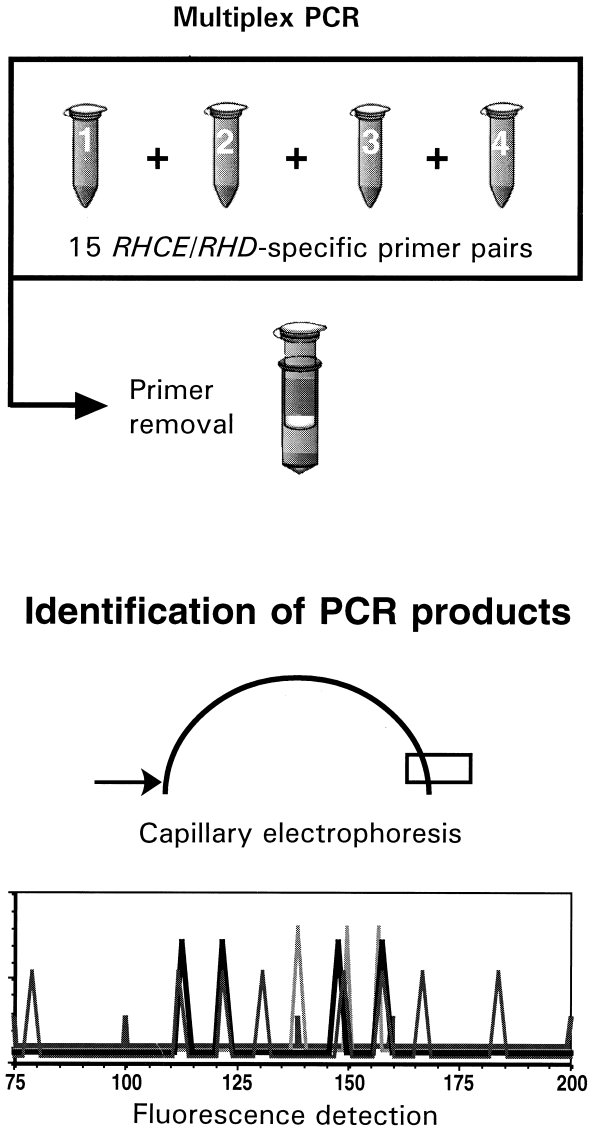


Fig. 4. Outline of a blood group genotyping strategy suitable for testing large numbers of blood samples. As an example, the application to multiplex *RHD*/*CE* DNA typing is shown. PCR amplification of many DNA fragments is performed in a modular fashion using fluorescence-labelled sequence-specific primers. After primer removal the amplicons can be specifically detected according to their fragment length and colour. The reading is achieved in an automated procedure by capillary electrophoresis followed by fluorescence detection. The procedure lends itself to automatic data retrieval, interpretation and documentation. Other large-scale genotyping procedures currently being developed are discussed in the text.

OTHER TECHNIQUES SUITABLE FOR DETECTING KNOWN SEQUENCE POLYMORPHISMS

Several alternatives to PCR-SSP are available to detect defined sequence polymorphisms. Some lack the versatility of PCR-SSP or have not yet been applied to *RH* DNA typing.

PCR amplicon hybridization with sequence-specific oligonucleotides (PCR-SSO)

PCR amplicon capturing to a solid phase, like filters and microtitre wells, followed by hybridization with sequence-specific oligonucleotides, or the 'reverse dot technique', is widely used for *HLA* genotyping. Although no applications for *RH* DNA typing have been established, the potential for automation, such as with enzyme-linked detection systems, is great. The knowledge of allele polymorphisms established by PCR-SSP can be easily applied to PCR-SSO.

Ligase chain reaction

The ligase chain reaction involves cyclic, sequence-dependent ligation of two oligonucleotides and is often used as a second step following PCR amplification. The combination of a biotin-labelled oligonucleotide with a digoxygenin-labelled oligonucleotide allows detection of the ligation product by ELISA, making this method amenable to automation. To date, a single application for *RH* DNA typing has been described (Maas *et al*., 1997).

PCR amplicon length polymorphism

In the introns of the *RH* genes, there are several insertions, deletions and repeat number polymorphisms. Amplification of these regions by flanking primers results in amplicons of gene or allele-specific lengths that can be separated by gel electrophoresis. *RHD*-specific sequences and *RHCE*-specific controls are amplified in the same tube by the same primer pair, obviating the need for additional controls. The first and a major application is *RHD* typing by an intron 4 deletion (Arce *et al*., 1993). The linkage of intron polymorphisms to *RH* phenotypes is excellent and some systems, like the detection of the *C* allele by virtue of an intron 2 insertion (Poulter *et al*., 1996), are more informative than exon-based assays. Technical limitations may be encountered because PCR conditions depend on the length of the investigated deletion. They tend to involve comparably long extension periods, which often prevents the technique from being suitable for modular systems. Then, PCR-SSP devised for the same polymorphism (Avent *et al*., 1997c; Gassner *et al*., 1997a) may be more useful.

PCR combined with restiction fragment length polymorphism (PCR-RFLP)

PCR amplicon digestion by restriction enzymes and analysis of the restriction fragment length polymorphism may detect single nucleotide substitutions located in restriction sites. A major application is c/D typing in intron 2 (Poulter *et al*., 1996). Enzyme digestion may take less than 2 min if it is performed by microwave heating (Poulter *et al*., 1996). Sufficient digestion should be controlled by an additional restriction site present in all amplicons. PCR-RFLP is easy to establish if a restriction site happens to incorporate the polymorphic site (Beckers *et al*., 1996b), but this is not the rule. Compared to PCR-SSP, there is more hands-on time and much less potential for modular set-ups.

TECHNIQUES SUITABLE TO DETECT UNEXPECTED SEQUENCE POLYMORPHISMS

PCR-SSP and the similar methods presented in the preceding sections are geared to detect distinct known polymorphisms and they will rarely detect other nucleotide sequence aberrations by chance. Several techniques do not aim for specific nucleotide polymorphisms but allow screening for aberrations occurring in longer stretches of a nucleotide sequence. Hence, for example, sporadic non-sense mutations may not escape detection, which furthers the test specificity. The enhanced information retrieval must be weighed against the increased complexity of the assays. Furthermore, routine application may be hampered by the detection of frequent (Stoerker *et al*., 1996), yet clinically irrelevant, silent or intronic mutations, which may incur clinically futile work-up. In the field of *RH* typing, denaturing gradient gel electrophoresis, heteroduplex analysis, single-strand conformation polymorphism and conventional sequencing have been utilized.

Denaturing gradient gel electrophoresis (DGGE)

In DGGE, double-stranded PCR amplicons are electrophoresed through a gradient of a denaturing agent of increasing concentration. At a characteristic concentration, part of the double-stranded DNA sequence begins melting, which results in an abrupt decrease of mobility and a characteristic sequence-dependent pattern (Fischer & Lerman, 1983). Electrophoresis usually takes 24 h and gel conditions must be specifically adapted for each amplicon, restricting DGGE largely to research questions. DGGE has been applied to *RH* exons 2 and 5 (Steers *et al*., 1996).

Heteroduplex analysis

Heteroduplexes containing single base mismatches can be separated from homoduplexes and other heteroduplexes by nondenaturing gel electrophoresis. Engineered DNA fragments with small deletions may ensure heteroduplex generation for almost any allele. Heteroduplex

systems have been developed for exons 2 and 5 (Stoerker *et al*., 1996) and applied for routine use (Rose *et al*., 1997).

Single-strand conformation polymorphism (SSCP)

The electrophoretic behaviour of single-stranded DNA in nondenaturing gels depends on sequence-specific secondary structures. This biological feature is utilized in SSCP. After denaturing by heat, electrophoresis can be performed in about 15 min. SSCP is hence much faster and simpler than DGGE or heteroduplex analysis. It is less sensitive and may miss about 20% of mutations. Recently, SSCP was utilized for screening for the G286A mutation determining the Rh:-26 phenotype (Faas *et al*., 1997c).

Conventional sequencing

The gold standard for nucleotide sequence determination is the full length sequencing of the desired stretch of the nucleic acid. The classical approach for the definition of aberrant *RH* alleles involves RNA isolation, reverse transcription, subcloning and plasmid sequencing. The procedure is very laborious and prone to mistakes because of splice variants, which are particularly abundant among *RH* transcripts. Identification of mis-sense mutations may be hampered by misincorparated nucleotides introduced by PCR and selected by subcloning of the plasmids.

Genomic sequencing has been used for screening for the D^{VII} mutation (Flegel *et al*., 1996). It is less laborious and obviates the need for subcloning, but the detection rate for heterozygotes may be less than 100%. Recently, we developed an *RHD*-specific genomic sequencing system for all exons obviating the need for subcloning (Wagner *et al*., 1998b). If hybrid alleles involving large gene conversions are excluded by PCR-SSP, this approach allows sequence determination of the full coding sequence with an expected detection rate of 100% within 3 working days. The utility of this system has been demonstrated by the identification of more than 15 alleles constituting the molecular cause of the weak D phenotype (Flegel *et al*., 1998).

FUTURE TECHNOLOGIES: BIOCHIPS

Biochips and PCR chips (Chee *et al*., 1996; Wodicka *et al*., 1997) could be used to determine the nucleotides at each of the ≈ 37 exon positions differing between the *RHCE* and the *RHD* genes. Presently it is doubtful if this resolution is really needed for clinically relevant *RH* DNA typing. Only population-wide sequence characterization of many *RHD* and *RHCE* alleles will adequately address this question. However, with much improved cost-efficiency the resolution of all cDNA nucleotide positions revealing all possible non-sense mutations might become feasible and would enhance the reliability for future *RH* genotyping to unprecedented levels clearly exceeding the precision of any serology-based method.

TOWARDS A RATIONAL TESTING STRATEGY

There is currently no optimal *RH* DNA typing strategy suiting all applications. DNA typing is performed for different populations and for different purposes. It is apparent from the previous sections that the techniques vary widely from few or multiple PCR reactions performed in separate tubes to multiplex reactions in single tubes. The use of two or more 'diagnostic sites' is recommended to limit the rate of false results. Furthermore, the equipment for and experience with genotyping systems, which may be available in a laboratory, will often guide the selection for a particular *RH* DNA typing system. For the broader application in clinical routine laboratories a PCR-based approach in conjunction with the specific detection of certain nucleotide positions by sequence-specific oligonucleotides is generally favoured.

Importance of the population tested

The polymorphisms (alleles) of a gene, their population frequencies and distribution in the examined population have critical importance for the practical application of DNA typing. Most *RH* DNA typing data derive from Caucasians. In African and Japanese people, there is a large fraction of RhD-negative alleles that harbour *RHD*-specific sequences and are not correctly recognized with almost any published strategy. The more frequent partial D differ between non-Caucasian (D^{IVa}) and Caucasian (D^{VII}) populations. Reliable *RH* DNA typing in non-Caucasian populations will have to await the identification of the more prevalent alleles in RhD-negative samples present in those populations. DNA typing in random samples without reference to the allele pool involved, i.e. the genetic derivation of the probands, is prone to mistakes. At the moment, the rate of false Rh phenotype predictions in populations other than Caucasians cannot be estimated reliably. Admixtures to the allele pool introduced by non-Caucasians may hamper *RH* DNA typing even in Western populations.

The Japanese RhD-negative samples may represent a special problem because they were reported to possess a normal *RHD* promoter, plentiful *RHD* mRNA, and an *RHD* coding sequence with a single mis-sense mutation (Okuda *et al*., 1997) that appeared not to be diagnostic. If high-precision DNA typing is attempted, the prevalence

of rare variants may become more important. Genotypes underlying partial D, for example D category VI, vary even within closely related Caucasian populations (Wagner *et al*., 1998a). The relevant questions may be answered by population-based approaches, which may, if properly conducted, yield interesting data for scientific problems.

Which polymorphism should be tested?

The expense of a genotyping system must be weighed against its residual failure rate in phenotype prediction to determine its cost-efficiency. In almost all current genotyping strategies rather short nucleic acid sequences are utilized. Full coverage of the cDNA is not practical with the available technology, and full coverage of a gene cannot even be attempted. Therefore, the phenotype prediction depends critically on the functional integrity of the detected allele (Flegel, 1997). To limit false positive *RHD* DNA typing, there is unfortunately no expedient alternative method to specifically detect the multiple *RHD* sequence aberrations occurring in RhD-negative phenotypes (Table 3). Because these alleles occur with moderate frequencies even in Caucasians, genotyping strategies should address this problem.

DNA typing experience with rare phenotypes (Avent *et al*., 1997c) and the phenotype frequency data (Wagner *et al*., 1995) indicated that the correct Rh phenotype is predicted in more than 99·5% of unselected samples from Caucasians even by testing of a single polymorphism only. The detection of a second polymorphism raises accuracy to about 99·9% (Table 4), which is currently the recommended approach (Bennett & Cartron, 1994; Simsek *et al*., 1994; Lighten *et al*., 1995). If two polymorphisms are to be tested, we favour two 'diagnostic sites' located in exon 4/intron 4 and in exon 7 (Table 1). By this approach, the clinically most relevant partial D, D^{VI} and D^{IV}, are recognized and the RhD-negative alleles due to *RHD–CE–D* hybrids involving substitutions in exons 4–7 (Table 3) are correctly predicted as RhD-negative. If three polymorphisms are to be tested, we recommend sites located in exons 4, 5 and 7 because this approach would test all three exofacial loops differing between *RHD* and the *C* allele of *RHCE*. To increase the specificity further, all *RHD*-specific exons can be tested (Table 1 and Fig. 2). However, the estimated frequency of partial D due to *RHD–CE–D* hybrid alleles other than D^{VI} and D^{IV} is less than 1 : 10 000 among unselected samples from Caucasians (Flegel & Wagner, 1996; Roubinet *et al*., 1996).

Importance of the application

For paternity testing, usually DNA of the mother, child and putative father is available. As long as results are obtained with the same method for all involved persons, mismatches between phenotype and DNA type will not affect paternity prediction. The frequency discrepancy between DNA-based and phenotypic haplotypes is usually too small to affect probability calculations significantly. Hence, a robust single polymorphism assay can be considered sufficient (van den Veyver *et al*., 1996). Methods, like DGGE, that allow the detection of sporadic aberrant *RH* alleles may enhance somewhat the predictive value of *RH* DNA typing in paternity testing if one of the persons possesses a rare aberrant allele. It remains doubtful whether this small advantage merits the additional effort of more complicated test systems.

For prenatal prediction of the Rh phenotype in Caucasians, typing errors due to technical problems including contamination with maternal blood are likely to influence the error rate more than the residual 0·1% of rare alleles that may be mistyped. Hence, for prenatal testing, a robust modular or multiplex system involving two or more polymorphisms may be chosen. Because of the lower rate of fetal loss incurred by the less invasive sampling procedures (van den Veyver & Moise, 1996), the *RHD* DNA typing is today clearly the method of first choice for predicting the fetal RhD phenotype.

On the other hand, a 99·9% accuracy is not sufficient if a replacement for routine serological methods is required. Contrary to a common perception, the major obstacle for Rh phenotype prediction is those RhD-negative phenotypes that carry *RHD*-specific DNA stretches (Table 3) rather than partial D. In Caucasians, such samples occurred with a frequency of about 5% among Ccddee and ccddEe samples (Avent *et al*., 1997c), resulting in an estimated population frequency of about 1 : 1000–2000. This rate exceeds by far that of clinically relevant partial D, like D^{VI} (Wagner *et al*., 1995). Hence, further advances in *RH* DNA typing of unselected individuals is unlikely to be achieved by adding more *RHD* exon-specific reactions only. Improvements will rather necessitate the identification and specific detection of nonfunctional *RHD* alleles. In this respect, modular approaches have the advantage that additional reactions may be easily incorporated.

A quite different situation concerns the screening of phenotypically abnormal samples, for example partial D and weak D. In partial D, an aberrant *RHD* sequence may almost always be detected, whereas a serological discrimination can be cumbersome or misleading. Exon scanning *RHD* PCR (Gassner *et al*., 1997b; Maaskant-van Wijk *et al*., 1997b) identifies those interesting samples of hybrid *RHD*/*CE* structure rapidly that merit further characterization by cDNA sequencing. For *RHD* alleles with point mutations, genomic sequencing (Wagner *et al*., 1998b) is today the most straightforward and rapid approach. The improved resolution achieved

by PCR detection of multiple *RHD*-specific products is useful for population surveys to elaborate the genetic basis of the various RhD and RhCE phenotypes.

Laboratory-specific considerations

Within the mentioned framework of *RH* DNA typing strategies, the selection of a specific DNA typing system may be guided by practical considerations specific for a particular laboratory. The more expedient of two otherwise comparable systems will generally be preferred. Often, it is advantageous to select an *RH* DNA typing system with thermocycling conditions already applied in the laboratory for other purposes, such as *HLA* genotyping. Investigators less experienced with DNA typing may prefer robust test systems, which are only marginally affected by variable DNA quality. ELISA- or fluorescence-based systems necessitate special equipment, but are likely to vastly outperform other systems if large-scale DNA typing is attempted.

CONCLUSION

Blood group phenotype prediction by molecular methods is feasible and for some applications are already superior to standard serological phenotyping. Examples for *RH* DNA typing are prenatal diagnostics and characterization of aberrant Rhesus proteins including partial D.

Personally, we will not be surprised if genotyping eventually replaces 'immunotyping' as the standard procedure in the blood group laboratory. This prediction is being questioned by some in the profession pointing to the many major problems that are – at the moment – undeniably associated with blood group genotyping. Many technical increments are required to achieve vast improvements in the reliability and cost-efficiency of genotyping, which are relatively low compared to standard serological methods. Once the many problems are addressed, they can be solved in a piecemeal approach, which will step by step reduce genotyping to routine practice for most applications currently tackled by serology. It is hoped that establishing the necessary population data will keep pace with the rapidly improving technologies in genotyping. Our profession could contribute considerably to the improvement of genotyping strategies that are vigorously developed in various medical fields.

ABO and RhD typing are among the most reliable diagnostic procedures. Being rather inexpensive is one reason that blood group serology is very cost-efficient, if applied prudently. In this situation, the demands required for blood group genotyping are exceptional compared to genotyping for other purposes. By striving to meet these demands for blood group genotyping, transfusion medicine may well contribute to the understanding of and the solutions for the biological problems associated with genotyping strategies in general.

AUTHORS' NOTE

We apologize for being unable to cite all of the papers that are relevant to this topic and thank many of the participants of the 3rd Annual Molecular Transfusion Medicine Seminars organized by Friedrich Schunter and held at the DRK-Blutspendezentrale Oldenburg on 3 September 1997 for interesting discussions that contributed to the ideas presented here. We are indebted to Petra A. Maaskant-van Wijk, Amsterdam, the Netherlands, for preparing and providing the gel image of Fig. 3. We are grateful to Catherine A. Hyland, Brisbane, Australia, Petra A. Maaskant-van Wijk and Brigitte H. W. Faas, Amsterdam, the Netherlands, for communicating data prior to their publication. We thank Zhu Zi-yan, Shanghai, China, for contributing a sample of D category VI type III with a strong anti-D alloantibody.

REFERENCES

Adinolfi, M., Sherlock, J., Kemp, T., Carritt, B., Southill, P., Kingdom, J. & Rodeck, C. (1995) Prenatal detection of fetal RhD DNA sequences in transcervical samples. *Lancet*, **345,** 318–319.

Andrews, K.T., Wolter, L.C., Saul, A. & Hyland, C.A. (1998a) Analysis of the RH D gene in an RH D negative donor (Abstract). *Vox Sanguinis*, **74** (Suppl. 1)**,** 53.

Andrews, K.T., Wolter, L.C., Saul, A. & Hyland, C.A. (1998b) The RhD-trait in a white blood donor with the RhCCee phenotype attributed to a four-nucleotide deletion in the RHD gene (Letter). *Blood*, in press.

Anstee, D.J. (1995) Blood group antigens definded by the amino acid sequences of red cell surface proteins. *Transfusion Medicine*, **5,** 1–13.

Arce, M.A., Thompson, E.S., Wagner, S., Coyne, K.E., Ferdman, B.A. & Lublin, D.M. (1993) Molecular cloning of RhD cDNA derived from a gene present in RhD-positive, but not RhD-negative individuals. *Blood*, **82,** 651–655.

Argall, C.I., Ball, J.M. & Trentelman, E. (1953) Presence of anti-D antibody in the serum of a D^u patient. *Journal of Laboratory and Clinical Medicine*, **41,** 895–898.

Aubin, J.T., Le Van Kim, C., Mouro, I., Colin, Y., Bignozzi, C., Brossard, Y. & Cartron, J.-P. (1997) Specificity and sensitivity of *RHD* genotyping methods by PCR-based DNA amplification. *British Journal of Haematology*, **98,** 356–364.

Avent, N.D. (1997) Human erythrocyte antigen expression: its molecular bases. *British Journal of Biomedical Sciences*, **54,** 16–37.

Avent, N.D., Jones, J.W., Liu, W., Scott, M.L., Voak, D., Flegel, W.A., Wagner, F.F. & Green, C. (1997a) Molecular basis of the D variant phenotypes DNU and D^{II} allows localization of critical amino acids required for expression

of Rh D epitopes epD3, 4 and 9 to the sixth external domain of the Rh D protein. *British Journal of Haematology*, **97,** 366–371.

Avent, N.D., Liu, W., Jones, J.W., Scott, M.L., Voak, D., Pisacka, M., Watt, J. & Fletcher, A. (1997b) Molecular analysis of Rh transcripts and polypeptides from individuals expressing the D^{VI} variant phenotype: an *RHD* gene deletion event does not generate all D^{VI}ccEe phenotypes. *Blood*, **89,** 1779–1786.

Avent, N.D., Martin, P.G., Armstrong-Fisher, S.S., Liu, W., Finning, K.M., Maddocks, D. & Urbaniak, S.J. (1997c) Evidence of genetic diversity underlying Rh D negative, weak D (D^u) and partial D phenotypes as determined by multiplex PCR analysis of the *RHD* gene. *Blood*, **89,** 2568–2577.

Beaudet, A.L., Antonarakis, S.E., Beutler, E., Cotton, R.G.H., Desnick, R.J., Kazazian, H.H., McAlpine, P.J., McKusick, V.A., Motulsky, A.G., Scriver, C.R., Shows, T.B., Tsui, L.-C. & Valle, D. (1996) Update on nomenclature for human gene mutations. *Human Mutation*, **8,** 197–202.

Beckers, E.A.M., Faas, B.H.W., Ligthart, P., Simsek, S., von Overbeeke, M.A.M., dem Borne, A.E.G.K., van Rhenen, D.J. & van der Schoot, C.E. (1996a) Characterization of the hybrid *RHD* gene leading to the partial D category IIIc phenotype. *Transfusion*, **36,** 567–574.

Beckers, E.A., Faas, B.H., Ligthart, P., von Overbeeke, M.A., dem Borne, A.E., van der Schoot, C.E. & van Rhenen, D.J. (1997) Lower antigen site density and weak D immunogenicity cannot be explained by structural genomic abnormalities or regulatory defects of the RHD gene. *Transfusion*, **37,** 616–623.

Beckers, E.A.M., Faas, B.H.W., Simsek, S., Overbeeke, M.A.M., van Rhenen, D.J., Wallace, M., von dem Borne, A.E.G.K. & van der Schoot, C.E. (1996b) The genetic basis of a new partial D antigen: D^{DBT}. *British Journal of Haematology*, **93,** 720–727.

Beckers, E.A.M., Porcelijn, L., Ligthart, P., Vermey, H., von dem Borne, A.E.G.K., Overbeeke, M.A.M. & van Rhenen, D.J. (1996d) The R_0^{Har} antigenic complex is associated with a limited number of D epitopes and alloanti-D production: a study in three unrelated individuals and their families. *Transfusion*, **36,** 104–108.

Beckers, E.A.M., Faas, B.H.W., von dem Borne, A.E.G.K., Overbeeke, M.A.M., van Rhenen, D.J. & van der Schoot, C.E. (1996c) The R_0^{Har} Rh: 33 phenotype results from substitution of exon 5 of the *RHCE* gene by the corresponding exon of the RHD gene. *British Journal of Haematology*, **92,** 751–757.

Bennett, P.R. & Cartron, J.-P. (1994) Prenatal determination of fetal RhD type (Reply). *New England Journal of Medicine*, **330,** 795–796.

Bennett, P.R., Le Van Kim, C., Colin, Y., Warwick, R.M., Cherif-Zahar, B., Fisk, N.M. & Cartron, J.-P. (1993) Prenatal determination of fetal RhD type by DNA amplification. *New England Journal of Medicine*, **329,** 607–610.

Beutler, E., McKusick, V.A., Motulsky, A.G., Scriver, C.R. & Hutchinson, F. (1996) Mutation nomenclature: nicknames, systematic names, and unique identifiers. *Human Mutation*, **8,** 203–206.

Bianchi, D.W., Zickwolf, G.K., Weil, G.J., Sylvester, S. & DeMaria, M.A. (1996) Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proceedings of the National Academy of Sciences USA*, **93,** 705–708.

Blunt, T., Daniels, G. & Carritt, B. (1994) Serotype switching in a partially deleted *RHD* gene. *Vox Sanguinis*, **67,** 397–401.

Busch, J., Huber, P., Holtz, J., Pfluger, E. & Radbruch, A. (1994) Simple and fast 'double-MACS' sorting of fetal erythroblasts from maternal blood for PCR-based paternity analysis. *Annals of the New York Academy of Science*, **731,** 144–146.

Carritt, B., Kemp, T.J. & Poulter, M. (1997) Evolution of the human RH (rhesus) blood group genes: a 50 year old prediction (partially) fulfilled. *Human Molecular Genetics*, **6,** 843–850.

Carritt, B., Steers, F.J. & Avent, N.D. (1994) Prenatal determination of fetal RhD type. *Lancet*, **344,** 205–206.

Cartron, J.P. (1994) Defining the Rh blood group antigens. Biochemistry and molecular genetics. *Blood Reviews*, **8,** 199–212.

Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X.C., Stern, D., Winkler, J., Lockhart, D.J., Morris, M.S. & Fodor, S.P.A. (1996) Accessing genetic information with high-density DNA arrays. *Science*, **274,** 610–614.

Cherif-Zahar, B., Bloy, C., Le Van Kim, C., Blanchard, D., Bailly, P., Hermand, P., Salmon, C., Cartron, J.-P. & Colin, Y. (1990) Molecular cloning and protein structure of a human blood group Rh polypeptide. *Proceedings of the National Academy of Sciences USA*, **87,** 6243–6247.

Cherif-Zahar, B., Le Van Kim, C., Rouillac, C., Raynal, V., Cartron, J.-P. & Colin, Y. (1994) Organization of the gene (RHCE) encoding the human blood group RhCcEe antigens and characterization of the promoter region. *Genomics*, **19,** 68–74.

Cherif-Zahar, B., Mattei, M.G., Le Van Kim, C., Bailly, P., Cartron, J.-P. & Colin, Y. (1991) Localization of the human Rh blood group gene structure to chromosome region 1p34.3–1p36.1 by in situ hybridization. *Human Genetics*, **86,** 398–400.

Cherif-Zahar, B., Raynal, V., Gane, P., Mattei, M.G., Bailly, P., Gibbs, B., Colin, Y. & Cartron, J.-P. (1996) Candidate gene acting as a suppressor of the *RH* locus in most cases of Rh-deficiency. *Nature Genetics*, **12,** 168–173.

Colin, Y., Cherif-Zahar, B., Le Van Kim, C., Raynal, V., van Huffel, V. & Cartron, J.-P. (1991) Genetic basis of the RhD-positive and RhD-negative blood group polymorphism as determined by southern analysis. *Blood*, **78,** 2747–2752.

Cooper, D.N. & Krawczak, M. (1993) *Human Gene Mutation*. Bios Scientific Publishers, Oxford.

Cossu, G., Angius, A., Gelfi, C. & Righetti, P.G. (1996) Rh D/d genotyping by quantitative polymerase chain reaction and capillary zone electrophoresis. *Electrophoresis*, **17,** 1911–1915.

Daniels, G., Green, C. & Smart, E. (1997) Differences between RhD-negative Africans and RhD-negative Europeans (Letter). *Lancet*, **350,** 862–863.

Dildy, G.A., Jackson, G.M. & Ward, K. (1996) Determination of fetal RhD status from uncultured amniocytes. *Obstetrics and Gynecology*, **88,** 207–210.

Eshleman, J.R., Shakin-Eshleman, S.H., Church, A., Kant, J.A. & Spitalnik, S.L. (1995) DNA typing of the human MN and Ss blood group antigens in amniotic fluid and following massive transfusion. *American Journal of Clinical Pathology*, **103,** 353–357.

Faas, B.H.W. (1998) *Rhesus blood group antigens: from postnatal phenotyping to prenatal genotyping*. PhD Thesis, Universiteit van Amsterdam, pp. 175–189.

Faas, B.H.W., Becker, E.A.M., Wildoer, P., Ligthart, P.C., Overbeeke, M.A.M., Zondervan, H.A., von dem Borne, A.E.G.K. & van der Schoot, C.E. (1997a) Molecular background of VS and weak C expression in blacks. *Transfusion*, **37,** 38–44.

Faas, B.H.W., Beckers, E.A.M., Simsek, S., Overbeeke, M.A.M., Pepper, R., van Rhenen, D.J., von dem Borne, A.E.G.K. & van der Schoot, C.E. (1996) Involvement of Ser103 of the Rh polypeptides in G epitope formation. *Transfusion*, **36,** 506–511.

Faas, B.H.W., Christiaens, G.C.M.L., Maaskant-van Wijk, P.A., Ligthart, P.C., Overbeeke, M.A.M., van Rhenen, D.J., von dem Borne, A.E.G.K. & van der Schoot, C.E. (1997b) Prenatal genotyping of Rh and Kell antigens on amniotic fluid DNA (Abstract). *Transfusion*, **37,** 100S.

Faas, B.H.W., Ligthart, P.C., Lomas-Francis, C., Overbeeke, M.A., von dem Borne, A.E. & van der Schoot, C.E. (1997c) Involvement of Gly96 in the formation of the Rh26 epitope. *Transfusion*, **37,** 1123–1130.

Faas, B.H.W., Simsek, S., Bleeker, P.M.M., Overbeeke, M.A.M., Cuijpers, H.T.M., von dem Borne, A.E.G.K. & van der Schoot, C.E. (1995) Rh E/e genotyping by allele-specific primer amplification. *Blood*, **85,** 829–832.

Ferguson-Smith, M.A., Zheng, Y.L. & Carter, N.P. (1994) Simultaneous immunophenotyping and FISH on fetal cells from maternal blood. *Annals of the New York Academy of Science*, **731,** 73–79.

Fischer, G.F., Fae, I., Petrasek, M. & Moser, S. (1995) A combination of two distinct in vitro amplification procedures for DNA typing of HLA-DRB and -DQB 1 alleles. *Vox Sanguinis*, **69,** 328–335.

Fischer, S.G. & Lerman, L.S. (1983) DNA fragments differing by single base-pair substitutions separated in denaturing gradient gels: correspondence with melting theory. *Proceedings of the National Academy of Sciences USA*, **80,** 1579–1583.

Flegel, W.A. (1997) *Häufigkeit sporadischer nicht-funktionaler Allele und ihre Bedeutung für die Genotypisierung am Beispiel des Polymorphismus im FUT1-Blutgruppengen.* Habilitationsschrift, Universität Ulm. <www.uni-ulm.de/~wflegel/HABIL>.

Flegel, W.A., Gassner, C., Müller, T.H., Schönitzer, D., Schunter, F. & Wagner, F.F. (1998) The molecular basis of weak D (Abstract). *Vox Sanguinis*, **74** (Suppl. 1)**,** 55.

Flegel, W.A., Hillesheim, B. & Wagner, F.F. (1996) RHD category VII is caused by a single molecular event (Abstract). *Transfusion Clinique et Biologique*, **3,** 33s.

Flegel, W.A. & Wagner, F.F. (1996) The frequency of RHD protein variants in Caucasians (Abstract). *Transfusion Clinique et Biologique*, **3,** 10s.

Flegel, W.A. & Wagner, F.F. (1998) *Rhesus Immunisierungsregister (RIR) [The Rhesus Immunization Surveillance]*. DRK-Blutspendedienst Baden-Württemberg, Ulm. <www.uni-ulm.de/~wflegel/RH/RIR>.

Fukumori, Y., Hori, Y., Ohnoki, S., Nagao, N., Shibata, H., Okubo, Y. & Yamaguchi, H. (1997) Further analysis of Del (D-elute) using polymerase chain reaction (PCR) with RHD gene-specific primers. *Transfusion Medicine*, **7,** 227–231.

Gänshirt-Ahlert, D., Börjesson-Stoll, R., Burschyk, M., Dohr, A., Garritsen, H.S., Helmer, E., Miny, P., Velasco, M., Walde, C., Patterson, D., Teng, N., Bhat, N.M., Bieber, M.M. & Holzgreve, W. (1993) Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *American Journal of Reproductive Immunology*, **30,** 194–201.

Gassner, C., Müller, T.H., Wagner, F.F., Flegel, W.A. & Schönitzer, D. (1997a) Rhesus C typing by polymerase chain reaction using sequence specific primers: realisation of 'medium resolution *RH*-DNA-typing' (Abstract). *Transfusion*, **37S,** 101S.

Gassner, C., Schmarda, A., Kilga-Nogler, S., Jenny-Feldkircher, B., Rainer, E., Müller, T.H., Wagner, F.F., Flegel, W.A. & Schönitzer, D. (1997b) RhesusD/CE typing by polymerase chain reaction using sequence-specific primers. *Transfusion*, **37,** 1020–1026.

Gassner, C., Schmarda, A., Nussbaumer, W. & Schönitzer, D. (1996) ABO glycosyltransferase genotyping by polymerase-chain reaction using sequence-specific primers. *Blood*, **88,** 1852–1856.

Geifman-Holtzman, O., Bernstein, I.M., Berry, S.M., Bianchi, D.W. & Holtzman, E.J. (1996a) Rapid molecular determination of fetal rhesus E type. *Prenatal Diagnosis*, **16,** 489–493.

Geifman-Holtzman, O., Bernstein, I.M., Berry, S.M., Holtzman, E.J., Vadnais, T.J., DeMaria, M.A. & Bianchi, D.W. (1996b) Fetal RhD genotyping in fetal cells flow sorted from maternal blood. *American Journal of Obstetrics and Gynecology*, **174,** 818–822.

Hamlington, J., Cunningham, J., Mason, G., Mueller, R. & Miller, D. (1997) Prenatal detection of rhesus D genotype (Letter). *Lancet*, **349,** 540.

Heid, C.A., Stevens, J., Livak, K.J. & Williams, P.M. (1996) Real time quantitative PCR. *Genome Research*, **6,** 986–994.

Hengstschlager, M., Holzl, G., Ulm, B. & Bernaschek, G. (1997) Raising the sensitivity of fetal RhD typing and sex determination from maternal blood (Letter). *Journal of Medical Genetics*, **34,** 350–351.

Hessner, M.J., McFarland, J.G. & Endean, D.J. (1996) Genotyping of KEL1 and KEL2 of the human Kell blood group system by the polymerase chain reaction with sequence-specific primers. *Transfusion*, **36,** 495–499.

Huang, C.H. (1996) Alteration of *RH* gene structure and expression in human dCCee and DC^{w}- red blood cells: phenotypic homozygosity versus genotypic heterozygosity. *Blood*, **88,** 2326–2333.

Huang, C.H. (1997a) Human D^{VI} category erythrocytes: correlation of the phenotype with a novel hybrid RhD-CE-D gene but not an internally deleted RhD gene. *Blood*, **89,** 1834–1839.

Huang, C.H. (1997b) Molecular insights into the Rh protein family and associated antigens. *Current Opinion in Hematology*, **4,** 94–103.

Huang, C.H., Chen, Y. & Reid, M. (1997) Human D^{IIIa} erythrocytes: RhD protein is associated with multiple dispersed amino acid variations. *American Journal of Hematology*, **55,** 139–145.

Hyland, C.A., Cherif-Zahar, B., Cowley, N., Raynal, V., Parkes, J., Saul, A. & Cartron, J.P. (1998) A novel single missense mutation identified along the RH50 gene in a composite heterozygous Rh_{null} blood donor of the regulator type. *Blood*, **91,** 1458–1463.

Hyland, C.A., Wolter, L.C. & Saul, A. (1994) Three unrelated Rh D gene polymorphisms identified among blood donors with Rhesus CCee ($r'r'$) phenotypes. *Blood*, **84,** 321–324.

Hyland, C.A., Wolter, L.C. & Saul, A. (1995) Identification and analysis of RH genes: application of PCR and RFLP typing tests. *Transfusion Medicine Reviews*, **9,** 289–301.

Ikemoto, S., Iwamoto, S., Tsuchida, S., Goto, K., Oyamada, T. & Kajii, E. (1996) Molecular genetic basis of red cell markers and its forensic application. *Forensic Science International*, **80,** 147–161.

Issitt, P.D. & Telen, M.J. (1996) D, weak D (D^{u}), and partial D: the molecular story unfolds. *Transfusion*, **36,** 97–100.

Jones, J. (1995) Identification of two new D variants, DHMi and DHMii using monoclonal anti-D. *Vox Sanguinis*, **69,** 236–241.

Jones, J.W., Finning, K.M., Mattock, R., Voak, D., Scott, M.L. & Avent, N.D. (1997) The serological profile and molecular basis of a new partial D phenotype, DHR. *Vox Sanguinis*, **73,** 252–256.

Jones, J., Scott, M.L. & Voak, D. (1995) Monoclonal anti-D specificity and Rh D structure: criteria for selection of monoclonal anti-D reagents for routine typing of patients and donors. *Transfusion Medicine*, **5,** 171–184.

Joyce, P. & Tavare, S. (1995) The distribution of rare alleles. *Journal of Mathematical Biology*, **33,** 602–618.

Kalinina, O., Lebedeva, I., Brown, J. & Silver, J. (1997) Nanoliter scale PCR with TaqMan detection. *Nucleic Acids Research*, **25,** 1999–2004.

Kemp, T.J., Poulter, M. & Carritt, B. (1996) A recombination hot spot in the Rh genes revealed by analysis of unrelated donors with the rare d – phenotype. *American Journal of Human Genetics*, **59,** 1066–1073.

Langley, C.H., Voelker, R.A., Leigh Brown, A.J., Ohnishi, S., Dickson, B. & Montgomery, E. (1981) Null allele frequencies at allozyme loci in natural populations of Drosophila melanogaster. *Genetics*, **99,** 151–156.

Le Van Kim, C., Mouro, I., Brossard, Y., Chavinie, J., Cartron, J.-P. & Colin, Y. (1994) PCR-based determination of Rhc and RhE status of fetuses at risk for Rhc and RhE haemolytic disease. *British Journal of Haematology*, **88,** 193–195.

Le Van Kim, C., Mouro, I., Cherif-Zahar, B., Raynal, V., Cherrier, C., Cartron, J.-P. & Colin, Y. (1992) Molecular cloning and primary structure of the human blood group RhD polypeptide. *Proceedings of the National Academy of Sciences USA*, **89,** 10925–10929.

Lee, S., Bennett, P.R., Overton, T., Warwick, R., Wu, X. & Redman, C.M. (1996) Prenatal diagnosis of Kell blood group genotypes: KEL1 and KEL2. *American Journal of Obstetrics and Gynecology*, **175,** 455–459.

Legler, T.J., Kohler, M., Mayr, W.R., Panzer, S., Ohto, H. & Fischer, G.F. (1996) Genotyping of the human platelet antigen systems 1 through 5 by multiplex polymerase chain reaction and ligation-based typing. *Transfusion*, **36,** 426–431.

Lighten, A.D., Overton, T.G., Sepulveda, W., Warwick, R.M., Fisk, N.M. & Bennett, P.R. (1995) Accuracy of prenatal determination of RhD type status by polymerase chain reaction with amniotic cells. *American Journal of Obstetrics and Gynecology*, **173,** 1182–1185.

Lin, Z., Cui, X. & Li, H. (1996) Multiplex genotype determination at a large number of gene loci. *Proceedings of the National Academy of Sciences USA*, **93,** 2582–2587.

Liu, W., Jones, J.W., Scott, M.L., Voak, D. & Avent, N.D. (1996) Molecular analysis of two D-variants, D^{HMi} and D^{HMii} (Abstract). *Transfusion Medicine*, **6** (Suppl. 2)**,** 21.

Lo, Y.M., Bowell, P.J., Selinger, M., Mackenzie, I.Z., Chamberlain, P., Gillmer, M.D., Elliott, P., Pratt, G., Littlewood, T.J. & Fleming, K.A. (1994a) Prenatal determination of fetal Rhesus D status by DNA amplification of peripheral blood of Rhesus-negative mothers. *Annals of the New York Academy of Science*, **731,** 229–236.

Lo, Y.-M.D., Bowell, P.J., Selinger, M., Mackenzie, I.Z., Chamberlain, P., Gillmer, M.D.G., Littlewood, T.J., Fleming, K.A. & Wainscoat, J.S. (1993) Prenatal determination of fetal RhD status by analysis of peripheral blood of rhesus negative mothers. *Lancet*, **341,** 1147–1148.

Lo, Y.M., Noakes, L., Bowell, P.J., Fleming, K.A. & Wainscoat, J.S. (1994b) Detection of fetal RhD sequence from peripheral blood of sensitized RhD-negative pregnant women. *British Journal of Haematology*, **87,** 658–660.

Lomas, C., Grässmann, W., Ford, D., Watt, J., Gooch, A., Jones, J., Beolet, M., Stern, D., Wallace, M. & Tippett, P. (1994) FPTT is a low-incidence Rh antigen associated with a 'new' partial Rh D phenotype, DFR. *Transfusion*, **34,** 612–616.

Lomas, C., Tippett, P., Thompson, K.M., Melamed, M.D. & Hughes-Jones, N.C. (1989) Demonstration of seven epitopes on the Rh antigen D using human monoclonal anti-D antibodies and red cells from D categories. *Vox Sanguinis*, **57,** 261–264.

Maas, J.H., Legler, T.J., Lynen, R., Blaschke, V., Ohto, H. & Köhler, M. (1997) [Rh-D genotyping for exon 2, 5 and 7 of German and Japanese blood donors with sequence specific

polymerase chain reaction]. *Beiträge zur Infusionstherapie und Transfusionsmedizin*, **34,** 203–209.

Maaskant van Wijk, P.A., Beckers, E.A.M., van Rhenen, D.J., Mouro, I., Colin, Y., Cartron, J.-P., Faas, B.H.W., van der Schoot, C.E., Apoil, P.A., von Blancher, A. & von dem Borne, A.E.G. (1997a) Evidence that the RHD (VI) deletion genotype does not exist. *Blood*, **90,** 1709–1711.

Maaskant van Wijk, P.A., Faas, B.H.W., de Ruijter, J.A.M., von Overbeeke, M.A.M., von dem Borne, A.E.G.K. & van Rhenen, D.J. (1997b) *RHD* genotyping by multiplex PCR analysis of all RHD specific exons (Abstract). *Transfusion*, **37,** 1S.

Maaskant van Wijk, P.A., Faas, B.H.W., de Ruijter, J.A.M., von Overbeeke, M.A.M., dem Borne, A.E.G.K., van Rhenen, D.J. & van der Schoot, C.E. (1998) Genotyping of RHD by multiplex polymerase chain reaction analysis of all *RHD*-specific exons. *Transfusion*, **38**, in press.

MacGeoch, C., Mitchell, C.J., Carritt, B., Avent, N.D., Ridgwell, K., Tanner, M.J. & Spurr, N.K. (1992) Assignment of the chromosomal locus of the human 30-kDal Rh (rhesus) blood group-antigen-related protein (Rh30A) to chromosome region 1p36.13–-p34. *Cytogenetics and Cellular Genetics*, **59,** 261–263.

Mallinson, G., Soo, K.S. & Anstee, D.J. (1995) The molecular basis of the Fy (a) and Fy (b) antigens (Abstract). *Transfusion Medicine*, **5,** 45.

Morris, T., Robertson, B. & Gallagher, M. (1996) Rapid reverse transcription-PCR detection of hepatitis C virus RNA in serum by using the TaqMan fluorogenic detection system. *Journal of Clinical Microbiology*, **34,** 2933–2936.

Mouro, I., Le Van Kim, C., Rouillac, C., van Rhenen, D.J., Le Pennec, P.Y., Bailly, P., Cartron, J.-P. & Colin, Y. (1994) Rearrangements of the blood group RhD gene associated with the D^{VI} category phenotype. *Blood*, **83,** 1129–1135.

Müller, T.H., Döscher, A. & Schunter, F. (1997a) Genotyping of the human platelet antigen-1 by ELISA detection of allele-specific amplicons. *Vox Sanguinis*, **73,** 185–188.

Müller, T.H., Gassner, C., Gran, S., Wagner, F.F., Flegel, W.A. & Schunter, F. (1997b) Semiautomated Rhesus DNA-typing by multiplex PCR: 4 reactions with 15 sequence-specific primer pairs (Abstract). *Transfusion*, **37,** 19S.

Ohnishi, S., Brown, A.J.L., Voelker, R.A. & Langley, C.H. (1982) Estimation of genetic variability in natural populations of Drosophila simulans by two-demensional and starch gel electrophoresis. *Genetics*, **100,** 127–136.

Okuda, H., Kawano, M., Iwamoto, S., Tanaka, M., Seno, T., Okubo, Y. & Kajii, E. (1997) The *RHD* gene is highly detectable in RhD-negative Japanese donors. *Journal of Clinical Investigation*, **100,** 373–379.

Olives, B., Merriman, M., Bailly, P., Bain, S., Barnett, A., Todd, J., Cartron, J.P. & Merriman, T. (1997) The molecular basis of the Kidd blood group polymorphism and its lack of association with type 1 diabetes susceptibility. *Human Molecular Genetics*, **6,** 1017–1020.

Pope, J., Navarrete, C., Warwick, R. & Contreras, M. (1995) Multiplex PCR analysis of RhD gene. *Lancet*, **346,** 375–376.

Poulter, M., Kemp, T.J. & Carritt, B. (1996) DNA-based Rhesus typing: simultaneous determination of RHC and RHD status using the polymerase chain reaction. *Vox Sanguinis*, **7,** 164–168.

Reubinoff, B.E., Avner, R., Rojansky, N., Manny, N., Friedmann, A., Laufer, N. & Mitrani-Rosenbaum, S. (1996) RhD genotype determination by single sperm cell analysis. *American Journal of Obstetrics and Gynecology*, **174,** 1300–1305.

Rose, N.C., Hurwitz, C., Silberstein, L., Andovalu, R. & Stoerker, J. (1997) Prenatal analysis of rhesus CcDEe blood groups by heteroduplex generator. *American Journal of Obstetrics and Gynecology*, **176,** 1084–1089.

Rossiter, J.P., Blakemore, K.J., Kickler, T.S., Kasch, L.M., Khouzami, A.N., Pressman, E.K., Sciscione, A.C. & Kazazian, H.H. (1994) The use of polymerase chain reaction to determine fetal RhD status. *American Journal of Obstetrics and Gynecology*, **171,** 1047–1051.

Roubinet, F., Apoil, P.A. & Blancher, A. (1996) Frequency of partial D phenotypes in the south western region of France. *Transfusion Clinique et Biologique*, **3,** 247–255.

Rouger, P. & Muller, J.-Y. (1996) Third International Workshop and Symposium on Monoclonal Antibodies against Human Red Cells and Related Antigens: Section RH. *Transfusion Clinique et Biologique*, **3,** 329–541.

Rouillac, C., Colin, Y., Hughes-Jones, N.C., Beolet, M., D'Ambrosio, A.-M., Cartron, J.-P. & Le Van Kim, C. (1995a) Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. *Blood*, **85,** 2937–2944.

Rouillac, C., Gane, P., Cartron, J.-P., Le Pennec, P.Y. & Colin, Y. (1996) Molecular basis of the altered antigenic expression of RhD in weak D (D^u) and RhC/e in R^N phenotypes. *Blood*, **87,** 4853–4861.

Rouillac, C., Le Van Kim, C., Beolet, M., Cartron, J.-P. & Colin, Y. (1995b) Leu110Pro substitution in the RhD polypeptide is responsible for the D^{VII} category blood group phenotype. *American Journal of Hematology*, **49,** 87–88.

Rouillac, C., Le Van Kim, C., Blancher, A., Roubinet, F., Cartron, J.-P. & Colin, Y. (1995c) Lack of G blood group antigen in D^{IIIb} erythrocytes is associated with segmental DNA exchange between *RH* genes. *British Journal of Haematology*, **89,** 424–426.

Salmon, C., Cartron, J.-P. & Rouger, P. (1984) *The Human Blood Groups*. Masson, New York.

Scott, M.L., Voak, D., Jones, J.W., Liu, W., Avent, N.D., Hughes-Jones, N. & Sonneborn, H.-H. (1997) A model for RhD – the relationship of 30 serologically defined epitopes to predicted structure. *Biotest Bulletin*, **5,** 459–466.

Sekizawa, A., Watanabe, A., Kimura, T., Saito, H., Yanaihara, T. & Sato, T. (1996) Prenatal diagnosis of the fetal RhD blood type using a single fetal nucleated erythrocyte from maternal blood. *Obstetrics and Gynecology*, **87,** 501–505.

Simsek, S., Faas, B.H.W., Bleeker, P.M.M., Overbeeke, M.A.M., Cuijpers, H.T.M., van der Schoot, C.E. & von dem Borne, A.E.G.K. (1995) Rapid Rh D genotyping by polymerase chain reaction-based amplification of DNA. *Blood*, **85,** 2975–2980.

Simsek, S., Bleeker, P.M.M. & von dem Borne, A.E.G. (1994) Prenatal determination of fetal RhD type (Letter). *New England Journal of Medicine*, **330,** 795–796.

Sonneborn, H.-H. & Voak, D. (1997) A review of 50 years of the Rh blood group system. *Biotest Bulletin*, **5,** 389–552.

Spence, W.C., Maddalena, A., Demers, D.B. & Bick, D.P. (1995a) Molecular analysis of the RhD genotype in fetuses at risk for RhD hemolytic disease. *Obstetrics and Gynecology*, **85,** 296–298.

Spence, W.C., Maddalena, A., Demers, D.B. & Bick, D.P. (1997) Prenatal determination of genotypes Kell and Cellano in at-risk pregnancies. *Journal of Reproductive Medicine*, **42,** 353–357.

Spence, W.C., Potter, P., Maddalena, A., Demers, D.B. & Bick, D.P. (1995b) DNA-based prenatal determination of the RhEe genotype. *Obstetrics and Gynecology*, **86,** 670–672.

Steers, F., Wallace, M., Johnson, P., Carritt, B. & Daniels, G. (1996) Denaturing gradient gel electrophoresis: a novel method for determining Rh phenotype from genomic DNA. *British Journal of Haematology*, **94,** 417–421.

Stoerker, J., Hurwitz, C., Rose, N.C., Silberstein, L.E. & Highsmith, W.E. (1996) Heteroduplex generator in analysis of Rh blood group alleles. *Clinical Chemistry*, **42,** 356–360.

Tanaka, M., Seno, T., Shibata, H., Okubo, Y., Okuda, H., Kajii, E. & Utsumi, R. (1997) Genotyping for RhC/c and RhE/e by PCR using allele-specific oligonucleotide primers. *Japanese Journal of Legal Medicine*, **51,** 32–38.

Tippett, P., Lomas-Francis, C. & Wallace, M. (1996) The Rh antigen D: partial D antigens and associated low incidence antigens. *Vox Sanguinis*, **70,** 123–131.

Tippett, P. & Sanger, R. (1962) Observations on subdivisions of Rh antigen D. *Vox Sanguinis*, **7,** 9–13.

Tippett, P. & Sanger, R. (1977) Further observations on subdivisions of the Rh antigen D. *Das Ärztliche Laboratorium*, **23,** 476–480.

Tonn, T., Westrup, D., Seidl, C., Kirchmaier, C.M. & Seifried, E. (1997) Sensitive determination of the RhD genotype in mixed samples using fluorescence-based polymerase chain reaction. *Vox Sanguinis*, **72,** 177–181.

van den Veyver, I.B., Chong, S.S., Cota, J., Bennett, P.R., Fisk, N.M., Handyside, A.H., Cartron, J.P., Le Van Kim, C., Colin, Y. & Snabes, M.C. (1995) Single-cell analysis of the RhD blood type for use in preimplantation diagnosis in the prevention of severe hemolytic disease of the newborn. *American Journal of Obstetrics and Gynecology*, **172,** 533–540.

van den Veyver, I.B. & Moise, K.J. (1996) Fetal RhD typing by polymerase chain reaction in pregnancies complicated by rhesus alloimmunization. *Obstetrics and Gynecology*, **88,** 1061–1067.

van den Veyver, I.B., Subramanian, S.B., Hudson, K.M., Werch, J., Moise, K.J. & Hughes, M.R. (1996) Prenatal diagnosis of the RhD fetal blood type on amniotic fluid by polymerase chain reaction. *Obstetrics and Gynecology*, **87,** 419–422.

Wagner, F.F. & Flegel, W.A. (1997) Polymorphism of the h allele and the population frequency of sporadic nonfunctional alleles. *Transfusion*, **37,** 284–290.

Wagner, F.F., Gassner, C., Müller, T.H., Schönitzer, D., Schunter, F. & Flegel, W.A. (1998a) Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohematologic features. *Blood*, **91,** 2157–2168.

Wagner, F.F., Gassner, C., Müller, T.H., Schönitzer, D., Schunter, F. & Flegel, W.A. (1998b) The molecular basis of weak D phenotypes. *Blood*, in press..

Wagner, F.F., Kasulke, D., Kerowgan, M. & Flegel, W.A. (1995) Frequencies of the blood groups ABO, Rhesus, D category VI, Kell, and of clinically relevant high-frequency antigens in South-Western Germany. *Infusionstherapie und Transfusionsmedizin*, **22,** 285–290.

Wallace, M., Lomas-Francis, C., Beckers, E., Bruce, M., Campbell, G., Chatfield, S., Nagao, N., Okubo, Y., Opalka, A., Overbeeke, M., Scott, M. & Voak, D. (1997) DBT: a partial D phenotype associated with the low-incidence antigen Rh32. *Transfusion Medicine*, **7,** 233–238.

Wenk, R.E. & Chiafari, P.A. (1997) DNA typing of recipient blood after massive transfusion. *Transfusion*, **37,** 1108–1110.

Wodicka, L., Dong, H., Mittmann, M., Ho, M.H. & Lockhart, D.J. (1997) Genome-wide expression monitoring in Saccharomyces cerevisiae. *Nature Biotechnology*, **15,** 1359–1367.

Wolter, L.C., Hyland, C.A. & Saul, A. (1993) Rhesus D genotyping using polymerase chain reaction. *Blood*, **82,** 1682–1683.

Woo, T.H., Patel, B.K., Smythe, L.D., Norris, M.A., Symonds, M.L. & Dohnt, M.F. (1998) Identification of pathogenic Leptospira by TaqMan probe in a lightcycler. *Analytical Biochemistry*, **256,** 132–134.

Yankowitz, J., Li, S. & Murray, J.C. (1995) Polymerase chain reaction determination of RhD blood type: an evaluation of accuracy. *Obstetrics and Gynecology*, **86,** 214–217.

Yankowitz, J., Li, S. & Weiner, C.P. (1997) Polymerase chain reaction determination of RhC, Rhc, and RhE blood types: an evaluation of accuracy and clinical utility. *American Journal of Obstetrics and Gynecology*, **176,** 1107–1111.