Molecular genetic blood group typing by the use of PCR-SSP technique

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BACKGROUND: DNA-based methods are useful for enhancing immunohematology typings. Ready-to-use Conformité Européenne (CE)-marked test kits based on polymerase chain reaction with sequence-specific priming (PCR-SSP) have been developed, which enable the examination of weak, unexpected, or unclear serologic findings.

DEVELOPMENT AND VALIDATION: Primers were designed according to established mutation databases. Proficiency testing for CE marking was performed in accordance with Directive 98/79EC of the European Parliament and of the Council of October 27, 1998 on in vitro diagnostic medical devices using pretyped in-house and external samples.

INTENDED USE: BAGene PCR-SSP kits are in vitro diagnostic devices. Genotyping of *ABO* and *RHD/RHCE* as well as *HPA* and *KEL*, *JK*, and *FY* specificities has to be performed after the conclusion of the serologic determination.

APPLICATION: Ready-to-use PCR-SSP typing kits allow the determination of common, rare, or weak alleles of the ABO blood group, Rhesus, and Kell/Kidd/Duffy systems as well as alleles of the human platelet antigens.

RESULTS: The investigations showed clear-cut results in accordance with serology or molecular genetic pre-typing.

CONCLUSION: PCR-SSP is a helpful supplementary technique for resolving most of the common problems caused by discrepant or doubtful serologic results, and it is an easy-to-handle robust method. Questionable cases in donor, recipient, and patient typing can be examined with acceptable cost.

INTRODUCTION

The molecular genetic basis of almost all blood group systems has been investigated and described in the literature. Deoxyribonucleic acid (DNA) typing is possible for many of the blood group antigens that are defined by single amino acid polymorphisms. An increased frequency of scientific literature dealing with molecular typing in immunohematology has appeared since about 1993.[1,2] In Germany, blood group genotyping, mainly the determination of *RHD* including weak D phenotypes, was implemented at the University Hospital in Ulm in 1998 to determine anti-D prophylaxis in the prenatal and postpartum settings.[3] Many other German university clinics and transfusion centers also apply molecular typing for the examination of different blood group systems. Since polymerase chain reaction with sequence-specific priming (PCR-SSP)[4,5] is well known and established for applications in transplantation medicine, a decision was reached to develop Conformité Européenne (CE)-marked test kits suitable for blood group genotyping using this technique, which is extensively described in the literature. Aspects of development and validation, as well as intended use and application of commercial available test kits for molecular genetic blood group typing, will be explained in this article.

ABBREVIATIONS: CE = Conformité Européenne; SSP = sequence-specific priming.

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DEVELOPMENT AND VALIDATION

Design dossier

The first step of development is the product idea, followed by creating the design dossier and required documents for CE marking in vitro diagnostics for blood group typing on a molecular genetic basis (e.g., kit design, specifications, milestones, standard operating procedures, product inserts, labeling, packaging, etc.).

Primer design

Primers were designed according to the Blood Group Antigen Database.[6] Published primers were first checked for sequence similarities with the use of BLAST[7] and subsequently adjusted for uniform melting temperature. After pilot experiments, alternative primers were designed and tested if necessary. The selection of clinically relevant alleles was decided following extensive discussions among scientists from Germany, Austria, and Switzerland as a joint working group of the German Society for Transfusion Medicine and Immunohematology. Pretyped DNA samples were used for verification of design.

Samples and validation

After the production of a prototype, a risk analysis and stability testing were carried out. Proficiency testing was performed using 1000 in-house and external samples, previously typed by serology or by molecular genetic methods (sequence analysis or real-time PCR).

Kit design

BAGene (BAG, Lich, Germany) ready-to-use PCR-SSP kits consist of PCR plates or strips with prealiquoted, dried, and colored reaction mixes containing allele-specific primers, internal control primers (specific for the human growth hormone gene[8]), and nucleotides. Furthermore, 10 × PCR buffer, PCR strip caps, worksheets, and evaluation diagrams as well as instructions for use are included. Taq polymerase is provided by the user.

INTENDED USE

The molecular determination of blood group antigens with the use of PCR-SSP kits is to be performed in conjunction with serology. These assays are available as a supplementary technique to investigate weak or discrepant serologic findings. The current assays are not intended to replace serology. In case of discrepant or unclear genotyping results, transfusion guidelines are formulated in accordance with serologic results. Final clarification by gene sequence analysis is recommended.

TABLE 1. Applications of PCR-SSP for the determination of blood groups

- Genotype multiply transfused recipients
- Genotype patients after ABO-incompatible BMT
- Determine *RHD* zygosity of partners from alloimmunized D-negative women before pregnancies
- Genotype D-negative donors with C or E to exclude the presence of the *RHD* gene and thus prevent anti-D alloimmunization of recipients caused by very weak Rh D variants in blood donors
- Identify genotype in case of weakly expressed Rh D (e.g., DEL) in donors
- Confirm weak D genotypes in recipients to avoid unnecessary use of D-negative blood units
- Quality control of serologic methods
- External quality assurance

APPLICATION

A summary of the different uses of BAGene PCR-SSP for molecular blood group typing is presented above (Table 1).

Determination of ABO blood groups

The genes for A and B transferase are located on the long arm of chromosome 9 (9q34).[9-11] They consist of seven exons with a total length of 1065 base pairs. The majority of clinical relevant polymorphisms (base substitutions, deletions, insertions) are located on exons 6 and 7.[12] Five common alleles are described in the literature: A^1, A^2, B^1, O^1, and O^2, and there are also numerous variants and subgroups. BAGene ABO-TYPE variant allows the molecular genetic determination of these five main alleles[13] as well as the common O^{1v} allele and the specific subgroup variants A^3, A^x, $A^{el}A^w$, B^3, B^x, B^w (Fig. 2).[14-18]

Determination of *RHD/RHCE* alleles

The two *RH* genes, *RHD* and *RHCE*, are located on the short arm of chromosome 1 (p34.3 to p36.1).[19] Their 3′ -ends are oriented to each other and separated by 30,000 base pairs.[20,21] The *RHD* gene encodes the antigen D; the *RHCE* gene encodes the antigens C, c, E, e. *RHD* and *RHCE* genes consist of 10 exons. Approximately 18 percent of Europeans are serologically D–. In almost all D– Caucasians, the *RHD* is completely deleted on both chromosomes.[22] In D– individuals from other ethnic groups (Africans, Asians), the inactive *RHD* associated with a Cde^s haplotype and *RHDΨ* are found.[23-25] BAGene RH-TYPE allows the molecular genetic determination of standard *RHD*/*RHCE* alleles[26,27] as well as the typing of a few *RHD* variants (DVI, DIV type 3, Cde^s, *RHDΨ*, *RHD*(W16X), *RHD-CE*(8-9)-*D*, *RHD-CE*(3-7)-*D*) and DEL (*RHD*(K409K), *RHD*(M295I), *RHD*(IVS3+1G>A)).[28] The determination of C^w [29] is included as well. *RHD* variants with a higher frequency in Asians (*RHD*(K409K), weak D type 15, 17)[30] can be detected with BAGene *RHD*-TYPE Asia.

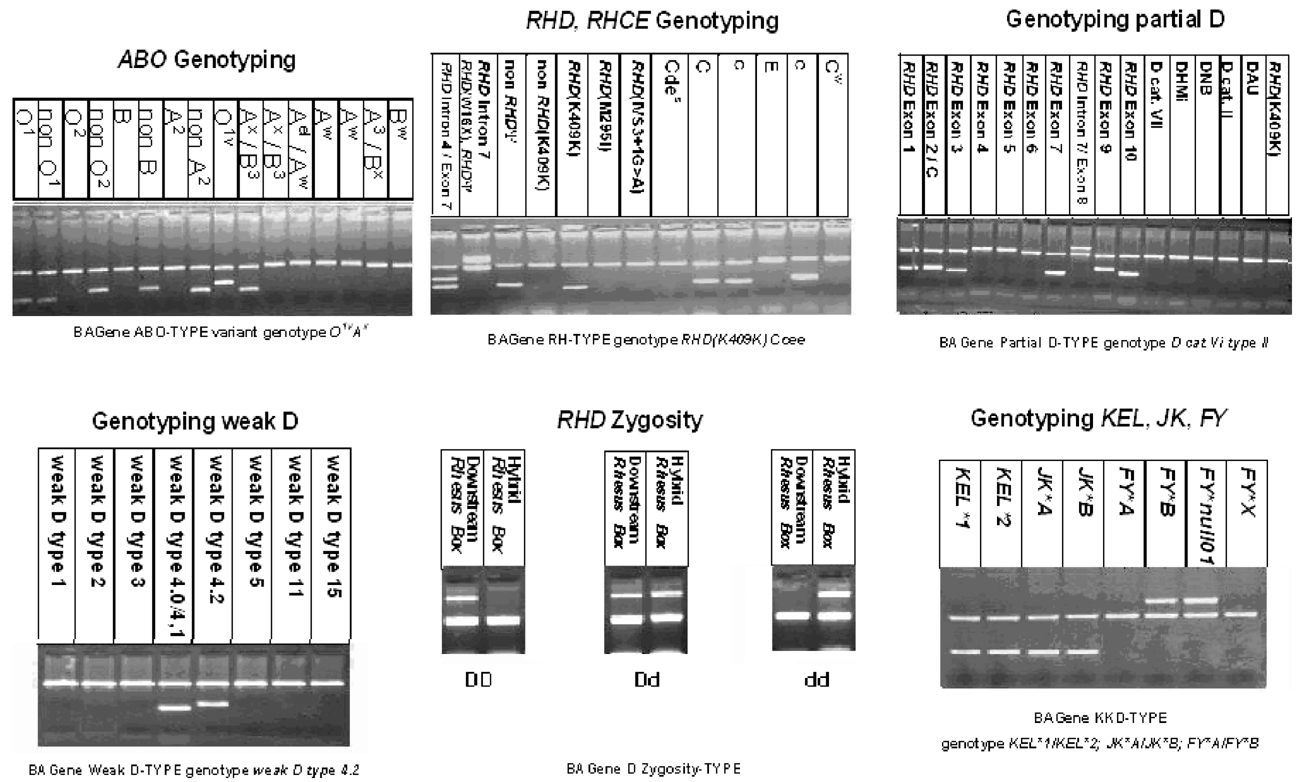

Fig. 1. Examples of gel pictures using BAGene PCR-SSP test kits for different applications.

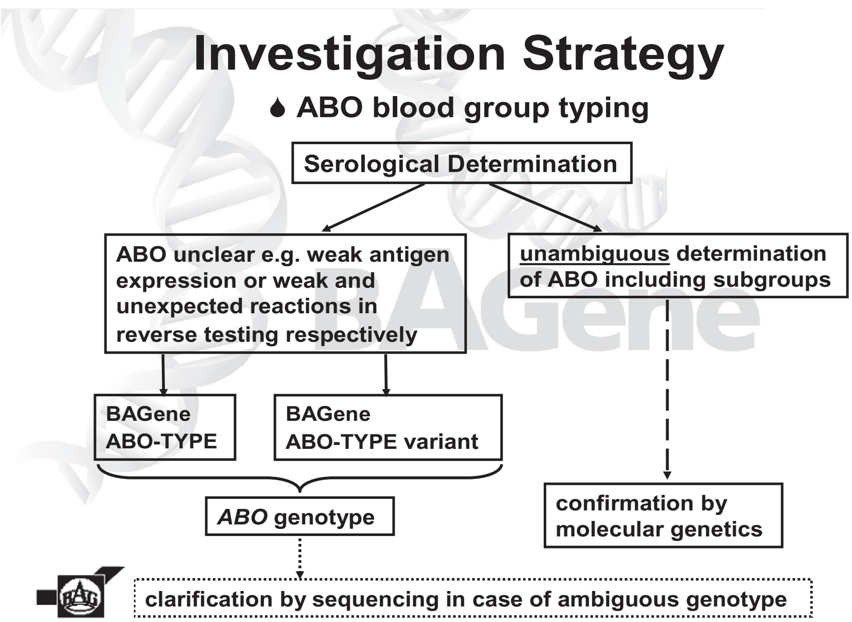

Fig. 2. Clear cut serologic determination of the ABO blood group cannot be achieved with samples of polytransfused recipients. Weak expression of A and B antigens, associated either with normal or with unexpected reverse typing, also hampers the evaluation of results. This flow chart depicts a strategy using the BAGene ABO-TYPE variant kit, which allows resolution of most unclear serologic findings.

Genotyping partial D and weak D

Variations of the antigen structure of RhD result either in a partial D or in a weak D phenotype. Molecular genetic examinations of these D variants have shown that weak D phenotypes as well as some partial D types are caused by point mutations. In other partial D, one or several exons of *RHD* are exchanged with the corresponding segments of *RHCE*, thus forming RhD-CE-D fusion proteins. In these fusion proteins, epitopes of the RhD protein are missing.[31] Therefore, individuals with partial D types (e.g., with the clinically relevant D category VI[32]) may be immunized by transfusion of erythrocytes expressing the normal RhD protein. According to the literature, the substitution of amino acids in partial D phenotypes are localized mainly extracellular. The substitution of amino acids in weak D is mainly limited to intracellular or transmembrane sections of the RhD protein.[33,34] BAGene Partial D-TYPE allows the molecular genetic determination of the D-categories II, III, IV, V,[35,36] VI, VII[37] as

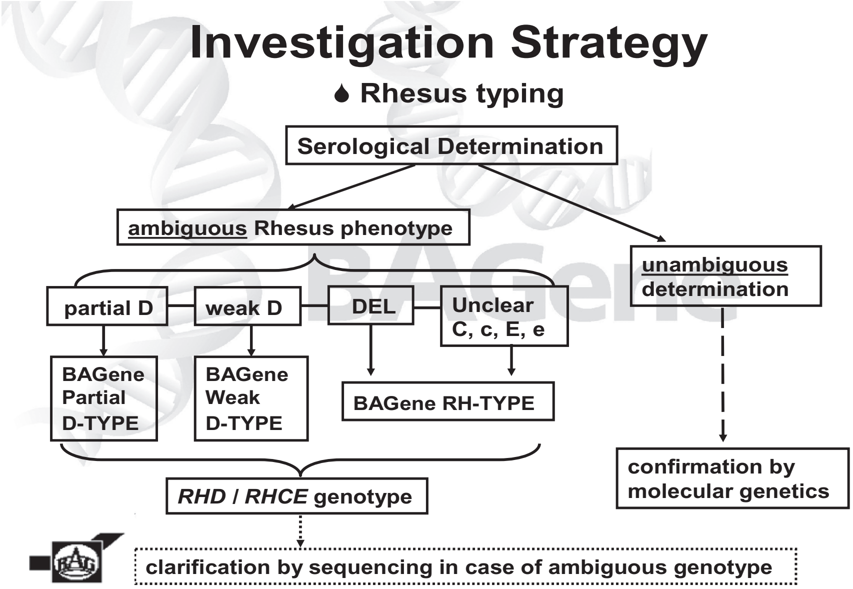

Fig. 3. Unclear Rh phenotypes can be investigated by selecting suitable SSP kits depending on specific purposes. This figure shows an approach, i.e., how to proceed in case of a questionable partial D pretyped by serology: Quite often, a weak D instead of a partial D is hidden behind. Testing for weak D first is recommended. In case that the most common weak D types can be excluded, typing with the Partial D-TYPE kit should follow. An additional kit is available to examine D-negative samples, for instance, with a big C or a big E. The RH-TYPE kit enables detection of D-negative *RHD* alleles, such as *DEL* types, *RHDψ*, or *Cde*[s]. RhCE antigens can also be crosschecked with this SSP kit.

well as partial-D DAU,[38] DBT,[39] DFR, DHMi, DHMii, DNB,[40] R_0Har (Rh33), and DEL (*RHD*(K409K)). BAGene Weak D-TYPE allows the molecular genetic determination of the weak D types 1, 2, 3, 4.0/4.1, 4.2, 5, 11, and 15 (Fig. 3).

Genotyping *RHD* zygosity

In the D+ haplotype, the *RHD* gene is flanked by two highly homologous DNA segments, the so-called *Rhesus boxes*, which are located 5′(upstream *Rhesus box*) and 3′(downstream *Rhesus box*) of *RHD*.[41] In D– Caucasians, the *RHD* gene is generally completely deleted on both chromosomes. This results in a hybrid *Rhesus box,* which comprises the 5′-end of the upstream *Rhesus box* and the 3′-end of the downstream *Rhesus box*. BAGene D Zygosity-TYPE with the use of PCR-SSP allows the determination of *RHD* zygosity (homozygosity or hemizygosity of D) by the amplification of the downstream *Rhesus box* (DD), or by the hybrid *Rhesus box* (dd), or by the downstream and the hybrid *Rhesus box* (Dd), respectively.[42]

Genotyping *KEL*, *JK*, and *FY*

The significant difference between *KEL1* und *KEL2* (serologic nomenclature K and k– Cellano) is caused by a single base substitution in exon 6 of the gene.[43,44] The Kidd system is located on chromosome 18 and consists of three different specificities Jk[a], Jk[b], Jk[null]. The alleles *JK*A* and *JK*B* of the Kidd system differ in one single nucleotide substitution at position 838 of the SLC14A1 gene.[45]

The *FY* gene is located on chromosome 1. It consists of the alleles *FY*A, FY*B, FY*X,* and *FY*null01*. Regarding serologic nomenclature, the *FY*A* allele corresponds to the Fy[a] antigen and the *FY*B* allele to the Fy[b] antigen.[46,47] The weakly expressed allele *FY*X* (Fy[X]) is serologically determined as Fy[b weak].[48] In the African population, the phenotype Fy(a–b–) can be observed with a frequency of 68 percent, whereas in Europeans, Fy(a–b–) is extremely rare (<0.1%). The most frequent cause of a Duffy negative, i.e., Fy(a–b–) erythrocyte phenotype in blacks, is a polymorphism in the GATA motif of the Duffy gene (DARC) promoter, disrupting a binding site for the GATA1 erythroid transcription factor. Individuals with this silent allele, also called *FY*null01*,[49,50] are resistant to *Malaria tertiana* (*Plasmodium vivax*).[51] BAGene KKD-TYPE allows a clear identification of the immunologic relevant alleles Fy[b weak] (*Fy*X*) and *FY*null01*. The kit consists of at least eight different PCR reaction mixes and enables the laboratory to perform the following assays: Kell (K, k), Kidd (Jk[a], Jk[b]), and Duffy (Fy[a], Fy[b], Fy[null], Fy[X]).[52]

RESULTS

The examination of 1000 in-house and external samples with the kits described here showed results that were in accordance with serology or molecular genetic pretyping. The diagnostic sensitivity and specificity of each primer mix was examined with DNA from reference samples. Those rare alleles that have not been tested because of unavailability are indicated on the worksheets and evaluation diagrams with the remark “n.t.” (i.e., not tested currently). Initial results from external studies comparing genotyping for *RHD* and *RHCE* with serology have been presented in several congresses.[53,54]

CONCLUSIONS

The PCR-SSP technique is helpful in resolving many of the problems caused by discrepant or doubtful serologic test results. It is easy to handle and is a robust method.

The current test kits are not intended to, nor designed to, replace serology and are not suitable for high throughput. Genotyping results for ABO must be interpreted with caution because of the serious clinical consequences of ABO major incompatible transfusion of red blood cells.

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REFERENCES

1. Müller TH, Hallensleben M, Schunter F, Blasczyk R. Molekulargenetische Blutgruppendiagnostik [Molecular genetic diagnosis of blood groups]. Dt Ärztebl 2001;98:A 317-322 [Heft 6].
2. Bennett PR, Le Van Kim C, Colin Y, et al. Prenatal determination of fetal RhD type by DNA amplification. N Engl J Med 1993;329:607-10.
3. Flegel WA. Blood group genotyping in Germany. Transfusion 2007;47(Suppl.):47S-53S.
4. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 1992;39:225-35.
5. Olerup O, Zetterquist H. DR “low-resolution” PCR-SSP typing—a correction and an update. Tissue Antigens 1993; 1:55-6.
6. Blood Group Antigen Gene Mutation Database. Available from: http://www.ncbi.nlm.nih.gov/projects/mhc/xslcgi.fcgi?cmd=bgmut/home
7. BLAST (Basic Local Alignment Search Tool). Available from: http://www.ncbi.nlm.nih.gov/BLAST/
8. Chen EY, Liao YC, Smith DH, Barrera-Saldana HA, Gelinas RE, Seeburg PH. The human growth hormone locus: nucleotide sequence, biology, and evolution. Genomics 1989;4: 479.
9. Ferguson-Smith MA, Aitken DA, Turleau C, de Grouchy J. Localisation of the human ABO. Np-1: AK-1 linkage group by regional assignment of AK-1 to 9q34. Hum Genet 1976; 34:35-43.
10. Chester MA, Olsson ML. The ABO blood group gene. A locus of considerable genetic diversity. Transfus Med Rev 2001;15:177-200.
11. Yip SP. Sequence variation at the human ABO locus. Ann Hum Genet 2002;66:1-27.
12. Seltsam A, Hallensleben A, Kollmann A, Burkhart J, Blasczyk R. Systematic analysis of the ABO gene diversity within exons 6 and 7 by PCR-screening revealed new ABO alleles. Transfusion 2003;43:428-39.
13. Gassner C, Schmarda A, Nussbaumer W, Schönitzer D. ABO glycosyltransferase genotyping by polymerase chain reaction using sequence-specific primers. Blood 1996;88: 1852-6.
14. Yamamoto F, McNeill PD, Yamamoto M, et al. Molecular genetic analysis of the ABO blood group system: 1. Weak subgroups: A^3 and B^3 alleles. Vox Sang 1993:64:116-9.
15. Yamamoto F, McNeill PD, Yamamoto M, Hakomori S, Harris T. Molecular genetic analysis of the ABO blood group system: 3. A^x and $B^{(A)}$ alleles. Vox Sang 1993;64:171-4.
16. Olsson ML, Irshaid NM, Hosseini-Maaf B, et al. Genomic analysis of clinical samples with serologic ABO blood grouping discrepancies: identification of 15 novel A and B subgroup alleles. Blood 2001;98:1585-93.
17. Seltsam A, Hallensleben M, Kollmann A, Blasczyk R. The nature of diversity and diversification at the ABO locus. Blood 2003;102:3035-42.
18. Seltsam A, Das Gupta C, Wagner FF, Blasczyk R. Nondeletional ABO*O alleles express weak blood group A phenotypes. Transfusion 2005;45:359-65.
19. Chérif-Zahar B. Localization of the human Rh blood group gene structure to chromosome region 1p34.3-1p36.1 by in situ hybridisation. Hum Genet 1991;86:398-400.
20. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA. Molecular basis of weak D phenotypes. Blood 1999;93:385-93.
21. Flegel WA, Wagner FF. Molecular genetics of *RH*. Vox Sang 2000;78(Suppl 2):109-15.
22. Blunt T, Daniels G, Carritt B. Serotype switching in a partially delected *RHD* gene. Vox Sang 1994;67:397-401.
23. Okada H, Kawano M, Iwamoto S, et al. The *RHD* gene is highly detectable in RhD-negative Japanese donors. J Clin Invest 1997;100:373-9.
24. Singleton BK, Green CA, Avent ND, et al. The presence of an *RHD* pseudogene containing a 37 base pair duplication and a nonsense mutation in Africans with the RhD-negative blood group phenotype. Blood 2000;95:12-8.
25. Wagner FF, Fromajer A, Flegel WA. *RHD* positive haplotypes in D negative Europeans. BMC Genet 2001; 2:10.
26. Gassner C, Schmarda A, Kilga-Nogler S, et al. RHD/CE typing by polymerase chain reaction using sequence-specific primers. Transfusion 1997;37:1020-6.
27. Flegel WA, Wagner FF, Müller TH, Gassner C. Rh phenotype prediction by DNA typing and its application to practice. Transfus Med 1998;8:281-302.
28. Shao CP, Maas JH, Su YQ, Köhler M, Legler TJ. Molecular background of RhD-positive, D-negative, Del and weak D phenotypes in Chinese. Vox Sang 2002;83:156-61.

29. Mouro I, Colin Y, Sistonen P, Le Pennec PY, Cartron J-P, Le Van Kim C. Molecular basis of the RhC^w (Rh8) and RhC^x (Rh9) blood group specificities. Blood 1995;86: 1196-201.
30. Luettringhaus TA, Cho D, Ryang DW, Flegel WA. An easy *RHD* genotyping strategy for D– East Asian persons applied to Korean blood donors. Transfusion 2006;46: 2128-37.
31. Rouillac C, Colin Y, Hughes-Jones NC, et al. Transcript analysis of D category phenotypes predicts hybrid Rh D-CE-D proteins associated with alteration of D epitopes. Blood 1995;85:2937-44.
32. Wagner FF, Gassner C, Müller TH, Schönitzer D, Schunter F, Flegel WA. Three molecular structures cause Rhesus D category VI phenotypes with distinct immunohematologic features. Blood 1998;91:2157-68.
33. Legler TJ, Maas JH, Blaschke M, et al. *RHD* genotyping in weak D phenotypes by multiple polymerase chain reactions. Transfusion 1998;38:434-40.
34. Wagner FF, Frohmajer A, Ladewig B, et al. Weak D alleles express distinct phenotypes. Blood 2000;95:2699-708.
35. Omi T, Takahashi J, Tsudo N, et al. The genomic organization of the partial D category DVa: the presence of a new partial D associated with the DVa phenotype. Biochem Biophys Res Commun 1999;254:786-94.
36. Legler TJ, Wiemann V, Ohto H, et al. D^{Va} category phenotype and genotype in Japanese families. Vox Sang 2000;78: 194-7.
37. Rouillac C, Le Van Kim C, Beolet M, Cartron J-P, Colin Y. Leu110Pro substitution in the RhD polypeptide is responsible for the DVII category blood group phenotype. Am J Hematol 1995;49:87-8.
38. Wagner FF, Ladewig B, Angert KS, Heymann GA, Eicher NI, Flegel WA. The *DAU* allele cluster of the *RHD* gene. Blood 2002;100:306-11.
39. Beckers EAM, Faas BHW, Simsek S, et al. The genetic basis of a new partial D antigen: D^{DBT}. Br J Haematol 1996;93: 720-7.
40. Wagner FF, Eicher NI, Jørgensen JR, Lonicer CB, Flegel WA. DNB: a partial D with anti-D frequent in Central Europe. Blood 2002;100:2253-6.
41. Wagner FF, Flegel WA. RHD gene deletion occurred in the Rhesus box. Blood 2000;95:3662-8.
42. Perco P, Shao CP, Mayr WR, Panzer S, Legler TJ. Testing for the D zygosity with three different methods revealed altered Rhesus boxes and a new weak D type. Transfusion 2003;43:335-9.
43. Lee S, Naime DS, Reid ME, Redman CM. Molecular basis for the high-incidence antigens of the Kell blood group system. Transfusion 1997;37:1117-22.
44. Lee S, Wu X, Reid M, Zelinski T, Redman C. Molecular basis of the Kell (K1) phenotype. Blood 1995;85:912-6.
45. Lucien N, Sidoux-Walter F, Olives B, et al. Characterization of the gene encoding the human Kidd blood group/urea transporter protein. Evidence for splice site mutations in Jknull individuals. J Biol Chem 1998;273:12973-80.
46. Neote K, Mak JY, Kolakowski LFJ, Schall TJ. Functional and biochemical analysis of the cloned Duffy antigen: identity with the red blood cell chemokine receptor. Blood 1994;84: 44-52.
47. Tournamille C, Le van Kim C, Gane P, Cartron JP, Colin Y. Molecular basis and PCR-DNA typing of the Fya/fyb blood group polymorphism. Hum Genet 1995;95:407-10.
48. Tournamille C, Le van Kim C, Gane P, et al. Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy(x) individuals. Blood 1998;92:2147-56.
49. Tournamille C, Colin Y, Cartron JP, Le van Kim C. Disruption of a GATA motif in the Duffy gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995;10:224-8.
50. Mallinson G, Soo KS, Schall TJ, Pisacka M, Anstee DJ. Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fya/Fyb antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a–b–) phenotype. Br J Haematol 1995;90:823-9.
51. Chitnis CE, Chaudhuri A, Horuk R, Pogo AO, Miller LH. The domain on the Duffy blood group antigen for binding Plasmodium vivax and P. knowlesi malarial parasites to erythrocytes. J Exp Med 1996;184:1531-6.
52. Rožman T, Dovč T, Gassner C. Differentiation of autologous ABO, RHD, RHCE, KEL, JK, and FY blood group genotypes by analysis of peripheral blood samples of patients who have recently received multiple transfusions. Transfusion 2000;40:936-42.
53. Legler TJ, Binder E, Smart E, Prager M, Maas JH. RHD and RHCE genotyping in South African blood donors with prepipetted PCR-SSP kits. Transfusion 2004;44(Suppl):114a (Abstract).
54. Thierbach J, Jung A, Hitzler WE. Retrospective, comparative typing of Rh(D) negative and weak D blood donors with serological and genotyping methods. Transfus Med Hemother 2006;33(Suppl 1):49 (P6.26).