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. 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. 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) 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.
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. Published primers were first checked for sequence similarities with the use of BLAST 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), 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 RH D 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). 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. Five common alleles are described in the literature: A1, A2, B1, O1, and O2, and there are also numerous variants and subgroups. BAGene ABO-TYPE variant allows the molecular genetic determination of these five main alleles as well as the common O allele and the specific subgroup variants A1, A*, A4Aw, B1, B*, Bw (Fig. 2).

### Determination of RH/D/RHCE alleles
The two RH genes, RHD and RHCE, are located on the short arm of chromosome 1 (p34.3 to p36.1). Their 3′-ends are oriented to each other and separated by 30,000 base pairs. The RHD gene encodes the antigen D; the RHCE gene encodes the antigens C, c, E, and E. RHD and RHCE genes consist of 10 exons. Approximately 18 percent of Europeans are serologically D−. In almost all D− Causians, the RHD is completely deleted on both chromosomes. In D− individuals from other ethnic groups (Africans, Asians), the inactive RHD associated with a Cde haplotype and RHDΨ are found. BAGene RH-TYPE allows the molecular genetic determination of standard RHD/RHCE alleles as well as the typing of a few RHD variants (DVI, DIV type 3, Cde, RHDS, RHD(W16X), RHDC(8-9)-D, RHDC(3-7)-D) and DEL (RHD(K409K), RHD(M295I), RHD(V53+1G=A)). The determination of Cw and Cw3 is included as well. RHD variants with a higher frequency in Asians (RHD(K409K), weak D type 15, 17) can be detected with BAGene RHD-TYPE Asia.
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. Therefore, individuals with partial D types (e.g., with the clinically relevant D category VI) 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.

\( RHD, RHCE \) Genotyping

Genotyping partial D

Genotyping weak D

\( RHD \) Zygosity

Genotyping KEL, JK, FY

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.
PCR-SSP TYPING FOR BLOOD GROUPS

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-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 RH alleles, such as DEL types, RHDE, or Cde. RhCE antigens can also be crosschecked with this SSP kit.

Investigation Strategy

![Diagram](image)

**Genotyping RH D zygoty**

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. 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-Weak TYPE with the use of PCR-SSP allows the determination of RH zygoty (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.

**Genotyping KEL, JK, and FY**

The significant difference between KEL1 and KEL2 (serologic nomenclature K and k– Cellano) is caused by a single base substitution in exon 6 of the gene. The Kidd system is located on chromosome 18 and consists of three different specificities JkA, JkB, and Jknull. The alleles JkA and JkB of the Kidd system differ in one single nucleotide substitution at position 838 of the SLC14A1 gene.

The FY gene is located on chromosome 1. It consists of the alleles FYA, FYB, FYX, and FYnull01. Regarding serologic nomenclature, the FYA allele corresponds to the FyA antigen and the FYB allele to the Fyb antigen. In the African population, the phenotype Fya(b–) can be observed with a frequency of 68 percent, whereas in Europeans, Fya(b–) is extremely rare (<0.1%). The most frequent cause of a Duffy negative, i.e., Fya(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 FYnull01, are resistant to Malaria tertiana (Plasmodium vivax).

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.

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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