Optimized sensitivity of allele-specific PCR for prenatal typing of human platelet alloantigen single nucleotide polymorphisms

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PCR using sequence-specific primers (PCR-SSP) is widely employed for the genotyping of single nucleotide polymorphisms (SNPs) in both routine diagnosis and medical research. The human platelet alloantigens (HPAs) represent SNPs in platelet-specific glycoproteins, and HPA-1, -2, -3, and -5 are the most relevant in immunohematology. In most protocols, the respective HPA-SNPs are analyzed in allele-specific reactions, each with at least 100 ng DNA. In many cases, prenatal HPA typing in the diagnosis of neonatal alloimmune thrombocytopenia is often limited by the restricted amounts of fetal DNA that are obtainable. We developed a novel PCR-SSP technique to achieve accurate HPA genotypes using only 1 ng DNA per reaction. The concentration of HPA-specific primers was increased to 1 μ M each and exhibited a higher sensitivity compared to a commercial PCR-SSP kit. The modified PCR-SSP technique enabled the identification of fetal HPA genotypes using only 0.5 mL amniotic fluid (from week 16 of gestation) and from a maternal plasma sample (from week 38 of gestation). The principle of the modified PCR-SSP technique may also be applied for the genotyping of other SNPs from limited amounts of DNA.

INTRODUCTION

Genotyping for single nucleotide polymorphisms (SNPs) is relevant to various research fields and in routine diagnosis. Today, allele-specific PCR amplification techniques using sequence-specific primers (PCR-SSP) are widely employed (1,2). For the typing of an SNP, two PCRs are needed, each containing an allele-specific primer and a common primer. After PCR and agarose gel electrophoresis, the genotyping result is evaluated by the presence or absence of an allele-specific PCR product. Compared to other techniques, PCR-SSP is rather DNA consuming because at least 100 ng genomic DNA are needed for each reaction. Other typing methods, such as PCR with allele-specific oligonucleotide (PCR-ASO) hybridization or with oligonucleotide ligation assay (PCR-OLA), can be carried out with much less DNA because the target sequences are amplified in a single multiplex PCR. However, these methods are more time-consuming, and addi-

tional reagents and laboratory equipment are needed.

Alloantigens primarily represent SNPs in the genes that encode immunogenic proteins. The status of alloantigens, such as human leukocyte antigens (HLAs), blood group antigens, or human platelet alloantigens (HPAs), is frequently investigated before organ transplantation or blood transfusion. Alloreactive antibodies against HPA may cause thrombocytopenic syndromes such as neonatal alloimmune thrombocytopenia (NAIT) and post-transfusion purpura (PTP). The most relevant antigens in Caucasian populations are HPA-1, -2, -3, and -5 (3). In a clinical environment, the rapid and accurate typing for the HPAs of patients and platelet donors is mandatory for patients supplied with HPA-matched platelet products. In the case of NAIT, typing the father provides important information on the risk of future pregnancies. Various PCR-based techniques have been described for the genotyping of the HPA-1, -2, -3, and -5 SNPs (2), and PCR-SSP is most commonly used

(4–7). Clinical samples such as whole blood samples from patients with low leukocyte counts or amniotic fluid from early weeks of gestation often contain limited amounts of DNA, and PCR-SSP typing does not provide reliable results.

Here we report a modified PCR-SSP technique suitable for the genotyping of SNPs from samples with low DNA content. We used the HPA-1, -2, -3, and -5 SNPs as a model. The optimal range of DNA amount per reaction was estimated by investigating dilution series of DNA.

MATERIALS AND METHODS

DNA Isolation from Reference Blood Samples and Clinical Samples

Blood samples from four individuals that had been previously HPA genotyped by sequencing of the SNP loci, representing the different allelic combinations, served as a reference. For the isolation of genomic DNA from 200 μ L whole blood, we used the QIAamp[®] DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's standard protocol. The DNA concentration was estimated from the absorbance at 260 nm using a UV-spectrophotometer (GeneQuant[™] pro; Amersham Biosciences, Freiburg, Germany). From the reference samples, we obtained 20–60 ng DNA/ μ L whole blood.

Plasma, amniotic fluid (especially obtained in early weeks of gestation), or buccal swabs represent clinical samples with potentially low DNA content. For this investigation, the clinical samples were provided by the Department of Obstetrics and Gynecology, University of Heidelberg, Faculty of Clinical Medicine Mannheim, after written consent. Amniotic fluid (0.5 mL) samples were taken from two women in week 16 of gestation. The amniotic cells were centrifuged for 5 min at 3000 $\times$ g and resuspended in 200 μ L phosphate-buffered saline (PBS) buffer. For DNA isolation, the same procedure as used for whole blood samples was carried out, except that we used only 50 μ L elution buffer to recover the DNA from the spin columns. The absorbance at 260 nm revealed DNA concentrations of approxi-

Table 1. Primer Sequences and Concentrations Used in PCR-SSP Genotyping for HPA-1, -2, -3, and -5

Primer Code	Primer Sequence	Primer Concentration (μM)	Product Size (bp)
HPA-1a	5'-GACTTACAGGCCCTGCCTCT-3'	1	190
HPA-1b	5'-ACTTACAGGCCCTGCCTCC-3'	1	189
HPA-1rev ^a	5'-GTGCAATCCTCTGGGGAC-3'	1	
HPA-2a	5'-CCCCCAGGGCTCCTGAC-3'	1	241
HPA-2b	5'-CCCCCAGGGCTCCTGAT-3'	1	241
HPA-2rev	5'-GCCAGCGACGAAAATAGAGG-3'	1	
HPA-3a	5'-GGACTGGGGGCTGCCCAT-3'	1	214
HPA-3b	5'-TGGACTGGGGGCTGCCCAG-3'	1	215
HPA-3rev	5'-ACAGCACACCCGGACCCC-3'	1	
HPA-5a	5'-GAAGGAAGAGTCTACCTGTTTACTATCAAAG-3'	1	258
HPA-5b	5'-AAGGAAGAGTCTACCTGTTTACTATCAAAA-3'	1	257
HPA-5rev	5'-CTCTCATGGAATGGCAGTA-3'	1	
β-glob-F ^b	5'-GGTTGGCCAATCTACTCCCAGG-3'	0.1	536
β-glob-R	5'-GCTCACTCAGTGTGGCAAAG-3'	0.1	

^aThe same reverse primers were used in both allele-specific PCRs.
^bPart of the human β-globin gene was used as an internal control.

mately 2 ng/μL in both samples. Approximately 1 mL plasma was obtained from the EDTA blood samples of four females before cesarean section. The standard protocol for the DNA isolation was adapted to a 1-mL sample volume, and DNA elution was performed in 50 μL buffer. The absorbance at 260 nm revealed DNA concentrations up to 4 ng/μL. DNA was also isolated from maternal leukocytes and corresponding umbilical cord blood samples as a source of fetal DNA.

Additionally, buccal swab samples of eight volunteer donors represented a third source with limited amounts of DNA. A standard protocol for the DNA isolation from buccal swabs was provided by the manufacturer of the DNA isolation kit and performed without modifications. The absorbance at 260 nm of the DNA solutions revealed amounts of 300–600 ng total DNA at concentrations of 2–4 ng/μL.

PCR-SSP Genotyping for HPA-1, -2, -3, and -5

Reactions of the modified PCR-SSP technique were carried out in a total volume of 10 μL. To reduce error rates caused by multiple pipetting steps, we prepared eight primer mixtures specific for the typing of the HPA-1ab, -2ab,

-3ab, and -5ab alleles. Each primer mixture contained 2 μM each of the allele-specific forward and common reverse primer and 0.2 μM each of the internal control (β-globin gene) primers (Table 1). Synthetic oligonucleotide primers were synthesized by MWG-Biotech AG (Ebersberg, Germany). Other PCR reagents were prepared as 10× concentrates: (i) 10× PCR buffer contained 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, and 0.1% bovine serum albumin (BSA); (ii) 10× dNTPs contained 2 mM each deoxynucleotide; (iii) 10× gel loading buffer contained 50% glycerol and 1 mg/mL cresol red. A ready-to-use reaction mixture was prepared by mixing equal volumes of each of the three 10× reagents and was named the red buffer mixture (RBX). Aliquots of the RBX could be stored at -20°C for at least 1 year without affecting the quality of the PCR results. The setup of the reactions was performed according to the protocol of a commercial PCR-SSP kit (HPA-SSP; Innotraining, Kronberg, Germany). The primer mixtures were dispensed in 5 μL into the 0.2 mL PCR tubes (8-tube strips or 96-well plates were used). For each sample, a PCR master mixture was prepared that contained 18 μL DNA solution, 26.3 μL RBX, and 0.7 μL *Taq* DNA polymerase (5 U/μL; Qiagen). Five microliters of

master mixture were dispensed into each primer mixture. Thus, each of the eight reactions contained 2 μ L DNA solution, 1 μ M each of the HPA-specific primers, 0.1 μ M each of the internal control primers, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.01% BSA, 5% glycerol, 0.1 mg/mL cresol red, and 0.4 U *Taq* DNA polymerase.

To compare the results, all DNA samples were HPA typed in parallel using the HPA-SSP kit. The kit's primer mixtures contained 0.2 μ M each of HPA-specific primer and 0.03 μ M each of the control primers (human growth hormone locus, 434 bp). The PCR was carried out according to the manufacturer's protocol with no modifications. Our modified PCR-SSP was performed with the following cycling program: a 2-min initial denaturation at 95°C, followed by 10 cycles of 15 s denaturation at 95°C and 1 min annealing/extension at 66°C, followed by 20 cycles of 15 s denaturation at 95°C, 1 min annealing at 62°C, and 30 s extension at 72°C. The amplification products were separated on 2% agarose gels containing 0.5 ng/mL ethidium bromide in a rapid agarose gel electrophoresis (RAGE®; Cascade Biologics, Portland, OR, USA) chamber for 5 min at 25 V/cm. The results were obtained by a visual inspection of the gels and documented using a UV documentation device with charge-coupled device (CCD) camera (UVP, Upland, CA, USA).

RESULTS AND DISCUSSION

Validation of the PCR-SSP Method

First-line validation was carried out by genotyping four reference samples that represented the three genotype variants for each SNP. Our modified PCR-SSP technique confirmed the genotypes of the reference samples (i.e., DNA1, HPA-1bb 2ab 3ab 5aa; DNA2, HPA-1aa 2aa 3aa 5aa; DNA3, HPA-1aa 2aa 3aa 5bb; and DNA4, HPA-1aa 2bb 3ab 5ab) (Figure 1). An additional amplification product of unknown origin was consistently found in the HPA-3b reaction. Because of its size, the PCR product could be easily distinguished from the internal control and the allele-specific PCR products.

The validation experiments were performed with 20 ng genomic DNA per PCR; thus, 160 ng DNA were needed for complete HPA genotyping. Additional validation of the technique could be achieved by the retyping of 92 blood donor samples that had been genotyped previously for HPA-1, -2, -3, and -5 using a commercial PCR-SSP kit. The modified PCR-SSP technique confirmed the HPA genotypes in all 92 samples (data not shown).

The primer sequences used in our modified PCR-SSP technique are similar to those supplied with the commercial kit and published previously (4–7), with slight modifications. The most significant difference was the increased primer concentrations (1 μ M each HPA-specific oligonucleotides) compared to the commercial kit (0.2 μ M) and to published protocols (4,6,7).

Estimation of the Optimal Range of DNA Concentration

The amount of template DNA is one of the most important parameters defining the specificity of the PCR-SSP techniques. Most of the published protocols suggest the use of 50–250 ng DNA per PCR (reviewed in Reference

2). To estimate the optimal range of DNA concentration for the modified PCR-SSP technique, we tested a linear dilution series of the four reference DNA samples with 125, 25, 5, 1, 0.2, and 0.04 ng per reaction. Unambiguous results could be obtained using 1–25 ng DNA per reaction (Figure 2). Higher amounts of DNA led to weak, unspecific amplification in the PCRs that was thought to be negative. Below 1 ng DNA per PCR, the DNA bands on the agarose gel were too weak for reliable reading of the typing results. With the use of a commercial PCR-SSP system, accurate typing results could only be achieved when 25 ng or more DNA were amplified per reaction (Figure 2). This was in accordance with the manufacturer's suggestion of 100 ng DNA per reaction.

Approximately 1 ng DNA per reaction was sufficient for accurate typing. Thus, a total of less than 10 ng DNA is required for PCR-SSP typing for HPA-1, -2, -3, and -5, which is comparable to other techniques such as PCR-ASO or PCR-OLA (reviewed in Reference 2). To use the modified protocol in routine HPA genotyping, the concentration of the DNA solutions should be approximately 10 ng/ μ L.

Figure 1. Validation of the PCR-SSP technique by typing four reference DNA samples. Genotyping results were as expected: HPA-1bb -2ab -3bb -5aa for DNA1; HPA-1ab -2aa -3aa -5ab for DNA2; HPA-1aa -2aa -3aa -5bb for DNA3; and HPA-1bb -2bb -3ab and -5ab for DNA4. The amplification product (536 bp) of the internal control is marked by an arrowhead. Twenty nanograms of template DNA were used per reaction.

Figure 2. Titration of the optimal range of template DNA concentration. A representative result from the genotyping of reference DNA3 using the modified (A) and the commercial PCR-SSP technique (B) is shown with given amounts of DNA per reaction. Approximately 1 ng DNA per reaction was sufficient in the modified PCR-SSP technique, whereas at least 25 ng DNA per reaction were needed for the commercial PCR-SSP technique.

Figure 3. HPA genotyping of clinical samples with low DNA content. The representative results were obtained from samples with DNA concentrations of approximately 2 ng/ μ L. (A) Fetal HPA genotypes could be identified from less than 0.5 mL amniotic fluid taken in week 16 of gestation only when the modified PCR-SSP technique was applied. (B) In one of the four maternal plasma samples, a mixed maternal/fetal HPA genotype was detectable. The fetal HPA-3a-specific PCR product is marked by a white arrow. (C) Unambiguous results could be obtained from buccal swab samples only using the modified PCR-SSP system.

Testing Clinical Samples with Low DNA Content

Prenatal HPA typing for the diagnosis of NAIT is an important clinical application. Amniotic fluid represents a common source for fetal cells and is widely used for prenatal genetic testing (8). The PCR-SSP and PCR-ASO techniques have been applied for SNP typing without amniocyte culture (9–11). Especially in the early weeks of gestation, the amount of fetal DNA that can be extracted from amniotic fluid is low because of the limited volume of amniotic fluid and the limited number of fetal cells in the fluid sample. We investigated whether the modified PCR-SSP technique is suitable to obtain reliable typing results from amniotic fluid at the early weeks of gestation. DNA was extracted from two amniotic fluid samples that were both from week 16 of gestation. Accurate results that represented the fetal genotypes could be obtained from both samples (Figure 3A). The genotyping of the DNA samples using the commercial PCR-SSP system failed (data not shown).

Another source of fetal DNA for genetic testing is maternal plasma that had been used in prenatal genotyping for Rhesus antigens (12,13). We investigated four samples of maternal plasma from weeks 38–40 of gestation. Previous genotyping of maternal leukocyte DNA and umbilical cord blood samples revealed fetal heterozygosity for one of the HPA-SNPs, whereas the maternal genotype was homozygous. Using the modified PCR-SSP technique, we could obtain a typing result from three of the four maternal plasma samples. In one plasma sample, no detectable amounts of DNA were found, and PCR-SSP typing failed. Only in one of the three cases where genotyping was successful could the fetus-specific allele be identified (Figure 3B). The commercial PCR-SSP kit failed to determine the genotypes from the plasma samples (data not shown). We conclude that the amount of DNA in plasma is individually different. Additionally, the maternal-to-fetal DNA ratio is variable and may be insufficient for prenatal SNP typing.

For a third example of samples with low DNA content, we investigated

buccal swabs from eight healthy individuals. In forensic investigations, buccal swabs are often the source of DNA (14) and, usually, short tandem repeat (STR) polymorphisms are analyzed after PCR amplification. The genotyping of anonymous SNPs is regarded as an alternative method in the determination of individual identity (15). All eight samples revealed total amounts of DNA in the range of 300–600 ng, with concentrations of 2–4 ng/ μ L. We could achieve typing results only when we used our modified PCR-SSP technique and not the commercial PCR-SSP technique (Figure 3C). The HPA genotypes previously determined from the blood samples of these individuals were confirmed.

In general, 1 μ M allele-specific primer is suitable to increase the sensitivity of PCR-SSP techniques. We could demonstrate that approximately 1 ng DNA per allele-specific reaction was sufficient and, thus, 2 ng DNA were needed to obtain the genotype of a SNP. Applying our modified PCR-SSP technique, the genotype of 50 SNPs may be achieved from only 100 ng DNA template.

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