TECHNICAL NOTE

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Efficient and Reliable PCR-Based Detection of the ABO Blood Group Alleles: Genotyping on Stamps and Other Biological Evidence Samples

ABSTRACT: PCR-based ABO genotyping was established using restriction enzyme digestion followed by horizontal polyacrylamide gel electrophoresis and silver staining. The method described here is fast, with results obtained within hours, not days, it obviates the need for radioisotopes and can be performed with 1–2 ng of extracted genomic DNA. ABO blood group determination was successful in various types of biological materials of forensic interest such as bloodstains, vaginal swabs, cigarette butts, and hair roots. Moreover, after preincubation in distilled water, DNA (2–8 ng) was extracted from 12 up to 10-years-old stamps and was correctly typed at the ABO locus. The results presented here indicate that the PCR-based ABO genotyping is a fast, sensitive, reliable, and economic method providing blood group determination in DNA from a variety of different types of specimens. It can provide determination from specimens of limited amount and/or with partially degraded DNA as well. Therefore, it is very useful for first-step suspect screening as well as in forensic research for the analysis of biological evidence.

KEYWORDS: forensic science, DNA, DNA typing, polymerase chain reaction, ABO blood group, stamps

The ABO blood group has been widely applied as a human genetic marker system in transfusion, paternity testing, anthropologic investigation and forensic identification. All the traditional methods in determining ABO types are designed to detect antibody or antigen materials. Because antigen substances are glycolipids or glycoproteins, there are several problems in discovering their activities that can affect the tests, such as, survival of antigen activity, contamination of microorganisms (1), nonspecific absorption etc. (2). The analysis of DNA, the much more stable genetic material within every nucleated cell, provides a valuable and powerful tool for overcoming the limitations of antigen detection. Thus, the cloning and characterization of alleles of genes encoding

classical serological markers make DNA-based genotyping possible, and the development of the polymerase chain reaction (PCR) for amplifying specific DNA fragments (3,4) enables the analysis of minute amounts and/or partially degraded low-molecular-weight DNA.

The gene encoding the ABO blood group glycosyltransferase, which catalyzes the transfer of *N*-acetylgalactosamine (group A) or galactose (group B) residues to the H-antigen, was recently cloned and sequenced (5). The A and B alleles differ by several base substitutions, whereas the O allele contains a single base deletion causing a reading frameshift that leads to an inactive gene product which is unable to modify the H-antigen. The identification of DNA sequence differences between the A, B and O alleles has made direct genotyping feasible, but the procedures described so far, requiring 50–100 ng template DNA (6,7), amplification times of 4 to 1.5 hours (6,7), gel electrophoresis of 3–19 hours (8,9) and labeled probes (8), are cumbersome and time consuming and not very sensitive.

We have therefore tried to define appropriate conditions allowing rapid and sensitive direct genotype determination, testing of minute amounts of DNA, and excellent separation and visualization on polyacrylamide gels. In this report we design a protocol for ABO genotyping on DNA isolated from various types of biological materials of forensic-science interest.

Materials and Methods

DNA Samples

DNA was extracted from 30 EDTA-blood samples obtained from random individuals, 20 blood stains, 12 sexual assault samples, 15 cigarette butts, 6 hair roots, as well as from 12 stamps affixed to postcards 1 month to 10 years ago by 12 known persons. The ABO phenotypes of these individuals and of the EDTA-blood samples as well as of 10 suspects and 4 victims of forensic cases and three additional control persons were previously determined by classical serology.

DNA Extraction

DNA was isolated from the blood samples, the blood stains, the cigarette butts, and the hair roots following proteinase K digestion and extraction by the phenol-chloroform method (10). A differential lysis procedure was used for the sexual assault samples, in

which epithelial cells were preferentially lyzed in a digestion buffer containing sodium dodecyl sulfate (SDS) and proteinase K (11). The sperm, which are resistant to digestion in the absence of reducing agent, were pelleted by centrifugation, three times washed in phosphate buffered saline (PBS) and resuspended in digestion buffer containing dithiothreitol (DTT) and proteinase K.

Material from the stamps was extracted by cutting a 1.0 cm^2 section into small pieces and incubating them for 5-h at 4°C in 1 mL of sterile H_2O. The shreds were then discarded and the tubes were centrifuged for 5 min at 13,000 × g. The supernatant was removed and the pellet was used for the DNA extraction by the phenol-chloroform method.

The extracted DNA was then ethanol precipitated, washed in 70% ethanol and finally redissolved in sterile distilled water. The DNA quantity and quality were assessed by ultraviolet visualization of ethidium-bromide-stained agarose minigels. In the case of very small samples, DNA was quantified by a slot blot hybridization procedure (12).

DNA Amplification

PCR amplification of 1–10 ng of genomic DNA was performed using a 9600 Perkin Elmer thermal cycler in a final volume of 50 μL, containing 10 mM Tris-HCl, pH 8.4, 50 mM potassium chloride (KCl), 1.5 mM magnesium chloride ($MgCl_2$), 200 μM each dNTP, 0.1% Triton X-100, 2 μL BSA (4-μg/μL), 2.5 units (U) of Taq polymerase, and 15 pmol of each primer.

Two DNA fragments of the ABO locus were amplified by the following two pairs of primers (6) that were synthesized on a abi DNA synthesizer (Microsynth) and purified on a 7 M urea polyacrylamide gel 20%:

primer 1 (forward): 5'- CACCGTGGAAGGATGTCCTC-3'
primer 2 (reverse): 5'- AATGTCCACAGTCACTCGCC-3'
primer 3 (forward): 5'- TGGAGATCCTGACTCCGCTG-3'
primer 4 (reverse): 5'- GTAGAAATCGCCCTCGTCCTT-3'

After 3 PCR cycles (94°C for 1 min, 58°C for 1 min, 72°C for 1 min) had been performed for primer 1 + 2 and primer 3 + 4 respectively, 28 additional cycles at 94°C for 25 s, 58°C for 20 s, 72°C for 45 s followed. Postamplification gels for the ABO fragments were run in order to check if the amplification was successful.

The amplification products of primer 1 + 2 were digested with 5 units of Kpn I and the products of primer 3 + 4 were cut with 5 units of Alu I. The digestion was performed at 37°C for 60 min.

A reaction mixture without DNA was used to detect contamination (negative control), whereas a DNA sample containing the blood group alleles B and 0 was used as a positive control.

Horizontal Gel Electrophoresis

The amplification products were separated on polyacrylamide gels ($T = 15\%$, $C = 3.1\%$) onto GelBond (FMC, Rockland, ME). The mix for one gel of 220 × 120 × 0.5 mm was: 2.5 g acrylamide, 0.08 g piperazine diacrylamide (PDA), 6.0 mL H_2O, 11 mL Tris-formate buffer (0.12 M, pH 9.0), 200 μL ammonium persulfate (APS) 10%, and 20 μL TEMED. The trailing ion, contained in soaked Whatman paper strips, was Tris-borate buffer (pH 9.0). Bromphenol-blue was added to the electrode buffer to serve as a dye marker for the discontinuous buffer boundary. The electrophoretic setup was 600 V, 20 mA, 20 W, 15°C for 1.5 h. We routinely loaded 2 μL of the digestion product as well as 2 μL of the size

marker V (Boehringer Mannheim) presenting with 22 defined fragments (587 to 8 bp).

Visualization of the DNA fragments was performed within 30 min by the silver staining method described by Budowle et al. (13).

Results

Yamamoto et al. (5) found that there are four nucleotide substitutions differentiating the A and B alleles (positions 523, 700, 793, 800) and that the A and O alleles are identical except for a single nucleotide deletion in the O allele at position 258, which is responsible for the lack of transferase activity by the O allele. To detect all possible ABO genotypes (OO, AO, AA, AB, BO, BB), the amplification of two DNA fragments including the critical nucleotide positions 258 and 700 is necessary. Primer 1 + 2 amplify a 200 bp fragment containing nucleotide 258 (G). Deletion of the 258th nucleotide results in a O allele specific 199 bp DNA fragment creating at the same time a Kpn I cleavable site (GGTACC). Digestion with Kpn I produces 171- and 28-bp (not visible) fragments in case of OO homozygosity (see Fig. 1, lane 7). The presence of 200-, 171- and 28-bp fragments demonstrates heterozygosity for the O allele and recognizes genotype AO and BO (see Fig. 1, lane 6). The detection of undigested 200-bp fragments proves the O allele to be missing indicating the presence of genotype AA, AB or BB (see Fig. 1, lane 8). The amplification product of primer 3 + 4 is a 128-bp fragment containing nucleotide 700 of the ABO locus. The base substitution G → A at position 700 is B allele specific and creates an Alu I restriction site (AGCT). The enzyme cuts the B allele into two fragments of 88 and 40 bp. Therefore, genotype BB is defined by the demonstration of 88- and 40-bp (not visible) fragments, genotypes BO and AB are characterized by the presence of 128-, 88- and 40-bp fragments (see Fig. 1, lane 2) and genotypes OO, AO and AA show only 128-bp fragments (see Fig. 1, lane 3, 4).

The appropriate conditions allowing amplification and digestion of the two specific ABO-DNA sequences were initially determined
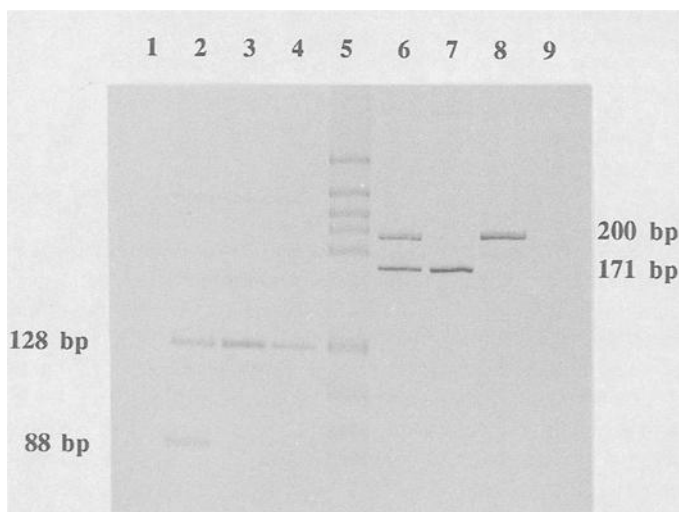

FIG. 1—Silver-stained polyacrylamide gel demonstrating ABO genotypes of DNA extracted from stamps. Lane 1 and 9: Negative control (reaction mixture without DNA). Lane 5: Size marker V (Boehringer) presenting DNA fragments of 267, 234, 213, 192, 184, 124, 104, and 89 bp. Lanes 1 to 4: AluI-digested amplification products of primer 3 + 4. Lanes 6 to 8: KpnI-digested amplification products of primer 1 + 2. Lane 2 and 6: Stamp number 1 with genotype BO (nine-year-old DNA). Lane 3 and 7: Stamp number 2 with genotype OO (ten-year-old DNA). Lane 4 and 8: Stamp number 3 with genotype AA (seven-year-old DNA).

TABLE 1—*ABO analysis of forensic casework samples and three control persons.*

Sample		Material (1–5 ng of DNA)		Geno* type	Blood† group
Case 1: Raid	Q1–Q5	Bloodstains from knife		AO	
	Victim	EDTA-blood		AO	
Case 2: Murder	Q1–Q3	Bloodstains from clothes		OO	
	Q4, Q5	Bloodstains from shoes		OO	
	Victim	EDTA-blood		OO	
Case 3: Burglary	Q1, Q2	Bloodstains from glass		AO	
	Suspect	EDTA-blood		AO	A
Case 4: Burglary	Q1–Q3	Bloodstains from paper		BO	
	Suspect	EDTA-blood		BO	B
Case 5: Murder	Q1, Q2	Bloodstains from gloves		AO	
	Q3	Hair		AO	
	Q4	Hair		OO	
	Q5	Hair		BO	
	Q6	Cigarette butt		OO	
	Victim	EDTA-blood		AO	A
Case 6: Murder	Q1–Q3	Bloodstains from towels & carpet		AO	
	Victim	EDTA-blood		AO	A
Case 7: Rape	Q1, Q2	Semen stains from linen		AO	
	Q6–Q8	Vaginal swabs	Female fraction	OO	
			Male fraction	AO	
	Victim	EDTA-blood		OO	O
	Suspect	EDTA-blood		AO	A
Case 8: Rape	Q1–Q3	Vaginal swabs	Female fraction	AB	
			Male fraction	AO	
	Q4–Q7	Cigarette butts		AO	
	Victim	EDTA-blood		AB	AB
	Suspect 1	EDTA-blood		AO	A
	Suspect 2	EDTA-blood		OO	O
Case 9: Rape	Q1, Q2	Semen stains from clothes		AO	
	Q3, Q4	Semen stains from towels		AO	
	Suspect	EDTA-blood		AO	
Case 10: Raid	Q1–Q4	Cigarette butts		BO	
	Q5–Q8	Cigarette butts		AO	
	Suspect 1	EDTA-blood		AO	A
	Suspect 2	EDTA-blood		OO	O
	Suspect 3	EDTA-blood		OO	O
	Suspect 4	EDTA-blood		BO	B
Case 11: Burglary	Q1–Q3	Cigarette butts		AO	
	Suspect	EDTA-blood		AO	A
Control person 1		Hair		AO	A
		Cigarette butt		AO	
Control person 2		Hair		OO	O
		Cigarette butt		OO	
Control person 3		Hair		BO	B
		Cigarette butt		BO	

*PCR-based ABO genotyping.
†Serological blood group analysis.

using DNA from whole blood samples taken from known individuals. The final conditions, described in Materials and Methods, permitted the amplification of two distinct DNA fragments of 200 and 128 bp. The sensitivity of the amplification systems was tested, and the experiment showed that as little as 0.1 ng of template DNA can be amplified and restricted and the resultant fragments can be genotyped using primer 1 + 2 (see Fig. 2, lane 7, 8, 9). The amplification products resulting from primer 3 + 4 were clearly detectable using 10 and 1 ng of template DNA (see Fig. 2, lane 2, 3). The ABO genotyping was successful in each of the 30 control DNA samples and corresponded to the known blood group of the donors.

The DNAs extracted from a variety of forensic samples including bloodstains, vaginal swabs, cigarette butts, and hair roots were also successfully amplified and ABO genotyped (see Table 1). The results of ABO genotype of the 12 stamps are summarized in Table 2 and the restriction enzyme digested amplification products of stamp 1, 2 and 3 are presented in Fig. 1.

To ensure that no contamination by external DNA or PCR products had occurred during the preparation of the samples and the PCR reagents, a negative control was included in the cycling of the PCR. All contamination controls were negative as judged by electrophoresis and genotyping. However, non-specific faint bands are visible in the DNA-containing PCR reactions as a consequence of the sensitivity of the detection method. Non-specific bands can occur due to heteroduplex formations and/or primer annealing to other loci that are partially (but not 100%) complementary to the primer sequence. As the ABO alleles are of defined sizes and as the nonspecific bands are constantly larger and apparently minor they can be neglected and do not interfere with the interpretation of the ABO system.

Discussion

The cloning of ABO and the identification of DNA sequence differences between the A, B and O alleles provides a means for
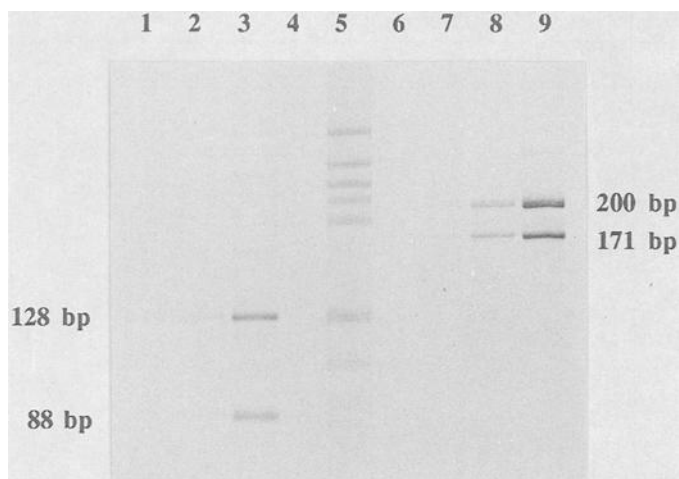
FIG. 2—*Sensitivity experiment using a dilution series of template DNAs presenting with the ABO genotype BO. Lane 4 and 6: negative control (reaction mixture without DNA). Lane 5: size marker V (Boehringer) presenting DNA fragments of 267, 234, 213, 192, 184, 124, 104, and 89 bp. Lanes 1 to 4: AluI-digested amplification products of primer 3 + 4. Lanes 6 to 9: KpnI-digested amplification products of primer 1 + 2. Lane 1 and 7: amplification products resulting from 0.1 ng of template DNA. Lane 2 and 8: amplification products resulting from 1 ng of template DNA. Lane 3 and 9: amplification products resulting from 10 ng of template DNA.*

TABLE 2—*ABO genotypes determined in DNA extracted from stamps.*

Stamp number	Canceled	Geno* type	Blood† group
1	February 1984	BO	B
2	October 1983	OO	O
3	August 1986	AA	A
4	February 1983	OO	O
5	September 1984	AO	A
6	February 1986	AO	A
7	September 1986	AO	A
8	December 1984	BO	B
9	October 1983	OO	O
10	April 1993	OO	O
11	April 1993	AO	A
12	October 1991	AO	A

*PCR-based ABO genotyping.
†Serological blood group analysis.

direct PCR-based genotyping. ABO genotyping is of course already extremely valuable in linkage analysis and gene mapping on human 9q34. Previously uninformative families can now be scored and the AO and BO heterozygotes can easily be distinguished from AA and BB homozygotes. Applications in the forensic field are important concerning paternity testing and criminal investigation because of the ease of admissibility of ABO-typing in the courtroom and the ability for the average juror to understand the ABO-typing system.

We have described a further alternative to classic serological ABO typing on forensic samples using PCR. In our study, 30 control sample have been analyzed. The results of this DNA-based typing were consistent with the ABO blood groups obtained by serotyping demonstrating that the PCR and electrophoresis conditions as well as the non-radioactive staining method described here, allow rapid, specific and sensitive ABO genotyping using not more than 1–2 ng of template DNA. Most of the more recently developed methods by other authors (6–9) are time consuming, need up to 100 ng of template DNA and use radioactive labeling of the amplification products. Ugozzoli and Wallace (8) and Crouse and Vincek (14) described also rapid (within hours) ABO genotyping protocols using, however, 50–100 ng of template DNA and ^{32}P-labeling (8) on the one side and 2 ng of template DNA and ethidium bromide staining (14) on the other side. The advantages of our conditions over the others previously reported are the increase of sensitivity (typing of even less than 1 ng of template DNA) using polyacrylamide gel separation and the non-invasive silver staining of the amplification products.

DNA isolated from bloodstains, body fluid stains, postmortem tissues, and other specimens of forensic science importance often contains not only a significant quantity of DNA from microbial and possibly other sources, but also degraded DNA. Our positive amplification and typing results of 20 blood stains, 12 sexual assault samples, 15 cigarette butts, and 6 hair roots prove the ABO-PCR test to be not only very sensitive but also very successful in amplifying degraded DNA. In rape cases, this method can, in opposition to the antigen test, correctly determine the blood group of the rapist. Moreover, the genotypes obtained from 12 stamps were identical to the blood group of the senders who attached the stamps. Enough DNA (2–8 ng) for more than one analysis could be extracted from the stamps after preincubation in water and following phenol-chloroform extraction. The correct genotyping of the stamps show that it is possible to identify the ABO blood group of up to ten-year-old DNA from saliva. Thus, in forensic cases involving mail bombs, extortion, kidnapping or threatening letters, biological evidence such as the saliva used to attach the stamp and seal the envelope can be analyzed at the ABO locus.

In conclusion, our studies indicate that the PCR-based ABO genotyping is a fast, sensitive, and economic method providing blood group determination in DNA from a variety of different types of specimens as well as from specimens of limited amount, thus, being very useful in forensic casework for first-step suspect screening as well as for the analysis of biological evidence.

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