TECHNICAL NOTE

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A PCR-Based Strategy for ABO Genotype Determination

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ABSTRACT: The ABO blood group system has been widely used in forensic serology. Several techniques have been developed which detect ABH antigens. To overcome the problems associated with conventional methods such as bacterial contamination, extreme environmental conditions, antigen activity, non-secretor issues, and non-specific absorption, several new strategies have been employed to detect ABO genotypes by PCR. We have developed improved amplimers for the glycosyl transferase locus on chromosome 9 and examined the suitability of PCR-based ABO genotyping for forensic identification. We show that the ABO system is primate specific and that DNA extracted from various tissues commonly encountered in criminal cases can be quickly and reliably typed by ABO-PCR. The results indicate that ABO genotyping by PCR and restriction enzyme digestion of the amplified product is a useful procedure for forensic analysis that can provide additional discriminating power compared to conventional immunological methods.

KEYWORDS: forensic science, criminalistics, DNA, PCR, ABO, ABO system. ABO blood groups, forensic DNA typing

The ABO blood group system, the first human genetic marker system characterized (1), has been a valuable tool in transfusion medicine, physical anthropology, disputed parentage testing, human identification, and in the forensic analysis of blood and physiological fluids in criminal investigation. Although the ABO system was described in terms of immunological properties at the turn of the century, the molecular basis for the polymorphism has only recently been characterized (2,3). A variety of serological techniques has been developed and employed for detection of the ABH antigens in human tissue, but there are a number of problems associated with ABO typing in specimens typically examined in forensic science laboratories (4,5). Typing strategies exploiting the ABO gene rather than its gene products should overcome many of these problems. Moreover, ABO typing at the DNA level yields genotypic rather than phenotypic information, thereby increasing the system's discrimination potential. A new DNA analysis strategy for amplifying regions of the ABO locus and detecting ABO genotypes was recently described (6). We have developed improved amplimers for the locus and examined the suitability of PCR-based ABO genotyping for forensic casework applications.

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Materials and Methods

DNA was extracted and purified from specimens typically or potentially encountered in forensic science laboratories including blood, sperm, hair root cells, bone, skin, intestine, and oral plus vaginal swabs, according to our standard laboratory protocols and TWGDAM recommended guidelines (proteinase K digestion at 56°C, phenol-chloroform extraction, and filtration-concentration through Microcon-100 microconcentrators (Amicon, Beverly MA). ABO phenotypes (determined by conventional serological methods) were known for all samples assayed. Samples were subjected to various environmental conditions (including −20°C, dry plus humid room temperature, and sea water immersion) prior to DNA extraction in order to examine the utility of the ABO-DNA typing procedure.

The ABO genotyping protocol requires two separate amplification reactions followed by digestion with diagnostic restriction enzymes, *Kpn* I and *Alu* I (see Fig. 1*B*). Primers 1 and 2 generate a 210/209 bp product (*Kpn* I digest); primers 3 and 4 generate a 175 bp product (*Alu* I digest). The digestion products were visualized by UV transillumination of ethidium bromide-stained 2.0% Metaphor gels following electrophoresis in 1X TBE for 380 Vh. Amplification reactions were performed in 50 μL final volumes in reaction mixtures containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.01% gelatin, 1.25 U Taq polymerase and 15 pmoles of each primer, in Model 480 or 9600 thermal cyclers (Perkin Elmer, Norwalk CT). The amplification parameters for both models are as follows: 95°C pre-denaturation for 2 m followed by 94°C (30 s), 60°C (2.0 m), 72°C (3.0 m), for 35 cycles.

Results and Discussion

Recently, sequence analysis of the ABO locus has revealed nucleotide-specific polymorphisms that fall within restriction endonuclease recognition sites (2,3). These single base changes provide a basis for PCR-based ABO genotyping, and theoretically

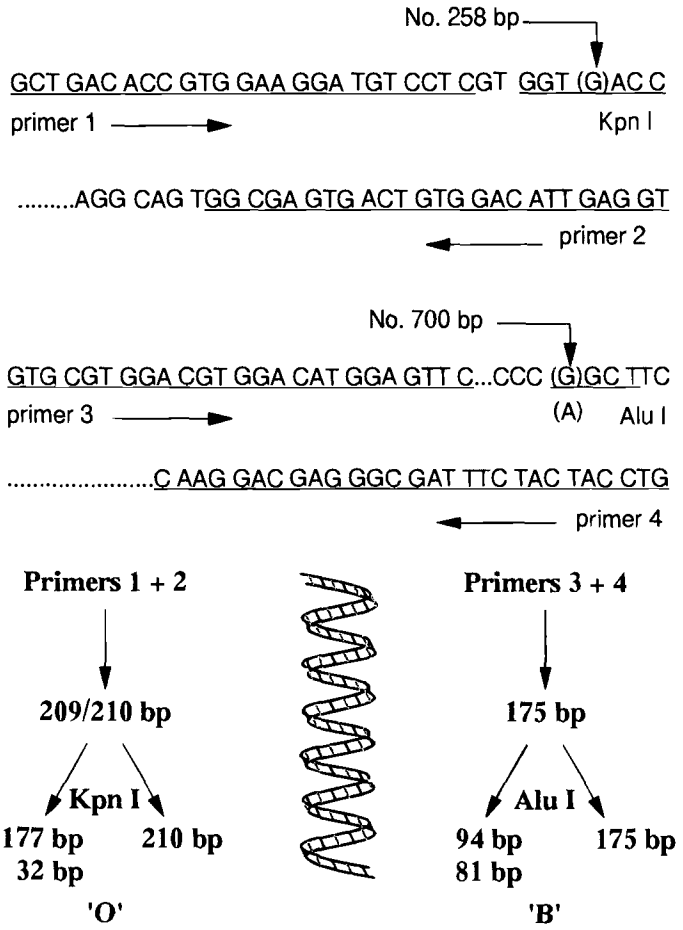

FIG. 1*A*—*A partial sequence of the ABO glycosyl transferase locus ('A' allele). Nucleotide 258 is deleted in the 'O' allele. The B allele is identified by the G to A transition at position 700. The four primers are underlined and the direction of extension is indicated by the arrows. Primer 1 (forward): 5'− GCT GAC ACC GTG GAA GGA TGT CCT C −3'. Primer 2 (reverse): 5'− ACC TCA ATG TCC ACA GTC ACT CGC C −3'. Primer 3 (forward): 5'− GTG CGT GGA CGT GGA CAT GGA GTT C −3'. Primer 4 (reverse): 5'− CAG GTA GTA GAA ATC GCC CTC GTC CTT G −3'. Predicted Tm's: 62–65°C. 1B. Summary of the ABO genotype detection protocol.*

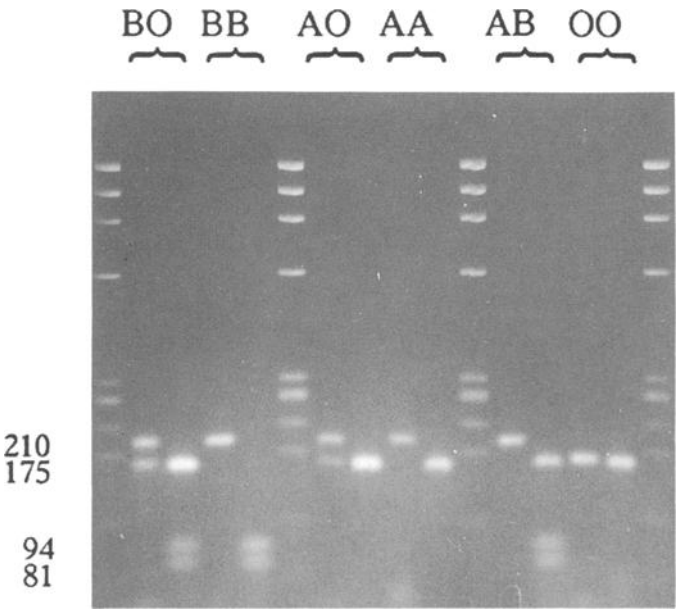

FIG. 2—*The six ABO genotypes. Phi X 174 DNA (*Hae *III digest) is used as the size standards. For each genotype, the 1 + 2 digestion (*Kpn *I) product is followed by the 3 + 4 (*Alu *I) product (left to right). The sizes of the DNA fragments are shown on the far left. One third of each amplification product is digested with 10 units of restriction enzyme.*

may be detected by three different strategies. First, one can amplify the polymorphic sites, digest the PCR products with appropriate restriction enzymes, and examine the digestion products by gel electrophoresis. This method, "PCR-RE" typing, was employed by Lee and Chang (6) and has been modified in the present work. In addition, this strategy has been utilized for typing the GC locus (7). Second, one can design allele-specific primer sets such that the amplification products are examined directly on gels (without restriction enzyme digestion) for genotype determination. This technique was called "PCR allele-specific amplification" (PASA) by Sarkar, Sommers and colleagues (8–10), and has recently been applied to ABO genotyping of forensic specimens by Crouse and Vincek (11). Third, one could amplify the polymorphic regions, and then determine the genotype using a dot-blot (or reverse dot-blot) format with labeled allele-specific oligonucleotide (ASO) probes. This last method has not been successfully adapted to ABO genotyping.

In the present study, ABO genotype detection exploits two nucleotide specific polymorphisms in the glycosyl transferase gene on chromosome 9 (2,3). The "O" allele results from a deletion at position 258, which eliminates transferase activity and creates a *Kpn* I recognition site. The "B" allele contains a G to A transition (relative to the "A" allele) at position 700, generating an *Alu* I recognition site (Fig. 1*A*). The genotype determination protocol is outlined in Fig. 1*B*. The 210/209 bp product contains an O allele-specific polymorphism. If the O allele is present, *Kpn* I digestion generates 177 bp and 32 bp fragments. The 175 bp product contains a B allele-specific polymorphism. If the B allele is present, *Alu* I digestion produces 94 bp and 81 bp fragments. The digestion pattern from both amplification reactions must be analyzed in concert for ABO genotype determination. We have developed new primer sets, optimized the PCR reactions previously described for

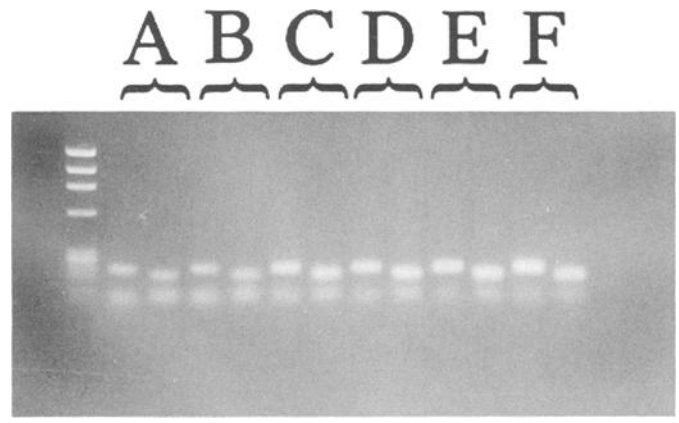

FIG. 3—*Template concentration profile. Far left Lane: Phi X 174 (*Hae *III digest) size standards. Lane A: 1 ng of human DNA (per reaction) used for amplification. Lanes B–F: 2 ng, 5 ng, 10 ng, 15 ng, and 20 ng, respectively. The 1 + 2 undigested amplification product is followed by the undigested 3 + 4 product (left to right). For all product gels, 10% of the amplified product was loaded for electrophoresis.*

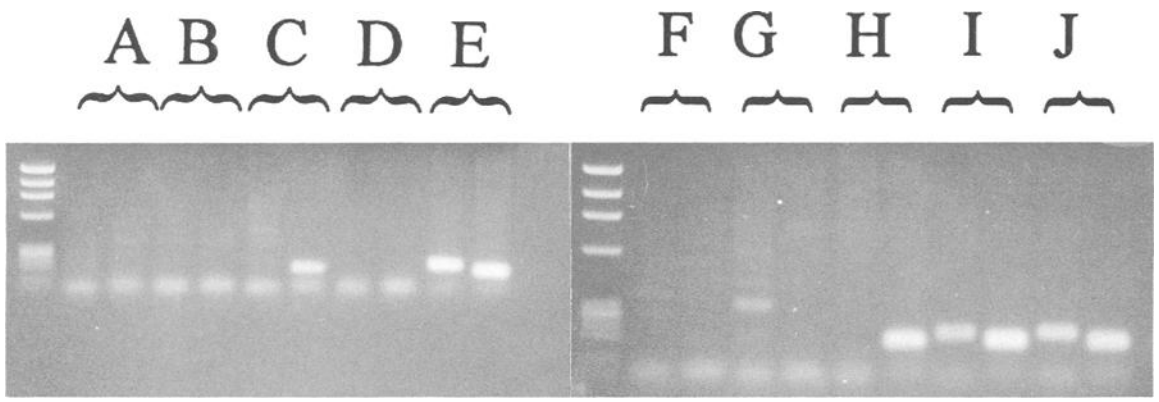

FIG. 4—*Species Specificity. Lanes A–J = 15 ng of template from the sources indicated. A: Deer. B: Dog. C: Cat. D: Cow. E: Chimp. F: Goat. G: Duck. H: Orangutan. I: Gorilla. J: Human. The undigested amplification product was loaded as in Fig. 3 (1 + 2, 3 + 4). Lanes immediately left of A and F: Phi-X 174/Hae III size standards.*

ABO gene amplification (6), and conducted a systematic validation study of the PCR-RE ABO typing protocol. Figure 2 shows examples of the six ABO genotypes.

First, the PCR reaction parameters were optimized. Having two different primer annealing temperatures, as described in (6), is inconvenient, and with our primers 60°C is an optimal primer annealing temperature for both reactions. In addition, we found that it was possible to significantly reduce the denaturation time (originally 2 m) (6) which improves the yield by extending Taq activity. The assay is also quite sensitive. Using the new primers, balanced amplification products are readily detected with as little as 1 ng of genomic template; 10 ng–20 ng of DNA are optimal for most casework samples (Fig. 3). All samples in Fig. 3 were successfully genotyped (data not shown). When the yield of amplification product is low, as in lanes A and B, all restriction fragments are readily detected by digesting a greater quantity of amplified DNA for the analytical gel.

To address the issue of species specificity, the ABO primers were tested against commonly encountered animal DNAs as well as against select primate DNA. Only primate DNA yielded both amplification products of the appropriate sizes (Fig. 4). Thus, the assay was primate-specific, at least within the universe of animal DNAs tested. Restriction endonuclease analysis of the (non-human) primate amplified products was not systematically pursued, as it was not directly relevant to these studies. The ABO gene homologies among human and higher primates may well be of some evolutionary interest. Conventional serological testing has indicated that chimpanzees are primarily "A" and "O," and that gorillas are "B." Variable typing results have been reported for orangutans (11,12).

Figure 5 shows the results of testing different tissues from the same individual. As expected, DNA from all of the tissues produced identical digestion patterns (type BO). Additional experiments using different tissues from several other individuals always produced concordant results for the same person (data not shown). The method has been tested with DNA from a variety of tissues, including blood, bone, skin, intestine, oral plus vaginal swabs, semen, and hair roots. The assay is also well-suited to typical forensic samples. Amplification products are easily generated from samples subjected to different environmental conditions prior to DNA extraction (Fig. 6). The correct genotype was detected after each treatment with the exception of sea water immersion (data not shown).

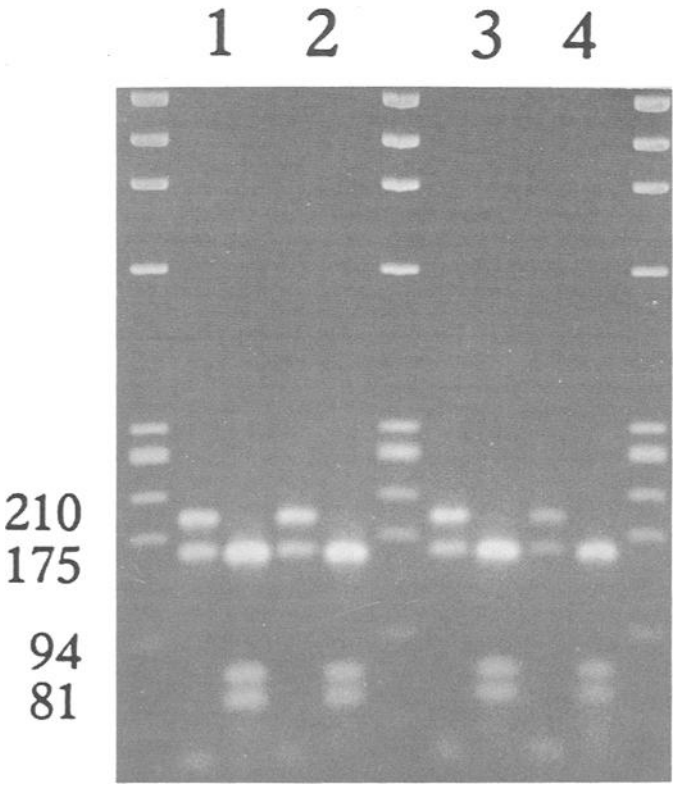

FIG. 5—*Digestion fragments from 4 tissues of the same individual (type BO). Lanes 1–4: Blood, Oral Swab, Skin, and Semen, respectively. The gel was loaded as described in Fig. 2. Phi-X 174/Hae III size standards in far left and far right lanes, and lane sections 2 and 3.*

Specimens from sexual assault cases are commonly submitted to forensic science laboratories. We thus conducted tests with vaginal/seminal mixtures which demonstrate that both the male and female ABO genotypes can be determined by this method following a differential DNA extraction protocol (data not shown). However, in some instances, differential extractions can be problematic for two reasons. First, it is difficult to achieve 100% separation. Second, the male ejaculate may contain a detectable quantity of epithelial cell DNA in addition to the much larger quantity of sperm DNA.

The results presented here indicate that the ABO genotyping by this protocol is robust and reliable for forensic analysis. The procedure overcomes some of the problems associated with immunological typing methods, and provides increased discrimination potential. An advantage of this method over other ABO typing strategies such as the two-step (PASA) ABO procedure (11), is that the digestion step serves as an additional internal control. This permits greater confidence in the results. Further work is underway in our laboratories to improve the procedure and make it more convenient. We are looking for new primer sites that will enable greater specificity, shorter cycling times, and that are compatible with multiplex-PCR. Additional desirable modifications include developing ASO probes that will make genotyping possible in simple direct or reverse dot blot formats, preferably using nonradioisotopic detection procedures. In addition, the ligase chain reaction (LCR) (13) might prove particularly useful for detecting the ABO and other sequence polymorphisms.

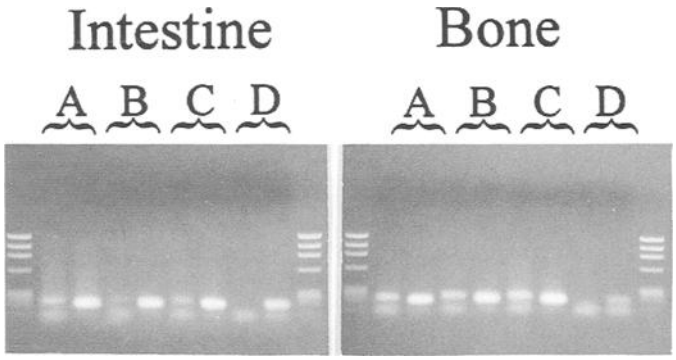

FIG. 6—*Effects of environmental conditions. DNA was extracted from intestine and bone of the same individual. Prior to extraction, the tissues were incubated for 1 month at the four conditions listed (A–D). A: −20°C. B: RT (25°C) dry. C: RT in a moisture chamber. D: Immersed in sea water. Amplification products are undigested. Phi-X 174/Hae III size standards in far left and far right lanes of each picture.*

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