TECHNICAL NOTE

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A Simplified Amplification Procedure for Two Regions of the Glycosyl Transferase (ABO Blood Group) Gene

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ABSTRACT: The ability to classify the ABO blood group of physiological stains has been an important tool for forensic scientists. A streamlined method for the determination of glycosyl transferase (ABO) genotypes using PCR amplification is described and validated here. Successful amplification and typing is possible with 1–2 ng template DNA. Concordance studies with samples of known types and nonprobative forensic casework samples were performed. Bloodstains from a single individual from one case produced results discrepant from those obtained by conventional serological ABO typing. All other samples produced results in complete agreement with expected ABO phenotypes. The method described here is relatively simple to perform, requires minimal template DNA and can be completed in less than one day.

KEYWORDS: forensic science, DNA, polymerase chain reaction, ABO blood group, glycosyl transferase, human identification

The ABO blood grouping system is widely used for the classification of physiological fluids. This system has proven to be one of the most versatile and long lasting in forensic serology because of the inherent stability of the antigens involved, the ability to readily obtain results from dried bloodstains, and the availability of known blood types from other records. With the determination of the molecular basis of the glycosyl transferase genes involved in the ABO system in 1990 (1,2,3), the possibility arose of obtaining the blood group genotype by examination of the DNA sequence involved in producing the transferase which adds the sugar molecules to the antigens. Fluids from non-secretor individuals in which no ABO antigens are present in the fluid could also be typed if nucleated cells were present within the fluid. Even though the basis of the polymorphism had been elucidated, no work was published applying this knowledge to forensic type samples until 1992 when Lee and Chang (4) presented a system for typing stains utilizing the Polymerase Chain Reaction (PCR) (5). In their system a set of four primers was used to amplify two separate regions on either side of an intron present within the transferase gene. Subsequent cleavage of these PCR products with the restriction enzymes *Kpnl* or *Alul* and examination of the resulting banding patterns allowed the determination of the blood group. The major drawback to this procedure was that large amounts of template DNA (much larger than those routinely found within evidentiary samples) were required to obtain reproducible results. This system also required that the products from the four primers be amplified in two separate amplifications, therefore doubling the amount of DNA template required. In 1995, Crouse and Vincek (6) published an alternative method of determining the blood group using a set of eight PCR primers which should amplify only specific alleles. Although this system did overcome some of the sensitivity problems originally found in the Lee and Chang (4) method and eliminated the need for the restriction digest, it still required that the amplifications be performed in separate tubes, again doubling the amount of DNA template utilized in the test procedure. It also requires extremely exacting conditions in order to prevent mispriming resulting in uninterpretable banding patterns. More recently, Ladd et al. (7) have presented a PCR method with a different set of four primers than originally published in (4), but in all other ways very similar. Again this latest proposal overcame the sensitivity problems, but retained the drawback of requiring separate amplifications for the two PCR products.

In this paper an alternative method for determining the glycosyl transferase (ABO) genotype of a physiological sample will be presented which produces results in a time frame comparable to that obtained with conventional absorption-elution methods, requires a minimal amount of DNA, and is performed using a single amplification reaction. This method is based on the method of Lee and Chang (4) with modifications to simplify and make the procedure as cost and time effective as possible for forensic laboratories.

Materials and Methods

DNA extractions were performed on bloodstains, buccal swabs or semen stains using the Chelex procedure (8) or by proteinase K-SDS digestion at 56°C for 1.5–2 hours, phenol:chloroform:isoamyl alcohol and chloroform extractions followed by washing in a Microcon 30 filtration unit. DNA quantification was performed using the Quantiblot kit (Roche Molecular Systems).

The four primers were HPLC purified and diluted to 25 pmol/μL stock solutions in 10 mM Tris-HCl pH 7.5, 1 mM EDTA. The sequences of the four primers (5′ to 3′) are as follows ABO1: CACCGTGGAAGGATGTCCTC; ABO7: AATGTCCACAGTCACTCGCC; ABO3: GTGGAGATCCTGACTCCGCTG; ABO10:

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CACCGACCCCCCGAAGAAC. The primers are positioned at positions 232, intra intron, 661 and 801 respectively from the sequences presented in earlier research (3,4).

The PCR reaction is carried out in a volume of 25 μL with 1.5 mM MgCl2, 1X PCR buffer (prepared using Perkin Elmer 10X PCR buffer), 200 μM dNTP, 200 μg BSA, 1.25 unit Taq Polymerase, 25 pmol each ABO1 and ABO7 and 12.5 pmol each ABO3 and ABO10. Up to 5 μL DNA template could be added to the reaction mix. Several reactions were also carried out using 400 μM dNTP with no adverse effects. Cycling parameters are 95°C 2 min, followed by 32 cycles of 95°C 10 sec, 60°C 10 sec, 72°C 10 sec in a Perkin Elmer GeneAmp 2400. No final extension at 72°C was used. Prior to amplification of organically extracted DNA these samples were denatured by boiling 4 minutes followed by quick chilling on ice to minimize renaturation. The boiling step has been found to be unnecessary and has been eliminated from subsequent studies.

Gel electrophoresis of PCR products and post restriction samples was performed using 2.75% ultrapure agarose (Gibco-BRL) in 1X TBE (9) at 150 V for approximately 30 minutes or until fragment separation was deemed suitable. DNA fragments were visualized by in-gel staining during electrophoresis using a 1:10000 dilution of SYBR1 (FMC Corporation) dye. Gels were photographed with Polaroid 667 film using 302 nm transillumination and a Tiffen #15 filter.

Restriction of PCR products was performed directly in the PCR reaction buffer using 2.5 units each of *KpnI* and *AluI* for 30 minutes to 1 hour at 37°C.

Results and Discussion

The conditions for amplification were evaluated using several different primer concentrations and annealing temperatures. Concentrations of each primer from 12.5 pmol to 25 pmol were examined and those indicated in the Materials and Methods chosen for producing the best product yield. Annealing temperatures from 60° to 63°C were utilized with 60°C producing the highest yield of desired product while still minimizing nonspecific priming (data not shown).

The reaction parameters were established using Chelex extracted DNA from buccal swabs of laboratory personnel. The ABO phenotype of each of these individuals was known prior to the testing performed in this study. In all instances, the glycosyl transferase (ABO) genotype obtained agreed with the known phenotype. Sperm samples from several of these individuals were also tested with the expected results being obtained (data not shown).

The primer set ABO1, ABO7 amplifies a region of the transferase gene 200 or 199 bp in length depending on the presence or absence of a single nucleotide. Digestion of this product with *KpnI* produces fragments of 171 and 28 bp. The primer pair ABO3, ABO10 amplifies a region 3′ to the intron with a length of 159 bp. *AluI* digestion of this product produces fragments of 118 and 41 bp. For ease of interpretation, the four possible bands visible after digestion have been designated 1 (200, 199 bp), 2 (171 bp), 3 (159 bp), and 4 (118 bp). The observation of band 2 indicates the presence of an O allele and the observation of band 4 indicates the presence of the B allele. Since bands 2 and 4 are derived from bands 1 and 3 respectively, careful attention should be paid to the intensity of these bands relative to each other when interpreting the photograph. If band 2 is significantly more intense than band 1 or band 4 is significantly more intense than band 3, a slight partial digestion by the enzyme or a potential mixture may be indicated. This situation of unequal intensity was noted only once in the approximately 80 samples typed in this study.

The ability to multiplex the amplification of both products in a single tube and then perform the double restriction digestion in that same tube greatly enhances the utility and ease of interpretation of this method. Figure 1 shows the expected pattern from each of the six possible genotypes in the glycosyl transferase (ABO) system.

The sensitivity of this system with regard to template DNA was investigated using an organically extracted sample of type BO. This sample was chosen since it produces only a single copy of each DNA fragment and thus optimal positive control for this system. Although in some instances as little as 500 pg template DNA produced interpretable results, the most consistent results were obtained with 1.0 to 5 ng DNA (Fig. 2). Amplification of DNA at levels above 10 ng resulted in a reduction of the clarity of the minigels used for interpretation as the lanes became overloaded and the DNA fragments became smeared and separation

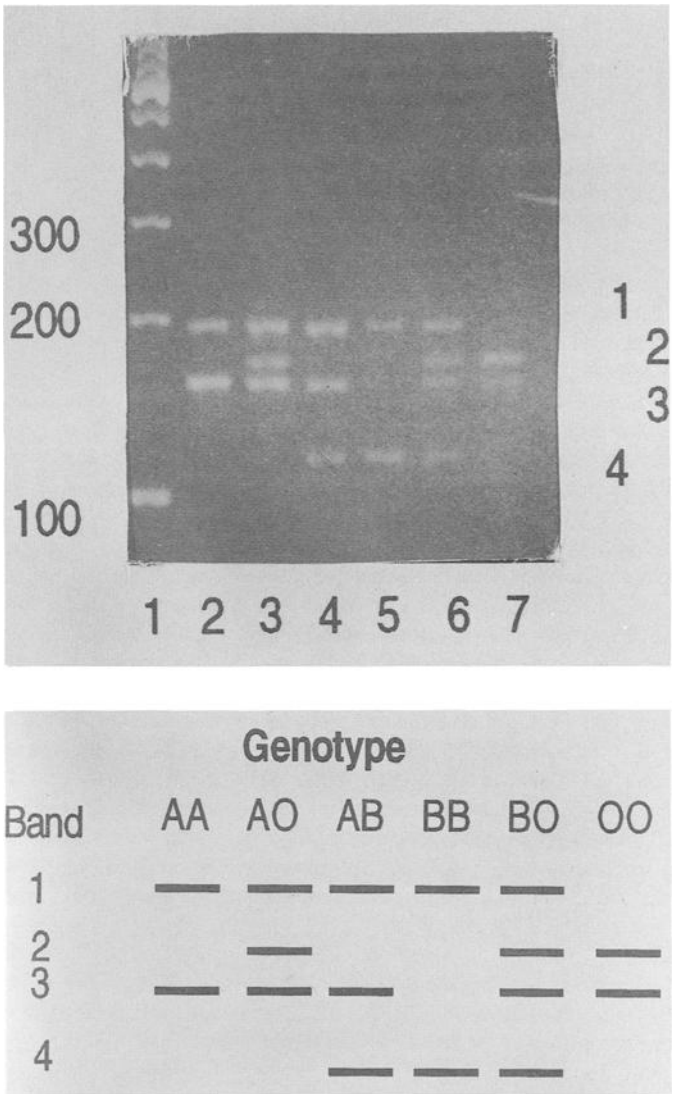

FIG. 1—*Lanes 1–7: 100 bp ladder, Genotypes AA, AO, AB, BB, BO and OO, respectively. The band identifications are shown on the side. A schematic of the expected banding patterns for each genotype.*

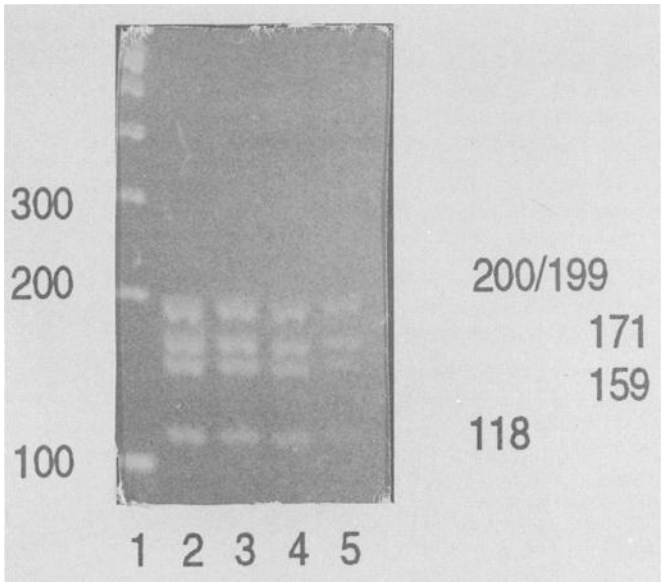

FIG. 2—*Sensitivity profile of the amplification of the transferase regions using a BO standard. Samples have been digested with KpnI and AluI. Lanes 1–5: 100 bp ladder, 10 ng DNA, 5 ng DNA, 2.5 ng DNA, and 1.2 ng DNA respectively.*

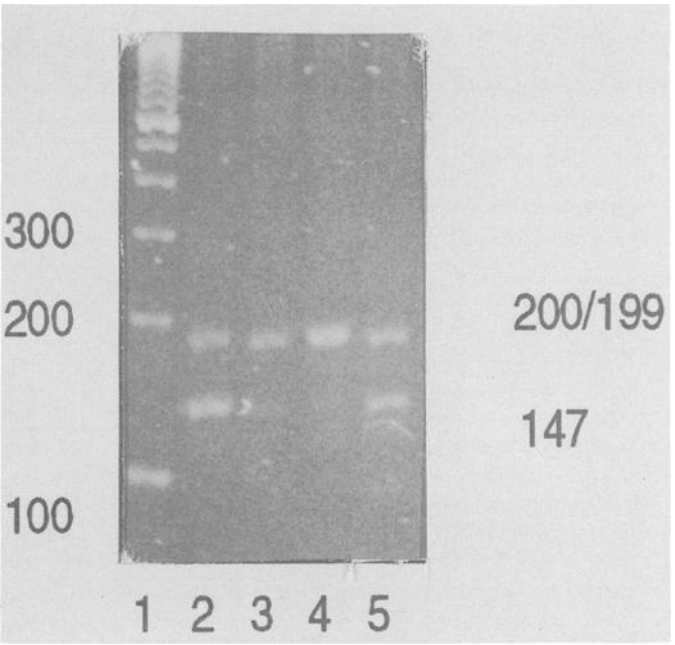

FIG. 3—*Allele specific primer reaction of discordant case sample. Primers specific for the B allele described in (6) and the A or O allele were utilized to determine if the discordant sample had the second B specific site in the transferase gene. The control samples were from individuals of known type. Lanes 1–5: 100 bp ladder, AB sample, AA sample, AO sample, and case sample respectively. The fragment at 147 bp represents a positive amplification of the B allele. No or minimal amplification from the B specific primer set was observed in the AA and AO samples used for controls. These samples are not digested with restriction enzyme and the PCR products are not identical to those obtained from the system described in detail in the study. All samples had been Chelex extracted.*

was reduced. Loading of a smaller aliquot of the sample can eliminate this problem.

In order to evaluate the usefulness of this method on casework samples, a concordance study was performed using bloodstains from casework received at the GBI-DOFS which had been typed using absorption-elution methods. These stains were deposited on a wide range of materials commonly encountered in forensic casework. A total of 81 different stains were extracted. Of these, 75 amplified successfully and 70 produced types in accordance with those expected (as measured by the type obtained from absorption-elution typing). The types obtained on these stains represented all six possible genotypes. Insufficient DNA was obtained from six of the stains to allow successful amplification.

In one sample, the post amplification yield gel proved extremely useful. Three to five ng DNA was used for amplification and a very weak nonspecific product band at approximately 120 bp was observed on both the amplification yield gel and the post-restriction gel. This band comigrated with the B specific band produced from AluI digestion of the ABO3, ABO10 primer pair. By examining both the yield gel and the difference in intensities between the non-specific band and band 3, the presence of the B allele was ruled out. This sample was correctly typed as OO.

The five stains which did not produce concordant results were all from a single case and originated from a single individual as determined by case information and additional conventional testing. These stains all produced a genotype of BO while conventional typing yielded type A, including forward and reverse typing performed on the liquid blood collected at autopsy from that case. A stain made from the liquid blood was also typed and produced the BO genotype. Currently there is no explanation for this deviation, but the presence of the B allele was confirmed using both the restriction method described here and the allele specific primers described in Crouse and Vincek (6) (Fig. 3). Although it is troubling that this result contrary to a conventional typing result was observed, it must be stressed that the glycosyl transferase (ABO) PCR system described here was internally consistent, with all stains which could be presumed to originate from the individual in that case producing the same type.

Mixtures of types were not examined, but would prove problematic to interpret unless significant differences in intensity between the bands were evident. Results obtained using this method should be used with care on sperm/non-sperm mixtures unless other tests are also used to confirm the efficiency of the differential extraction procedure employed. Extensive species testing was not performed, but tests using DNA isolated from dog and cat did not produce expected DNA fragment sizes.

In conclusion, a method has been described here which allows the quick and easy determination of glycosyl transferase (ABO) genotypes from small forensic samples through the PCR amplification of two regions of the glycosyl transferase gene. Until a mechanism can be proposed for the types obtained from the discrepant sample, it may be prudent to classify the results obtained using this method as glycosyl transferase types rather than ABO types to prevent potential misunderstandings when comparing results to those obtained using conventional typing procedures. The DNA may be extracted by Chelex or organic methods and as little as 1–2 ng is required for successful, unambiguous results. Utilization of the DNA sequence to determine the glycosyl transferase types of forensic casework samples should eliminate some of the problems associated with conventional typing such as interference and weak antibody binding. The total time required for obtaining the transferase genotype is 8–9 hours, including DNA extraction, if short incubation times with proteinase K are used. This allows relatively

rapid screening of samples, with sufficient DNA remaining in most instances for additional testing to be performed when necessary.

Both conventional and PCR typing of a bloodstain from the discordant sample presented here has been repeated by another laboratory, with the results duplicating those obtained in this study. Sequencing the amplified DNA from this sample is being pursued in an effort to clarify the mechanism behind this unexpected result.

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