TECHNICAL NOTE

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Multiplex Amplification and Typing Procedure for the Loci D1S80 and Amelogenin

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ABSTRACT: A method has been developed that enables multiplex amplification and simultaneous typing of the loci D1S80 and amelogenin using discontinuous polyacrylamide gel electrophoresis and silver staining. The protocol is sensitive, simple, rapid, and relatively inexpensive. The results of the multiplex analysis of the D1S80 and amelogenin loci were comparable to those obtained when each locus was analyzed individually. A small validation study was undertaken to evaluate the forensic applicability of this multiplex system. The data demonstrate that DNA exposed to a variety of environmental insults yields reliable multiplex typing results.

KEYWORDS: forensic science, DNA typing, validation studies, polymerase chain reaction, multiplex amplification, D1S80, amelogenin, sex determination

Amplification of DNA sequences by the polymerase chain reaction (PCR) has enabled the forensic application of molecular biology analytical methods that are rapid, simple, and possess a high degree of detection sensitivity. The most characterized forensic amplified fragment length polymorphism (AMP-FLP) is the D1S80 locus (1–19). After the D1S80 alleles are amplified by PCR, they can be resolved by discontinuous polyacrylamide gel electrophoresis and subsequently detected by silver staining (2,4,5). The alleles are based on the number of repeats contained within them and more than 25 different D1S80 alleles have been observed (5). Moreover, as little as 100 pg of template DNA can be typed at the D1S80 locus (4,12).

The advantages of a multiplex system are that less template DNA is consumed than when analyzing each locus independently, less reagents are consumed, and labor is reduced. The amelogenin gene is a locus that also has been demonstrated to be valid for forensic applications and potentially may be analyzed in a multiplex fashion with the D1S80 locus (20–25). Typing the amelogenin gene enables determination of the sex of the contributor of a biological sample.

This study describes a multiplex method for typing the D1S80 and amelogenin loci simultaneously. The system is simple, rapid, and sensitive, and there is no apparent compromise in the efficacy of typing compared with single system methods for the D1S80 or amelogenin locus.

Materials and Methods

Sample Preparation

Whole blood samples were collected in EDTA Vacutainer tubes from donors at the FBI Academy in Quantico, Virginia. The samples were air-dried on cotton cloth. The validation samples used in this study were prepared similarly to those in a D1S80 validation study described previously (4). The DNA was extracted according to the method described by Comey et al. (26). The quantity of DNA in each sample was estimated using the slot-blot procedure described by Waye et al. (27).

Validation Study

Body fluid samples from three different donors were used for the validation study. The D1S80 loci types of the donors and their sex are 28–25, male; 24–20, female; and 28–24, female.

The small validation study consisted of the following analyses: 1) sensitivity and detection of mixed samples; 2) effects of chemical contaminants (such as, dirt, bleach, acid, and unleaded gasoline) on the DNA in blood and semen samples; 3) the effect of typing DNA from blood and semen samples deposited on denim; 4) the effect of sunlight exposure and storage in shaded conditions up to 20 weeks on DNA typing; 6) determination of the sensitivity of the D1S80 and amelogenin multiplex system by typing genomic DNA from two samples at different quantities. The quantities of DNA amplified for these two samples were 4 ng, 2 ng, 1 ng, 500 pg, 250 pg, and 125 pg; 7) cross-reactivity of DNA from species other than human. DNA from the following organisms was analyzed: gorilla, Japanese macaque, orangutan, spider monkey, Celebes ape, Debrazza monkey, pig, sheep, burro, horse, goat, dog, chicken, cat, steer, and deer. These samples were analyzed using 30 ng of template DNA. The quantity of nonhuman DNA was

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determined by ethidium bromide staining of DNA separated in miniature agarose gels; and 8) DNA derived from various tissues, including blood, semen, saliva, and hair.

In addition, this study examined the effect of varying the primer concentration, the PCR buffer, the volume of the PCR, and the number of PCR cycles versus quantity of template DNA.

The DNA samples were typed for D1S80 according to the method of Budowle et al. (5) with slight modifications. Primers specific for the amelogenin locus (supplied by Promega Corp., Madison, WI) (25) were amplified simultaneously with the D1S80 primers. In addition, to the PCR buffer (pH 8.3) described previously (4,5) (Perkin-Elmer Corp.), the pH of the PCR buffer was increased to 9.0 (Promega Corp.). The volume of the PCR was either 25 or 50 μL. To accommodate the amelogenin fragments (212 base pairs for the X and 218 base pairs for the Y fragment) the polyacrylamide gel was 8.5% T and 2% C.

Results and Discussion

The procedure described in this study enables the simultaneous typing of the D1S80 and amelogenin loci (Fig. 1). The typing results attained with this multiplex system are consistent with types derived from single system analyses. There was no apparent compromise in the quality of the results with a multiplex approach compared with single system protocols. The only difference in the protocols for typing the D1S80 locus individually (4,5) and in a multiplex fashion with the amelogenin locus was the acrylamide concentration in the analytical gel. In order to ensure that the amelogenin did not migrate off the end of the polyacrylamide gel the acrylamide concentration was increased from 7.5% T to 8.5% T. Thus, results obtained from previous validation studies would apply to this multiplex protocol and support its forensic utility.

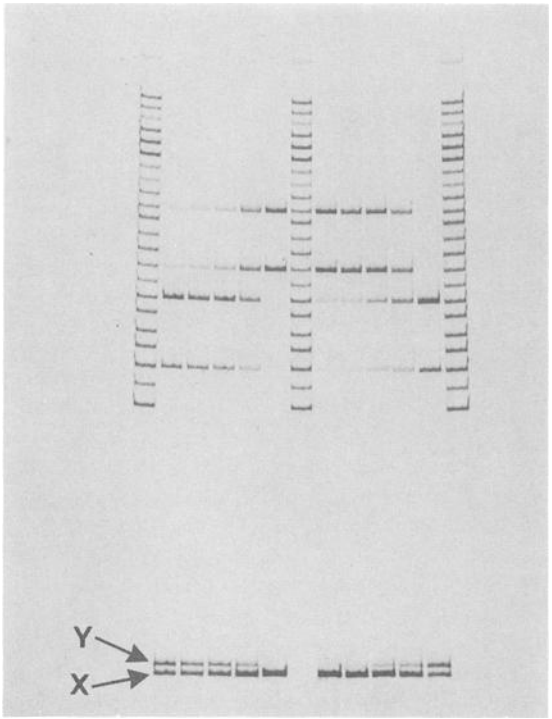

FIG. 1—*A silver stained discontinuous polyacrylamide gel displaying D1S80 and amelogenin types. The samples (a 50 μL PCR at 27 cycles) are mixtures. From left to right the samples (male:female ratio in mass amounts of DNA added together) are: allelic ladder; 15:1; 7:1; 3:1; 1:1; 0:1; allelic ladder; 1:15; 1:7; 1:3; 1:1; 1:0; and allelic ladder. The* arrow *indicates the position of the amelogenin product. The cathode is at the top.*

Environmental Insult Study

Both the D1S80 and the amelogenin loci have been validated previously for forensic applications (3–25). Moreover, there exists now a good understanding of the reliability of typing DNA derived from environmentally-insulted samples. Therefore, only a small validation study was performed.

DNA extracted from blood and semen exposed to a variety of environmental insults was amplified by multiplex PCR and typed successfully at the D1S80 and amelogenin loci. Approximately one ng DNA template samples were analyzed. The results are summarized in Table 1. D1S80 and amelogenin types were obtained for 40 and 45, respectively, of the 50 samples. Not surprisingly, there were five samples that could be typed for the amelogenin locus and not the D1S80 locus (for example, see sunlight/shade study); most likely this is due to the smaller size of the amelogenin amplicons and/or the higher yield of amelogenin amplicons.

The samples in the sunlight/shade study were analyzed twice—using 27 cycles or 30 cycles of PCR. In general, it appears that DNA derived from blood was more stable than DNA derived from semen. All blood samples, exposed to sunlight or stored in the shade up to 20 weeks were typeable; while some semen samples exposed to sunlight were not typeable. There were four samples that could not be typed at the D1S80 locus using 27 cycles; however, one of the samples was typeable using 30 PCR cycles. Also, one of the two sunlight/shade samples that was not typeable for amelogenin at 27 PCR cycles was typeable at 30 PCR cycles.

Body Fluid Mixtures

The presence of two or more contributors to a sample generally is inferred by the presence of more than two D1S80 bands and/or noticeably different intensities of bands in the profile. While this observation holds true for the D1S80 locus, the same inference was not possible for the amelogenin locus. Even at various mixtures of male:to:female blood samples or semen:to:female saliva samples (i.e., more doses of Y to X), mixtures were not clearly elucidated. Thus, with the procedure described here, the presence of a mixture generally should be evaluated cautiously at the amelogenin profile. When there is only one band, with a length of 212 base pairs, it can be stated that only female DNA can be detected; when both bands (212 and 218 base pairs) are observed, the presence of male DNA can be deduced. The presence of mixtures will be determined more easily at the D1S80 portion of the multiplex profile.

Tissue Study

Since there would be no expectation that D1S80 and amelogenin profiles from a healthy individual would differ in DNA derived from various tissues, only four different tissue samples were tested. DNA from blood, semen, buccal cells, and follicular sheath material yielded the correct types for both the D1S80 and the amelogenin loci.

Cross-Reactivity with Other Species

As described previously, only DNA from higher primate species yielded D1S80 products, and only gorilla DNA products were in the size range of human D1S80 amplicons (4,6). Amelogenin products were detected for all species tested except macaque,

TABLE 1—*Results of the D1S80 and amelogenin multiplex environmental insult study.**

Specimen/substrate or condition	No. typed for D1S80	No. typed for amelogenin
Denim/blood	+	+
Denim/semen	+	+
Dirt/blood	+	+
Dirt/semen	+	+
Bleach/blood	+	+
Bleach/semen	−	−
Acid/blood	−	+
Acid/semen	−	−
Gasoline/blood	+	+
Gasoline/semen	+	+
Sunlight/semen/27 cycles/1 week	+	+
Sunlight/semen/30 cycles/1 week	+	+
Sunlight/semen/27 cycles/3 weeks	−	+
Sunlight/semen/30 cycles/3 weeks	+	+
Sunlight/semen/27 cycles/4 weeks	−	+
Sunlight/semen/30 cycles/4 weeks	−	+
Sunlight/semen/27 cycles/10 weeks	−	−
Sunlight/semen/30 cycles/10 weeks	−	+
Sunlight/semen/27 cycles/20 weeks	−	−
Sunlight/semen/30 cycles/20 weeks	−	−
Sunlight/blood/27 cycles/1 week	+	+
Sunlight/blood/30 cycles/1 week	+	+
Sunlight/blood/27 cycles/3 weeks	+	+
Sunlight/blood/30 cycles/3 weeks	+	+
Sunlight/blood/27 cycles/4 weeks	+	+
Sunlight/blood/30 cycles/4 weeks	+	+
Sunlight/blood/27 cycles/10 weeks	+	+
Sunlight/blood/30 cycles/10 weeks	+	+
Sunlight/blood/27 cycles/20 weeks	+	+
Sunlight/blood/30 cycles/20 weeks	+	+
Shade/semen/27 cycles/1 week	+	+
Shade/semen/30 cycles/1 week	+	+
Shade/semen/27 cycles/3 weeks	+	+
Shade/semen/30 cycles/3 weeks	+	+
Shade/semen/27 cycles/4 weeks	+	+
Shade/semen/30 cycles/4 weeks	+	+
Shade/semen/27 cycles/10 weeks	+	+
Shade/semen/30 cycles/10 weeks	+	+
Shade/semen/27 cycles/20 weeks	+	+
Shade/semen/30 cycles/20 weeks	+	+
Shade/blood/27 cycles/1 week	+	+
Shade/blood/30 cycles/1 week	+	+
Shade/blood/27 cycles/3 weeks	+	+
Shade/blood/30 cycles/3 weeks	+	+
Shade/blood/27 cycles/4 weeks	+	+
Shade/blood/30 cycles/4 weeks	+	+
Shade/blood/27 cycles/10 weeks	+	+
Shade/blood/30 cycles/10 weeks	+	+
Shade/blood/27 cycles/20 weeks	+	+
Shade/blood/30 cycles/20 weeks	+	+
Totals	40	45

*Each line of table represents one specimen subjected to duplex D1S80 and amelogenin typing. + denotes correct result; − denotes weak or no detectable results.

chicken, goat, and deer. Other than for higher primates, the amelogenin profiles were weak, and only dog and cat amelogenin products migrated to a similar position on the gel where human bands reside. Because of the low intensity of the animal amelogenin products, the absence of D1S80 products, and the rarity of cases where one has to consider cat and dogs as DNA sources, there should be little impact when nonhuman DNA is present on forensic interpretations when typing the amelogenin locus.

Sensitivity Study

Successful typing of D1S80 has been reported with as little as 100 pg of genomic template DNA (12) and for amelogenin as little as 20 pg of template DNA were typeable (23). Genomic DNA at quantities of 4 ng, 2 ng, 1 ng, 500 pg, 250 pg, and 125 pg from two different individuals was analyzed. As little as 125 pg of template DNA could be typed readily for the D1S80 and amelogenin loci at 27–30 PCR cycles. Generally, the bands at the amelogenin locus are slightly more intense than at the D1S80 locus profile—suggesting that the sensitivity of detection at the amelogenin locus is slightly better than at the D1S80 locus.

PCR Buffer, Primer Concentration, and PCR Volume

Currently, there are two commercially available buffers for the PCR—the standard (50 mM KCl, 10 mM Tris-Cl, and 1.5 mM $MgCl_2$) at pH 8.3 and the same buffer but at pH 9.0. Both were evaluated to determine whether or not there was a difference in the amplification yield, or quality, of the multiplex amplicons. Generally, the yield of PCR products was not substantially different between the two buffers.

The concentration of D1S80 primers as a single system PCR had been determined previously (4,5), and the results were consistent with the multiplex system. Ranges of primer concentration from 0.2 μM to 0.3 μM had little effect on the amplification process. Thus, the D1S80 primer concentration for the multiplex amplification of D1S80 and amelogenin was maintained at 0.25 μM. With the amelogenin, half and twice the recommended primer concentration (as recommended by Promega Corp.—see manufacturer's manual) yielded slightly less and more amelogenin product, respectively. Although there was an increase in amelogenin product with increased primers, the amelogenin primer concentration suggested by the manufacturer is recommended. The sensitivity of amelogenin already is sufficient and better than for D1S80 detection; increasing the sensitivity of detection could result in stochastic effects; an increase in primer concentration could at times result in mispriming events; and an increase in primer concentration would be more costly.

When the quantity of template DNA and the concentration of reagents are held constant, generally there will be an increase in the yield of PCR using a PCR volume of 25 μL instead of 50 μL. Although the increase in sensitivity of detection is not substantial, the same trend holds for the D1S80 and amelogenin multiplex system described here. However, when the volume of PCR is 25 μL, there are more restrictions on the volume of the DNA template sample that can be used for the PCR (that is, no more than 16.25 μL of template DNA can be added to the PCR using our protocol). Moreover, when 4 ng of template DNA are used in the PCR, artifact bands can be observed at the D1S80 locus, but not at the amelogenin locus. The choice of a 25 or 50 μL PCR should be considered based on the laboratory needs.

Effect of Multiplex Amplification on D1S80 or Amelogenin Typing

When the D1S80 and amelogenin products from the multiplex amplifications were typed and compared with those analyses where each locus was amplified and analyzed separately, there was little difference in the efficiency of typing. The typing results were the same, and there was no compromise in the yield of PCR products when the samples were subjected to multiplex analysis.

Conclusion

A multiplex analysis of the D1S80 and amelogenin loci can be used to amplify and type successfully DNA derived from human biological specimens. The current study demonstrates that the procedure is robust and valid. In addition, the study shows that some samples that contain little DNA, or when the DNA in the sample is somewhat degraded, can be typed successfully by increasing the number of PCR cycles from 27 to 30. Since the method is relatively easy to perform and does not require additional costly equipment, multiplex typing of the D1S80 and amelogenin loci should be able to be implemented into most application oriented laboratories already performing PCR.

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