TECHNICAL NOTE

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Sex Determination of Dried Blood Stains Using the Polymerase Chain Reaction (PCR) with Homologous X-Y Primers of the Zinc Finger Protein Gene

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ABSTRACT: The ability to "sex" unknown dried bloodstains is frequently of evidentiary value in forensic casework. Chelex-extracted DNA from 115 specimens (105 dried blood standards and 10 casework samples) was amplified for specific ZFX and ZFY regions of the X and Y chromosomes and subsequent restriction enzyme digestion. The polymerase chain reaction (PCR) yielded a 209 base pair (bp) product containing a polymorphic position with a Y chromosome portion including an additional Hae III restriction site while the X chromosome portion contains only one. The digested PCR product was separated by polyacrylamide gel electrophoresis (PAGE) and detected by silver staining. Female samples exhibit bands of 172 bp and 37 bp only while male samples (XY) exhibit 2 additional bands appearing as an 88/84 bp doublet. Initially the 105 known bloodstains were typed according to the procedure discussed here and correct gender determination was achieved for all samples therefore establishing the reliability of this method. The 10 casework samples yielded the expected results as well. This assay demonstrates potential in both presumptive and confirmatory capacities.

KEYWORDS: forensic science, sex-determination, forensic serology, polymerase chain reaction (PCR), zinc-finger protein gene

Forensic serology has advanced tremendously since Landsteiner's discovery of the ABO system in 1900 (1). Excluding hormones and some temporary pathological factors, more than 160 antigens, 150 serum proteins, and 250 cellular enzymes have been found in human blood (2). The tremendous potential for the use of molecular biological techniques in forensic investigations has been recognized and accepted in recent years and is continually expanding. PCR is currently applicable to a wide variety of biological evidence including bloodstains on any imaginable surface, semen mixed with vaginal fluids, hairs, bone fragments, tissue from under a victim's fingernails, etc. (3,4).

Deoxyribonucleic acid (DNA) recovered from biological evidence is of use in cases involving homicide, paternity, sexual assault, and identity testing (5,6). A breakthrough using trace amounts of recovered DNA is the polymerase chain reaction (PCR), an amplification technique to enzymatically multiply a specific segment of DNA up to a million-fold. Since the first report of specific DNA amplification using the PCR in 1985 the number of applications and modifications of the basic method has grown steadily (3,7). Previously a technique for gender determination has been applied to biological evidence (8).

Humans have a heteromorphic system of chromosomes to determine the sex of the developing embryo. The male is XY, while the female is XX. Only a small portion on the Y chromosome determines gender. A marker on the Y chromosome near the gender determinant encodes for a protein named ZFY. ZFY consists of 13 zinc finger motifs for binding nucleic acids (9). A homologous portion of the X chromosome, ZFX, similarly encodes for a zinc finger protein that is capable of cross-hybridization with ZFY under stringent conditions (10).

A sex dependent Hae III restriction fragment length polymorphism (RFLP) exists between ZFY and ZFX making this locus an excellent probe for gender determination. A modification of the Aasen and Medrano assay (11) is described in the accompanying paper (12) and we used this method for gender determination of unknown dried bloodstains.

Materials and Methods

Sample Preparation

Strips of Seri Cotton Cloth (Product # S233) were exposed to a Sylvania G30T8 30W Germicidal Lamp for 20 minutes and cut into pieces approximately 2.5 cm square. Each cutting was then placed into a separate sterile petri dish (Falcon #1006) and sealed individually in plastic bags (Cole Palmer Zip-Lip). Blood samples, along with non-confidential demographic information such as race and gender, were provided by a local volunteer blood donation center. Routinely EDTA vacutainer tubes are collected with each unit of blood for disease testing and the remainder discarded. These purple-top tubes were less than 24 hours old upon receipt and already labeled with laboratory identification numbers. The swatches were then labeled with the corresponding lab number, stained with the known blood, air-dried and subsequently stored frozen at −14°C. These bloodstains are subsequently referred to as known dried blood standards. Unstained cuttings were retained

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to serve as negative cloth control samples. One of these negative controls was ran with each batch of bloodstains and treated identically to the other stains throughout the entire process. This was included to indicate any improper preparation, handling, and/or contamination of samples and reagents throughout the procedure. In addition, 10 casework samples consisting of bloodstains and corresponding negative substrate controls were also tested.

DNA Extraction

Chromosomal DNA was isolated by the method of Walsh et al. (13). Briefly, cuttings of approximately 3 mm square from the dried bloodstain and negative cloth control samples were placed in 1.5 mL Eppendorf tubes and 1.0 mL of sterile, distilled water (dH_2O) was added. The samples were incubated for 30 minutes at room temperature with occasional mixing by inversion. Centrifugation for 3 minutes at 10 15,000 × g separated the supernatant from a cell debris pellet containing the cloth substrate. The supernatant was discarded without disturbing the cell/cloth pellet and 200 μL of 5.0% Chelex [Sigma C-7901 Chelating Resin (Iminodiacetic Acid)] was gently added at the top of each tube and allowed to run down onto the pellet. Without vortexing or mixing, the samples were incubated for 30 minutes in a 56°C water bath. Upon removal, the specimens were vortexed for approximately 10 seconds, boiled for 8 minutes in a water-filled heating block and centrifuged for 3 minutes at 10,000–15,000 × g. Ten μL of each supernatant containing the extracted DNA was used for the PCR analysis. DNA quantitation was not performed prior to amplification and therefore the sensitivity of this method was not addressed. The remaining Chelex-extracted DNA was stored at 2–4°C. These samples can be used repeatedly for PCR analysis provided that they are vortexed for 10 seconds and centrifuged for 3 minutes at 10–15,000 × g immediately prior to use.

PCR Amplification

Each 50 μL amplification reaction contained 6 μL of 10× reaction mix consisting of the following: 0.42M KCl, 20 mM $MgCl_2$, 83 mM Tris-HCl pH 8.4, 1.6 mM each dNTP's, 0.21U/μL AmpliTaq, 0.83 μM RR 10 primer, 0.83 μM RR 12 primer. The primer sequences are 1) RR10:5′—CTG GAG AGC CAC AAG CTG AC—3′ and 2) RR12:5′—TTG CTG TGG ACT GCC AAG AG—3′. In addition, 34 μL dH_2O and 2 drops of mineral oil were added and 10 μL of template DNA was added last through the oil. Each set of amplification reactions contained a negative cloth control which had been extracted as a sample and 10 μL of this supernatant was used for the PCR reaction. Amplification was performed on a Perkin-Elmer TC-1 DNA Thermal Cycler using the following parameters: 1) 94°C, 1 minute; 2) 60°C, 1 minute; and 3) 72°C, 1 minute. Following the completion of 32 cycles, an additional extension step of 72°C for 15 minutes was performed. The thermal cycler was then linked to a continuous 4°C soak file to preserve the reactions until they could be analyzed further or stored at −20°C.

Restriction Endonuclease Digestion and Gel Analysis

Ten μL of product from each amplification reaction was digested overnight at 37°C with 10 U of restriction endonuclease Hae III in 1× NEB Buffer 4 and 0.3 μL dH_2O (New England BioLabs). The entire digestion reaction was analyzed on a 1 mm thick, 16 cm long × 18 cm wide, 9% nondenaturing polyacrylamide gel using a Hoeffer SE 600 vertical gel apparatus (14). The gel was electrophoresed in 1× Tris-borate buffer (TBE) at 200 volts for approximately 2 hours or until the tracking dye was 10 cm from the bottom of the well (15). PhiX 174 digested with Hinf I (Promega #G1751) was used as a molecular weight marker. Gels were silver stained according to the protocol in the Perkin Elmer AmpliFLP™ D1S80 PCR Amplification Kit, Part No. N808-0054, p. 14.

Results and Discussion

Gender determination from dried bloodstains was achieved utilizing a sex dependent, Hae III restriction fragment length polymorphism that exists between the ZFX and ZFY loci. Specific amplification of the ZFX and ZFY regions of the X and Y chromosomes using the PCR gave a 209 base pair (bp) product spanning the polymorphic region (Fig. 1*A*). The amplification products were subjected to Hae III digestion followed by PAGE analysis and the DNA visualized by silver staining. Figure 1*B* shows that following digestion all amplified samples should contain fragments of 172 bp and 37 bp originating from the X chromosome-specific PCR product. Samples of female origin (XX) had only these two bands present. Samples of male origin (XY) exhibit an additional 88 and 84 bp doublet resulting from digestion of the Y chromosome-specific PCR product.

Genomic DNA from 115 specimens (58 of male origin, and 47 of female origin) was subjected to PCR amplification of specific ZFX and ZFY regions. Initially 95 of the 105 known dried blood standards, and all of the casework specimens yielded amplifiable

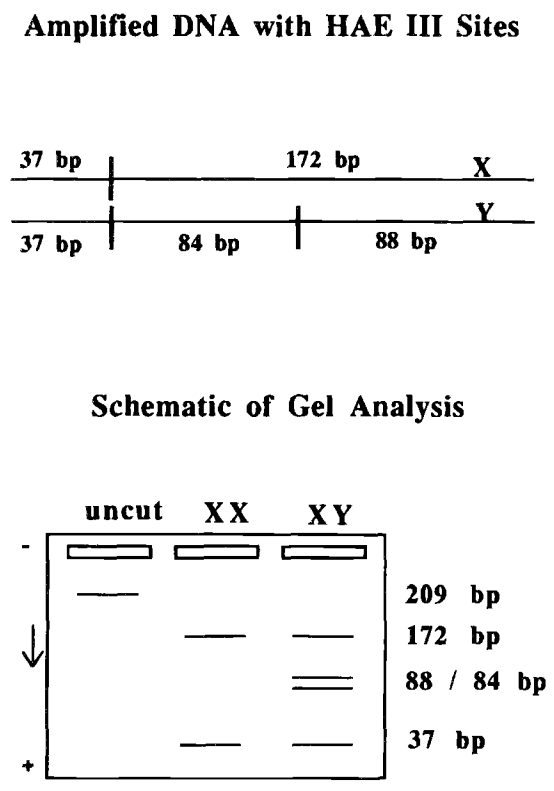

FIG. 1—(A) *Diagram of the Hae III restriction endonuclease cleavage sites exhibited in the 209 base pair (bp) PCR product from both the X and Y chromosomes are indicated as* (I). (B) *Schematic of gender determination using silver stained polyacrylamide gel electrophoresis of the Hae III restriction fragments as demonstrated in part* (A). *Expected female (XX) and male (XY) banding patterns are shown along with an undigested PCR product (uncut). The direction of migration is indicated by the arrow.*

DNA following Chelex extraction. A second extraction of a new cutting from the 10 remaining known standards yielded amplifiable DNA. It was not determined why the first extraction was unsuccessful. After Hae III digestion and PAGE analysis all specimens demonstrated the expected 172 bp and 37 bp bands from the contributing X-chromosomal portion. The appearance of the diagnostic, Y-chromosomal 88/84 bp doublet, in conjunction with the X-specific 172 bp and 37 bp bands, indicated male specimens. Figure 2 is a representation of 7 samples from which sex was ascertained. Successful gender determination was achieved for all specimens, each analyzed as an "unknown," therefore establishing the reliability of this procedure. In addition, this method was applied to 10 casework samples. Eight of these specimens (4 bloodstains and 4 corresponding substrate negative controls) were collected based on location at the crime scene and the patterns of the stains. They were suspected of belonging to the deceased female victim while the male defendant claimed them to have originated from his accidental injury. All 8 of these samples yielded the expected results; the 4 stains being of female origin and the 4 negative controls yielding no detectable DNA. The remaining 2 casework specimens were procured from an external proficiency test and the correct gender results were obtained.

Previous efforts to characterize biological material as to gender have included the radioimmunoassay of hormones, cytogenetic methods, and more recently DNA methods including sex-specific banding patterns, amplification reactions of alpha-satellite sequences of the human X and Y chromosomes, and PCR analysis applied to the X-Y homologous gene amelogenin (9,16–27). Some of the DNA methods rely on the assumption of female origin based on the absence of a Y-chromosomal amplification product (21,22). The method we describe here uses the presence of both X- and Y-chromosomal-specific regions to conclusively determine male gender and female origin is assumed based on the absence of the Y-chromosomal-specific product. It is important to consider that the diagnostic products result from enzyme digestion. The presence of the two X-chromosomal-specific products (172 bp and 37 bp) signify a successful enzymatic restriction. Therefore, the absence of the Y-specific 88/84 bp doublet is more likely due to the fact that no Y-chromosomal-specific product is present as opposed to not being visualized due to incomplete Hae III digestion. This occurrence indicates that it would be less likely to erroneously assume female gender identification using this method.

In addition to other disadvantages, previous methodology for determining the gender of biological evidence were rather time consuming (9,16–22) in comparison to the method we describe which can easily be completed in 2 days. In the study presented here, procedures containing variables as to time, temperature, etc. were performed using the maximum parameters. For example, the Hae III enzyme restriction was incubated overnight to ensure a complete digestion. However, this assay could be optimized by addressing such issues as assay sensitivity, the number of PCR cycles required, and restriction enzyme concentration in relation to incubation time.

The ability to "sex" biological evidence at the molecular level is yet another opportunity to associate evidence between victims, perpetrators, and crime scenes. In addition to the obvious advantages of DNA and especially PCR-based analysis including sample quantity and quality, this determination is advantageous in that it has both presumptive and confirmatory potential. Also this method may be applied in a variety of forensic situations including the following: the sexing of skeletal remains or other biological evidence when non-DNA methods fail or are not available; to eliminate evidence not requiring further DNA characterization; and prevent repetitive typing of evidence.

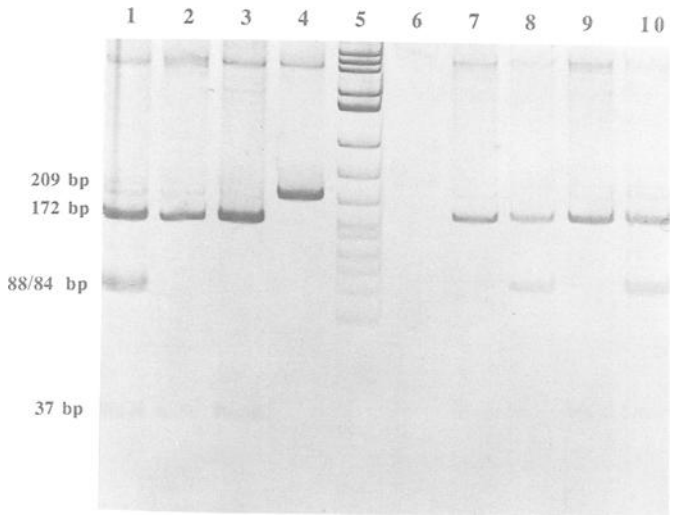

FIG. 2—*Silver-stained polyacrylamide gel showing gender determination from 7 different bloodstains. Samples in lanes 1, 8, and 10 are of male origin: lanes 2, 3, 7, and 9 indicate female origin: lane 4 is undigested PCR product: lane 5 is PhiX 174 (Hinf I) molecular size marker: lane 6 denotes a negative cloth control.*

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