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Sex Determination of Forensic Samples by Simultaneous PCR Amplification of α-Satellite DNA from Both the X and Y Chromosomes

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ABSTRACT: Simultaneous amplification of the alphoid repeated sequences clustered in the centromeric regions of both the human X and Y chromosome was performed. Modification and improvement of the polymerase chain reaction conditions resulted in detectable amplification products from less than 1 ng of genomic DNA template. Sex determination was successful in various types of biological materials of forensic interest as bloodstains, vaginal swabs, cigarette butts, bones, and hair roots. The authors suggest that the coamplification of both X- and Y-sequences in a unique reaction mixture is a fast, human specific, sensitive and reliable method providing internal reaction control and sex 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 research for the analysis of biological evidence.

KEYWORDS: forensic science, DNA, polymerase chain reaction, sex determination, alpha-satellite sequences, X chromosome, Y chromosome

The polymorphisms of the human genome provide excellent means for discrimination between individuals at the DNA level. However, restriction fragment length polymorphism (RFLP) analysis shows some limitations such as sensitivity, analysis time and the failure of typing degraded samples. The development of the polymerase chain reaction (PCR) for amplifying specific DNA fragments [*1,2*] has greatly simplified the determination of genetic markers. The value of PCR for forensic analysis is that it allows the genetic typing of samples with very little or partially degraded DNA.

Sex determination is an important element in the analysis of biological evidence submitted to forensic laboratories. It can be useful in criminal cases in general, but above all the X-Y test becomes very relevant in sexual assault cases when a male genotype can be obtained even though spermatozoa are microscopically absent. In contrast, microscopical identification of only a few spermatozoa does not always lead to the extraction of sufficient male DNA and the detection of a female genotype identifies the vaginal cell DNA of the victim. In such cases, further investigations are not indicated.

There are a number of reports on the sex determination using DNA analysis techniques. Male and female genotypes have been identified on agarose gels after simultaneous amplification of the Y-specific repeat sequence from the DYZ1 locus and the X-specific DNA repeat sequence from the DXS424 locus [*3*], by dual PCR amplification of the X-Y homologous amelogenin gene [*4*], and by PCR amplification of the centromeric alphoid repeat sequences from both the X and Y chromosomes [*5,6*]. Y chromosome sequences have also been detected on polyacrylamide gels following low stringency PCR amplification of the ZFY locus [*7*].

As there is a requirement in some laboratories for an internally controlled sex determination method with a high reliability and sensitivity for the analysis of forensic samples, we tried to define appropriate conditions allowing coamplification of both Y-specific and X-specific DNA sequences, testing of minute amounts of DNA, excellent separation and visualization on polyacrylamide gels. In this report we design a protocol for sex determination of DNA isolated from various types of biological materials of forensic science interest.

Materials and Methods

DNA Extraction

DNA was isolated from 30 sex-known peripheral blood samples, 20 blood stains, 15 cigarette butts, and 6 hair roots following proteinase K digestion and extraction by the phenol-chloroform method [*8*]. Bone specimens from two 20 to 25 year-old human skeletal remains were prepared according to previously described methods [*9*]. A differential lysis procedure as described by Sajantila et al. [*10*], but with some modifications, was used for the 20 sexual assault samples, in which epithelial cells were preferentially lysed in a digestion buffer containing sodium dodecyl sulfate (SDS) and proteinase K. 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. 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 [*11*].

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Primers

Witt and Erickson constructed primers that could be used to amplify the centromeric alphoid repeat sequences from both the X and Y chromosome [*12*]. We have used for our studies the oligonucleotides modified and reported by Gaensslen et al. [*5*]:

X3 5′ - TATTTGGACTCTCTCTGAGGA
X4 5′ - TTCTACTACAAGGGTGTTGCA
Y3 5′ - GTGTATTCACCTCCGGGAG
Y4 5′ - ACAAAAGGTTCAATTCTGTGAG

The X primers amplify a 157-bp product, the Y primers amplify a 200-bp product.

DNA Amplification

PCR amplification of 0.1–1 ng of genomic DNA was performed by modification of previously described procedures [*5,6*]. The samples were amplified using a 9600 Perkin Elmer thermal cycler in a final volume of 50 μL, containing 10 mM Tris-HCl, pH 8.4, 3.5% formamide, 50 mM potassium chloride (KCl), 200 μM each dNTP, 2 mM magnesium chloride ($MgCl_2$), 2 μL BSA (4 μg/μL), 2 units (U) of Taq polymerase, and 0.1 μM each primer. The X and Y amplifications were carried out simultaneously in the same reaction tube.

The thermal cycle profile was 3 cycles 94°C for 2 min, 60°C for 2 min, 72°C for 2 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min.

A reaction mixture without DNA as well as a sample containing female DNA were used to detect contamination, whereas a male DNA sample was used as a positive control.

Electrophoresis

The amplification products were separated on polyacrylamide gels (T = 12%, C = 2.9%) cast onto GelBond (FMC, Rockland, ME). The mix for one gel of 220 × 120 × 0.5 mm was: 2.0 g acrylamide, 0.06 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. We routinely loaded 2 μL of the 50 μL-reaction mix 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 by the silver staining method decribed by Budowle et al. [*13*].

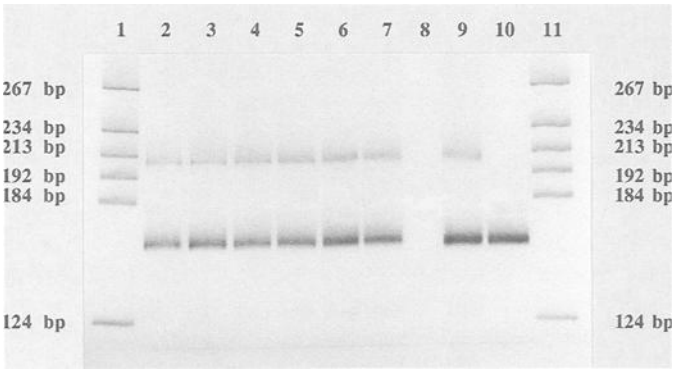

FIG. 1—*Silver-stained polyacrylamide gel displaying X- and Y-specific DNA sequences. Lane 1 and 11: size marker V with fragment lengths of 267, 234, 213, 192, 184, and 124 bp; lanes 2 to 7: presentation of both the X-specific 157 bp-fragment and the Y-specific 200 bp-fragment amplified from hair roots (lanes 2, 3), cigarette butts (lanes 4, 5), bloodstains (lane 6), and a blood sample (lane 7) from a male victim; lane 8: reaction negative control; lane 9: male control; lane 10: female control.*

Results

The appropriate conditions allowing coamplification of both Y-specific and X-specific DNA sequences were initially determined using DNA from whole blood samples taken from known individuals. The final conditions, described in Materials and Methods, permitted simultaneous amplification of two distinct X and Y sequences (X = 157 bp; Y = 200 bp) in male DNA, whereas female DNA presented only with the X-specific 157 bp-ragment missing the Y-specific 200 bp-amplification product. The sex-determination was successful in each of the 30 DNA samples and corresponded to the known sex of the donors.

The DNA extracted from a variety of forensic samples including bloodstains, vaginal swabs, cigarette butts, bones, and hair roots, gave clear sex-specific amplification bands and the results were in agreement with the known sex of the samples (Figs. 1, 2).

Figure 2 (lanes 10–12) demonstrates that both the X- and Y-specific primers showed visible bands even when less than 1 ng of template DNA was amplified. By exercising some precautions, such as using sterile solutions, wearing gloves, and pipetting in a separate PCR working area, contamination can usually be avoided. However, the possibility that the samples themselves may be a mixture of two tissues or contaminated by multiple handling cannot be eliminated. The primate specificity of the X- and Y-sequences presented here has recently been tested and confirmed by Gaensslen et al. [*5*].

Discussion

It would be expected that amplification of single-copy regions would require more template DNA than multiple-copy sequences to yield sufficient PCR product for detection on an ethidium bromide-stained gel. Witt and Erickson detected human specific centromeric alphoid repeats of X and Y chromosomes using PCR for sex determination [*12*]. Since this PCR analysis tests for the presence of repetitive sequences, the target DNA is abundant, and a few DNA molecules are sufficient to obtain positive signals. Gaensslen et al. succeeded in reducing the required DNA amount to 10 to 20 ng per PCR reaction and in the coamplification of X and Y sequences [*5*]. The PCR and electrophoresis conditions as well as the staining method described here, allow successful coamplification of both X- and Y-specific DNA sequences using less than 1 ng of template DNA. Fattorini et al. reported clearly detectable amplification products from 10 pg of genomic DNA [*6*]. However, their results are based on the reduction of the stringency conditions of the annealing temperature increasing the risk of producing non-specific extra bands.

DNA isolated from bloodstains, body fluid stains, postmortem tissues, and other specimens of forensic science importance contains not only a significant quantity of DNA from microbial and possibly other sources but also degraded DNA. Our results showed

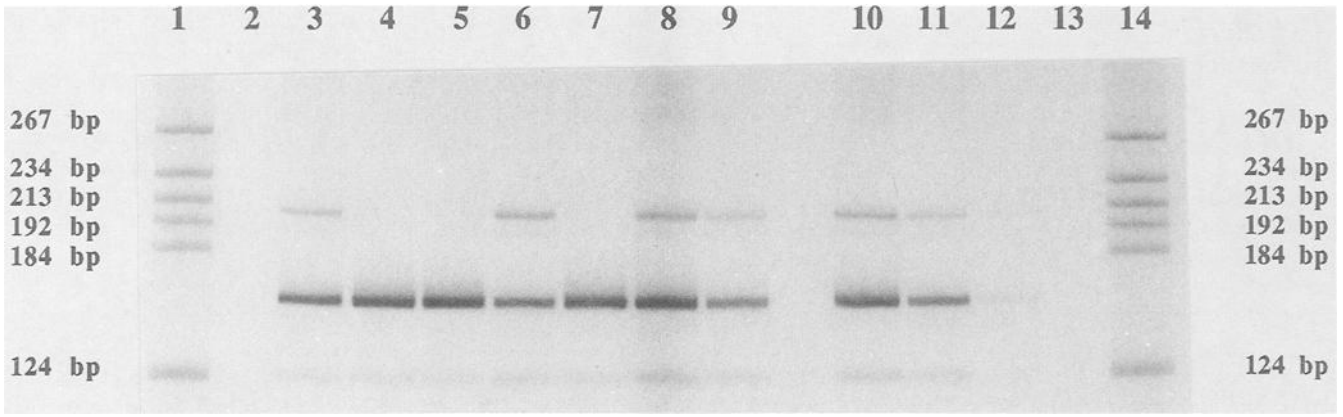

FIG. 2—*Sex determination in various biological samples and in different amounts of template DNA. Lane 1 and 14: size marker V with fragment lengths of 267, 234, 213, 192, 184, and 124 bp; lane 2 and 13: reaction negative control; lane 3: male control; lane 4: female control; lane 5: vaginal swab, female fraction; lane 6: vaginal swab, male fraction; lane 7: bone (femur) from a 20-year-old human female skeleton; lane 8 and 9: bone (femur and humerus) from a 20-year-old human male skeleton; lanes 10 to 12: amplification products resulting from 1 (lane 10), 0.5 (lane 11), and 0.1 ng (lane 12) of template DNA.*

that it was possible to identify the sex of 20-year-old bone DNA proving the X-Y-PCR test to be not only very sensitive but also very successful in amplifying degraded DNA.

Moreover, in a simultaneous amplification reaction of both X- and Y-specific DNA sequences, the successful amplification of X sequences serves as an internal control demonstrating the absence of polymerase inhibitors and the presence of sufficient target DNA.

Finally, the PCR procedure, in combination with polyacrylamide gel electrophoresis and silver staining is a technique that provides sufficient DNA for analysis and eliminates the need for isotopic detection.

In conclusion, our studies indicate that the coamplification of both X- and Y-sequences in a unique reaction mixture is a fast, human specific, sensitive and reliable method providing sex 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 research for the analysis of biological evidence.

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