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Sex determination of forensic samples: co-amplification and simultaneous detection of a Y-specific and an X-specific DNA sequence

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Summary. The detection of restriction fragment length polymorphisms (RFLP) (1) in DNA extracted from forensic samples remains impossible in a significant number of cases due to deterioration and contamination of the biological material and the extremely low quantities of DNA isolated. The polymerase chain reaction (PCR) is a recent and particularly convenient method for analysing and typing very small amounts (10–20 ng) of degraded human DNA (2, 3). DNA analysis at the level of a few cells present in forensic samples such as bloodstains, semen stains, vaginal swabs and head hair bulbs now appears possible using DNA amplification. A PCR protocol [4, 5] was adapted to simultaneously amplifiy a Y-specific DNA repeat sequence from the DYZ1 locus [6] and an X-specific DNA repeat sequence from the DXS424 locus [7]. The co-amplified Y-specific DNA fragment (102 bp) and X-specific DNA fragments (181–199 bp) were visualized on an ethidium bromide-stained 4% agarose gel. The male or female type of the amplified DNA extracted from blood samples, bloodstains, semen stains, vaginal swabs, brain tissue and 1, 2, 5, or 10 head hair bulbs was determined.

Key words: DNA – PCR – Co-amplification – DYZ1 locus – DXS424 locus – Sexing

Zusammenfassung. Der Nachweis von Restriktionsfragmentlängen-Polymorphismen (RFLP's) (1) aus DNA, welche von forensischen Proben extrahiert wurden, bleibt häufig unmöglich wegen Verschlechterung, Kontamination des biologischen Materials and extrem geringer Mengen, welche isoliert werden können. Die Polymerase-Kettenreaktion (PCR) ist eine neue und ganz besonders geeignete Methode, um sehr kleine Mengen (10–20 ng) degradierter DNA zu analysieren und zu typisieren (2, 3). DNA-Analysen auf der Ebene weniger Zellen in forensischen Proben wie Blutspuren, Samenspuren, Scheidenabstrichen und Haarwurzeln erscheint nunmehr möglich mit Hilfe der DNA-Amplifikation. Ein PCR-Protokoll (4, 5) wurde adaptiert, um gleichzeitig eine Y-spezifische DNA-Sequenz vom DYZ1-Locus (6) und eine X-spezifische DNA-Sequenz vom DXS424-Locus (7) zu amplifizieren. Das co-amplifizierte y-spezifische DNA-Fragment (102 Bp) und das X-spezifische DNA-Fragment (181–199 Bp) wurden mit einem Ethidiumbromid gefärbten 4% igen Agarosegel sichtbar gemacht. Der männliche oder weibliche Typ der amplifizierten DNA, welche von Blutproben, Blutspuren, Spermaspuren, Vaginalabstrichen, Hirngewebe und 1, 2, 5 oder 10 Haarwurzeln extrahiert worden war, wurde bestimmt.

Schlüsselwörter: DNA – PCR – Co-Amplifikation – DYZ1 locus – DXS424 locus – Geschlechtsbestimmung

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Introduction

Sex determination constitutes one of the first relevant steps during the identification analysis of forensic samples. Typing of male [X-Y] genotypes from stains present on exhibits is particularly informative when dealing with sexual assaults but can also be useful in criminal cases in general. In fact, obtaining a male genotype with DNA extracted from vaginal swabs constitutes a preliminary positive result in order to proceed with amplification of other informative and polymorphic DNA sequences such as HLA-DQ-Alpha (8), APO-B (9) and D1S80 (10) loci or typing of samples using single (11) and multi (1) -locus systems.

In recent years recombinant DNA techniques have been adopted to resolve forensic problems such as paternity determination, personal identification [1] and sex determination [12, 13]. Up to now positive identification of male or female genotypes was generally based on the detection of DNA restriction fragments with Y or X specific probes. Hybridization with Y-specific probes identifies relatively high molecular weight (HMW) DNA fragments of about 2–5 Kb [13, 14, 15 16].

In the present study we propose a very simple and rapid method for sex determination of forensic samples from various origins: blood samples, blood and semen

stains, vaginal swabs and head hair bulbs. We successfully co-amplified a Y-specific DNA repeat sequence located on the DYZ1 locus [6] and X-specific DNA repeat sequence located on the DXS424 locus [7] using the polymerase chain reaction (PCR) [4, 5]. The X and Y DNA fragments amplified are human specific and are visualized on ethidium bromide-stained agarose gels.

Materials and methods

DNA extraction and purification. DNA extraction was carried out by incubating each sample in standard lysis buffer containing 8 *M* Urea, 2% SDS, 10 m*M* EDTA, 0.3 *M* NaCl, 10 m*M* Tris-HCl pH 8.0 and 300 μg/ml proteinase K [17]. The incubation time and temperature of intermediate shaking were dependent of the origin of the sample (blood, bloodstains, semen stains, vaginal swabs or hair bulbs). Samples containing semen (vaginal swabs, semen stains) were differentially extracted: after a first incubation in standard lysis buffer and centrifugation, sperm nuclei were collected in the pellet and lysed by treatment with 2% SDS, 300 μg/ml proteinase K, 10 m*M* EDTA and 10 m*M* dithiothreitol (DTT) mixture (18, 19) for 2 hours at 65°C. DNA purification was performed by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v) to the sample for two 15 min periods, with constant agitation. The DNA extracted from blood or hair bulbs was then ethanol precipitated and pelleted by centrifugation.

The DNA solution obtained from blood or semen stains was dialysed against TE buffer and concentrated using Centricon™ AMICON 30 microconcentrator tubes. DNA aliquots were analysed for quality and quantity using ethidium bromide–stained agarose gel analysis but in the case of very low quantities of DNA (0.1 ng/μl) undetectable on yield gel we used the DNA DIPSTICK kit (Invitrogen Corp.) to evaluate the DNA concentration.

PCR Amplification. Purified DNA (20 ng) was amplified in a 25 μl reaction mixture containing 10 m*M* Tris-HCl pH 8, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* of each dNTP, 1 U Taq DNA polymerase, 0.1 μ*M* of each Y_{1-3} and Y_{1-4} primer and 0.4 μ*M* of each X_{145} and X_{146} primer.

The sequences of the Y-specific primers were two 21-mers [20]:

Y_{1-3}: 5′ ATT ACA CTA CAT TCC CTT CCA 3′
Y_{1-4}: 5′ AGT GAA ATT GTA TGC AGT AGA 3′

The sequences of the X-specific primers were two 20-mers [7]:

X_{145}: 5′ ACC TAG TTG GAG GCT ATG CA 3′
X_{146}: 5′ CCC AGT TAC TAA CAT CTA TG 3′

A denaturation step of 5 min at 94°C was followed by 40 cycles consisting of a denaturation step at 94°C for 1 min, an annealing step at 53°C for 1 min and an extension step at 72°C for 2 min. A final extension step of 10 min at 72°C ended the amplification reaction. PCR reactions were performed using a Perkin Elmer™ thermocycler (Cetus Cor., CA, USA).
Under these conditions, the X and Y DNA fragments amplified well and contamination problems never appeared.

Amplified DNA fragment analysis. The X (181–199 bp) and Y (102 bp) DNA fragments synthesized by PCR amplification were visualized and identified under UV light in an ethidium bromide stained 4% Nusieve agarose gel. A 10 μl aliquot of the reaction mixture was loaded into each well of the agarose gel.

Results

The appropriate conditions allowing co-amplification of both Y-specific and X-specific DNA sequences were initially determined with DNA extracted from blood samples (Fig. 1). Human DNA was prepared from blood samples taken at random from a French population. The final conditions, described in Materials and methods, permitted us to synthesize 2 distinct X and Y fragments (Y = 102 bp and X = 181–199 bp) when amplifying male DNA (Fig. 1, lanes M) and a single band corresponding to the X-specific DNA sequence (X = 181–199 bp) when amplifying female DNA (Fig. 1, lanes F).

However some females showed 2 slightly separated X-specific DNA fragments situated between 181 and 199 bp (Fig. 1 first lane F; Fig. 2, lane 4) due to the polymorphism (8 alleles) existing on the DXS424 locus [7].

Our [X-Y] PCR results were always in agreement with the known sex of the donors. A blind trial was carried out with a total of 117 individuals including 61 female samples and 56 male samples. Only 3 [X-Y] tests had to be repeated because of the absence of amplifica-

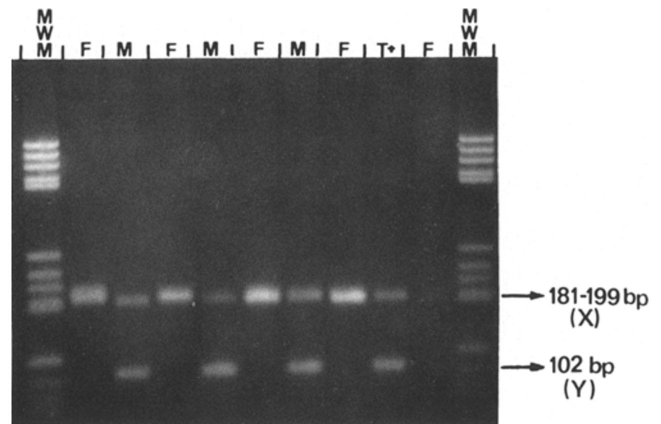

Fig. 1. [X-Y] PCR amplification of human DNA extracted from blood samples. The X and Y amplified DNA fragments are visualized on a 4% nusieve agarose gel. MWM = DNA molecular weight marker, Hae III digested pBR_{322}; lanes F = female DNA; Lanes M = male DNA. T^+ known male human DNA

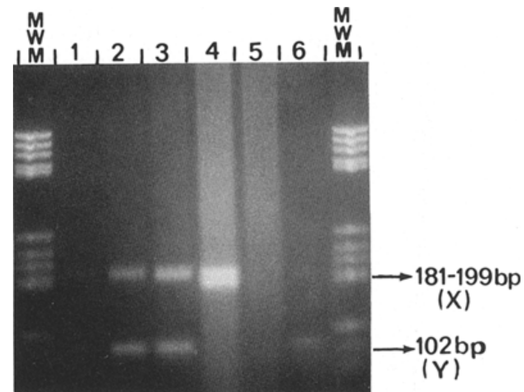

Fig. 2. [X-Y] PCR amplification of DNA extracted from forensic samples. The X and Y amplified DNA fragments are visualized on a 4% nusieve agarose gel. MWM = DNA molecular weight marker, Hae III digested pBR_{322}; lane 1 = vaginal swabs; lane 2 = bloodstain; lane 3 = semen stain; lane 4 = brain tissue; lane 5 = bloodstain determined as non-human; lane 6 = bloodstain

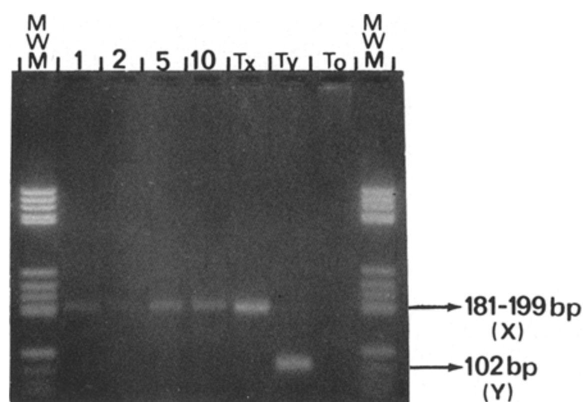

Fig. 3. [X-Y] PCR amplification of human DNA extracted from head hair bulbs. The X and Y amplified DNA fragments are visualized on a 4% nusieve agarose gel. MWM = DNA molecular weight marker, Hae III digested pBR_{322}; 10 µl aliquots of [X-Y] amplified DNA after extraction from one head hair bulb (lane 1); 2 head hair bulbs (lane 2); 5 head hair bulbs (lane 5); 10 head hair bulbs (lane 10). Lane Tx = X amplification control; Ty = Y amplification control; To = absence of DNA

tion which was due to in-appropriate DNA quality or DNA concentrations.

In each [X-Y] amplification experiment positive and negative controls were run simultaneously with the test samples. As a positive control, known DNA was amplified for the X and Y-specific fragments (Fig. 1; lane T^+; Fig. 3 lanes Tx and Ty). Negative controls were performed in the absence of DNA (Fig. 3; lane To) in order to detect possible contamination problems.

The human specificity of our [X-Y] test was also confirmed by testing different kinds of non-human male and female DNA from sheep, rabbit, pig, cow, mouse and monkey. None of these DNAs could be amplified (data not shown) in the presence of the X and Y-specific primers used in this study (see under Materials and methods).

In current forensic investigations, results were obtained from 65 vaginal swabs or semen stains, 36 bloodstains, 12 muscle samples, 4 brain samples, 3 bone samples and 1 hair sample.

Sex determination of forensic samples in sexual assault cases can be very relevant and even essential when the first step analysis of microscopical identification of spermatozoa is confirmed by the [X-Y] PCR test realised on the further extracted DNA (Fig. 2, lane 3). Furthermore, the [X-Y] PCR test becomes informative in sexual assault cases when a male genotype can be obtained even though spermatozoa are microscopically absent. Such an example is shown on Figure 2, lane 1 where no spermatozoa were detected but a male [X-Y] profile was observed on the agarose gel. The two X and Y DNA bands are faint but are easily visualized under U.V. light.

In contrast, microscopical identification of only a few spermatozoa does not always lead to the isolation of sufficient male DNA and the detection of a female [X-X] genotype characterizes the DNA of the victim (vaginals cells). In such cases, further investigations are superfluous.

As an example, female DNA was isolated from a single head hair bulb and a fraction was used for a [X-Y] PCR test (Fig. 3, lane 1). Enough DNA was extracted from the same single head hair bulb to carry out 2 additional amplification tests for the HLA.DQ-Alpha and the D1S80 loci.

Discussion

The sensitivity of the [X-Y] PCR test is high and the amplified fragments can be obtained from less than 20 ng of purified DNA corresponding to 30–50% of that isolated from a single hair bulb (Fig. 3). This also corresponds to the lowest quantity of DNA used for sex determination compared to the 1.5 µg of HMW DNA necessary for Southern hybridization methods (15, 16) or the 50 ng of DNA used in dot hybridization procedures (13). A second interesting aspect of this PCR test is the possibility to use degraded genomic DNA for amplification owing to the short fragment size of the analyzed sequences in contrast to fragment sizes between 3 and 5 Kb when studying restriction DNA fragments [14, 15, 16].

Furthermore, the human specificity of the test allows us to determine, in the first step of the investigations, the human or non-human origin of stains present on forensic samples. The [X-Y] PCR method is also a means to avoid the problems of specificity often encountered when identifying DNA sequences with DNA probes where the specificity can change depending on the stringency conditions or concentrations of DNA. Low stringency conditions or excess DNA in dot blot experiments can lead to mistakes and false male positives [14]. Nevertheless the dot hybridization procedure can be a rapid (1 to 2 days) and sensitive (detection of 45 ng of genomic DNA) hybridization method but still requires the labelling of probes and relatively HMW DNA (13).

Co-amplification of the X and Y sequences in a unique amplification reaction offers advantages such as saving of time, reagents and DNA in order to have enough DNA left for additional amplification reactions and AMP-FLP studies. In fact the coamplification method eliminates the need for multiple PCR tests to amplify each of the two X and Y DNA fragments as in recent reports [21].

Finally, co-amplification of both X an Y DNA sequences in a unique reaction mixture using only one DNA sample is a fast, low cost, human specific and reliable sex determination method provided that appropriate controls are carried out and that each step of the amplification experiment is run carefully and scrupulously.

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