TECHNICAL NOTE

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PCR Amplification of Animal DNA with Human X-Y Amelogenin Primers Used in Gender Determination

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ABSTRACT: The X-Y homologous gene amelogenin has been described for use as a PCR sex typing test for humans. Previous studies have revealed that appropriate primers yield a 106 and 112 bp fragment from the human X and Y chromosome respectively. Our studies have found that a PCR product is also obtained from DNA isolated from a number of common animals. This animal PCR product is very close in size to that of the human female PCR derived product and may be confused as human under routine agarose gel electrophoresis. In this report we detail the variety of animals examined and a method that can maximize the usefulness of this system.

KEYWORDS: criminalistics, amelogenin, sex testing, PCR, conserved sequence

The gender determination of the donor of a body fluid stain can be an important piece of information in the analysis of forensic case work. A number of PCR based methods are available to identify a sample as originating from a human male or female [*1–5*]. Most PCR sex typing systems rely on two primer sets, one to amplify a portion of the Y chromosome if present, while the other acts as a control and amplifies a portion of the X chromosome. A method recently described by Sullivan et al. [*6*]. uses one set of primers to amplify a small region of the X-Y homologous amelogenin gene, yielding a 106 bp fragment from the X chromosome and a 112 bp fragment from the Y chromosome. The amplification of a portion of an X-Y homologous gene offers a sex typing system with an internal positive control from the X chromosome having exactly the same amplification requirements as the diagnostic fragment from the Y chromosome. In addition, this system amplifies a short DNA sequence which should be useful for forensic samples containing highly degraded DNA. Mannucci et al. [*7*] suggest that this primer set can be used for sex and species identification by co-amplification with DQ alpha primers.

Amelogenin is an important protein in the developing mammalian tooth enamel matrix [*8–10*]. It is a hydrophobic protein that constitutes about 90% of the organic matrix found in developing tooth enamel and may regulate the size and growth of calcium hydroxyapatite crystals [*11*]. The gene for amelogenin has been studied in murine [*12–14*], bovine [*15–18*], porcine [*19*] and human [*8,9,20,21*] species. While the human [*19*] and bovine [*18*] gene is found on the X and the Y chromosomes, it appears to be located only on the X in mice [*13*] and on the autosomes of monotremes and marsupials [*22*]. Large portions of the protein sequence are highly conserved among the species examined [*19*]. The use of a cDNA probe indicates that within vertebrates large portions of the amelogenin gene has been conserved during evolution [*23*].

We have found that the area of the gene amplified by the primers detailed by Sullivan et al. [*6*], while part of the noncoding region of the gene [*20*], is also highly conserved. A PCR product is generated from the DNA of a number of common animals that is similar in size to that obtained from the human X chromosome. This animal PCR product could be mistaken for human during routine agarose gel electrophoresis. In this report we discuss the animals surveyed and some measures that can be taken to minimize misinterpretations.

Materials and Methods

The primers previously described [*6*] were synthesized at the University of Vermont's Zoology Department using an Applied Biosystems model 931 EP DNA synthesizer employing the phosphoramidite method. The primers were used after a butanol purification [*24*]. DNA was extracted from blood patched on cotton cloth, dried and stored at −20°C or muscle tissue stored at −20°C (kindly provided by the Vermont Fish and Wildlife Department and local veterinarians) using the proteinase K digestion and phenol-chloroform extraction method [*25*]. The aqueous phase from the organic extraction was concentrated using a microcon 100 mini-concentrator (Amicon). The concentrate was washed three successive times with 450 μL of 10 mM Tris/hydrochloric acid, 1 mM sodium (Na_2) ethylenediaminetetraacetic acid (EDTA), pH 7.5 (TE), to yield a final volume of 40 μL. The DNA concentration was estimated by ultraviolet visualization of DAPI (4′,6-diamidino-2-phenylindole) [*26*] or ethidium bromide stained DNA minigels [*25*]. Generally, a sample containing 10 ng of DNA was used per amplification. Each 50 μL reaction tube contained 0.2 μM of each primer, 0.2 mM of each deoxyribonucleoside triphosphate (Sigma), 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin (Sigma) and 2 units Taq polymerase (BRL). The amplification was slightly different than that described in the original work

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[6] to accommodate the difference in thermocyclers. The following PCR conditions were used for a Perkin Elmer 9600 thermocycler: 94°C for one minute, followed by 35 cycles of a 15 second denaturation at 94°C, 15 second annealing at 60°C, 15 second extension at 72°C, followed by an 8 minute final extension at 68°C. Samples were then held at 4°C until analysis.

The PCR products were analyzed using a 4.5% Metaphor™ (FMC) agarose minigel (6 × 8 cm) in 45 mM Tris-HCl, 45 mM boric acid and 1 mM EDTA, pH 8.0 (TBE) with 0.1μg/mL of ethidium bromide in the gel and tank buffer [27]. Twelve microliters of sample were combined with a loading buffer containing 15% ficol [24] and the tracking dye xylene cyanol. Electrophoresis was conducted at 125 volts for one hour and twenty minutes. The products were observed with UV transillumination.

Results and Discussion

The amplification of human DNA always gave the expected PCR fragments depending on the sex of the donor (data not shown). No anomalies in the size of the PCR products were observed in any human sample. The amplification and electrophoresis of a number of common animal DNA samples produced a single fragment very similar in size to the 106 bp fragment expected from the human X chromosome (Table 1, Fig. 1). Of those animals yielding a PCR product, only a single band of 102–103 bp was obtained regardless of the sex of the animal, bovine and equine male and female product are shown (Fig. 2). As Table 1 details, no PCR product was obtained from some of the animals examined.

To confirm that the animal and human PCR products were indeed different, animal and human male PCR products were mixed and subjected to electrophoresis (Fig. 2). Three clearly defined

TABLE 1—*Results of amplification and size of PCR product of animal DNA.*

Animal	Amplification (+ or −)	Size
Racoon (P)	+	102–103 bp
Mouse (P)	−	NA
Squirrel (P)	−	NA
White-Tail Deer Male (T)	+	102–103 bp
White-Tail Deer Female (T)	+	102–103 bp
Domestic Cat Male (P)	+	102–103 bp
Domestic Cat Female (P)	+	102–103 bp
Otter (P)	−	NA
Moose Male (T)	+	102–103 bp
Moose Female (T)	+	102–103 bp
Domestic Turkey Male (P)	−	NA
Swine (P)	+	102–103 bp
Domestic Dog Female (P)	+	102–103 bp
Bovine Female (P)	+	102–103 bp
Bovine Male (P)	+	102–103 bp
Bobcat (P)	+	102–103 bp
Bear Male (T)	+	102–103 bp
Domestic Horse Male (P)	+	102–103 bp
Domestic Horse Female (P)	+	102–103 bp
Porcupine (P)	+	102–103 bp
Chicken (P)	−	NA
Sheep (P)	+	102–103 bp
Beaver (P)	−	102–103 bp
Rabbit (P)	−	NA
Goat (P)	+	102–103 bp

NOTE: P = Blood preserved on a cotton patch. T = Meat, shaving from frozen sample. Unless otherwise noted, the morphological sex of the animal is not known.

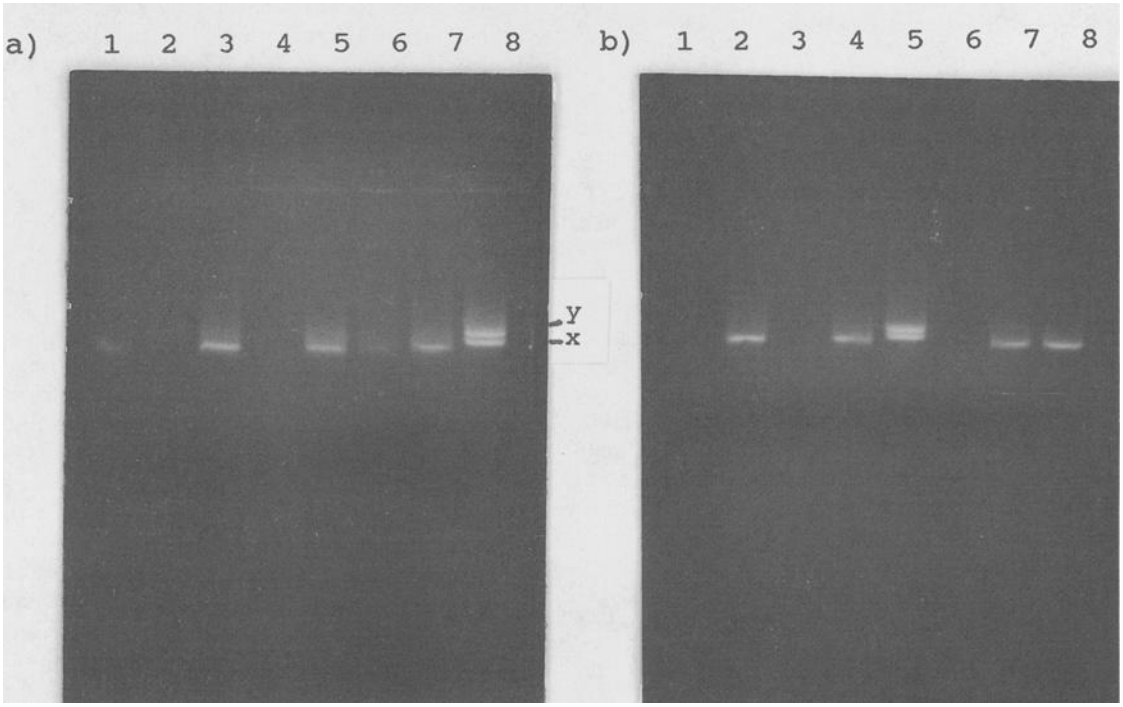

FIG. 1—*Agarose minigels of PCR products from human male and various animal DNA templates. Gel a) lanes 1–8 contain goat, porcupine, bobcat, mouse, racoon, cat female, dog female and human male respectively. Gel b) lanes 1–8 contain turkey male, swine, beaver, sheep, human male, rabbit, deer male and deer female respectively. In all DNA samples evaluated, 10 ng of template DNA was amplified and 12 μL of the amplified product was loaded per well. Human male DNA is used as the molecular weight marker, "y" marking the 112 bp band from the Y chromosome and "x" marking the 106 bp band from the X chromosome.*

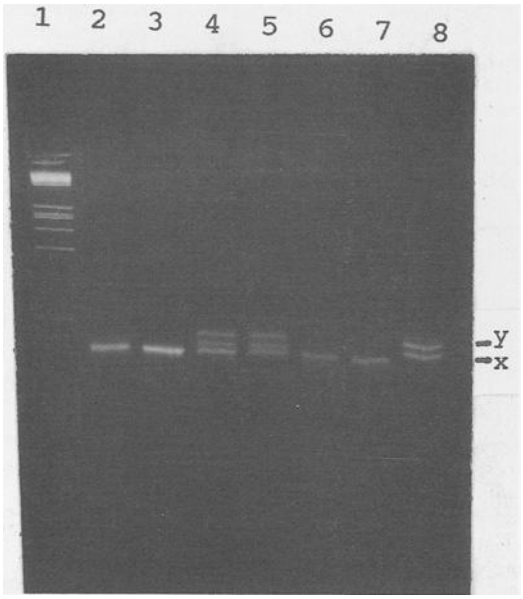

FIG. 2—*Agarose minigel of PCR products from known male and female bovine and equine DNA. Lanes 1–8 contain ØX174-HaeIII, horse male, horse female, mixture of human male and horse male, mixture of human male and bovine female, bovine male, bovine female and human male. Mixtures contain 6 μL of each amplification. The human male sample is used as the size marker for comparison, "y" marking the 112 bp band from the Y chromosome and "x" marking the 106 bp band from the X chromosome.*

bands were observed after electrophoresis demonstrating that the animal band is definitely different in size (Figure 2, lanes 4 and 5). This animal fragment is estimated to be only 2 or 3 base pairs smaller than the human 106 bp band.

An effort was made to eliminate the animal derived products by raising the annealing temperature of the PCR reaction and by limiting the amount of template DNA used for amplification. An increase in the annealing temperature from 60°C to 62°C and then to 64°C did not eliminate the animal products. An increase from 60°C to 66°C eliminated the production of any products, including those from the human samples. Restricting the amount of animal template DNA still resulted in animal PCR products. Successful amplification of male and female bovine and equine DNA at the 1 ng level was obtained (data not shown).

These results suggest that the animal band is indeed a specific product of the primers derived from the human sequence and the amelogenin homolog in the animals. The single size fragment in female and male suggests that in species such as bovine, shown to have X and Y homologs [*18*], the region amplified shows no sex differences that can be exploited for gender identity. It may be that in a number of these animals the gene is only found on the X chromosome, which would also result in the lack of gender differences.

The amelogenin gene is highly conserved, such that the bovine, porcine, murine and human amino acid sequences show identity in the first 28 N-terminal amino acids [*19*]. Nakahori [*20*] used a probe in Southern blot analysis that spanned a large portion of the amelogenin gene and found that it hybridized to primate, mouse, rat and cattle DNA. The small region of the gene examined in our study is in a non-coding region [*20*] but is obviously still well conserved. To date sequencing of animal amelogenin has been confined to the analysis of cDNA in a few model systems [*12,15,17,18*], so direct comparison of the sequence of the intron region amplified by these primers is not possible.

The generation of a PCR product so similar to the human X chromosome product complicates but does not render the system unusable for forensic work. Contamination of a forensic specimen with animal DNA could lead to confusion or misinterpretation. Even if a specimen is judged to be of human origin or at least contain human protein/DNA it may also harbor trace quantities of animal DNA that could yield an animal PCR product through the very sensitive PCR procedure. By optimizing electrophoretic conditions, one can achieve resolution of the PCR products to reveal well separated bands. Single band products must be adequately evaluated using gel systems that can resolve the small 2–3 base pair differences observed between the human X chromosome and the animal product. To ensure that this resolution is attained, we routinely include a mixture lane on each gel consisting of human male and animal products as demonstrated in Fig. 2, lanes 4 and 5. Comigration of unknowns with animal PCR product could also be a useful means for positive identification. We have tried acrylamide gel systems as an alternative to agarose but have not been satisfied with the resolution compared to that obtained with Metaphor™. A new product from AT Biochem (RF7) is a proprietary blend of acrylamide type monomers that offers an enhanced resolution of small DNA fragments. We have had some success with this product, it may be a useful alternative to the agarose system described here.

The PCR examination of forensic samples to identify the gender of the donor is a worthwhile endeavor. With the appropriate standards and electrophoretic conditions, bands arising from forensic samples can be correctly identified allowing one to use this system to its full potential.

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