CASE REPORT

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Origin and Gender Determination of Dried Blood on a Statue of the Virgin Mary*

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ABSTRACT: In Italy, blood exudation from objects of worship recurs frequently in ancient chronicles and literature, in popular beliefs, and even in modern mass-media reports. This phenomenon, that was associated with epochal or catastrophic events, has roots that reach classical antiquity. In the last few years, several events connected with the detection of bloody "tears" on statues of the Virgin Mary required forensic medicine investigations. In the present report we describe genetic investigations conducted on dried blood of unknown derivation found on a statuette representing the Virgin Mary. To test the human or animal origin of the blood, we amplified *Alu*-specific sequences from DNAs obtained from the unknown sample and from humans, large apes, various Old and New World monkeys, a prosimian, mouse, common domestic artiodactyls and chicken. This investigation restricted the range of possible origin of the statue blood to humans, apes and Old World monkeys. To test the male or female origin of the blood, we used a multiplex nested polymerase chain reaction method, that allows the simultaneous amplification of the X-specific locus DXZ4 and of the Y-specific locus SRY. Considering the unlikelihood of an origin from simian Old World primates, the exclusive amplification of the X-specific product from the unknown sample and from human female blood controls, compared to the amplification of distinct X- and Y-specific bands from human male blood controls, strongly supports a human female origin of the statue blood.

KEYWORDS: forensic science, DNA typing, polymerase chain reaction, *Alu* DNA sequences, primates, SRY, DXZ4, Virgin Mary, statue, dried blood, gender determination

Mention of phenomena related to the exudation of blood from objects of worship, including in particular statues or icons representing divinities or saints, recur frequently in ancient chronicles, literature, historical documents, popular beliefs, and even in press or modern mass-media reports from Western countries, and particularly Italy. As many other exterior manifestations of popular religious beliefs, this phenomenon has deep roots that reach classical antiquity and perhaps even preclassical cults of the Mediterranean area (1–3). In the past, blood exudation from cult images was associated with epochal or catastrophic events, including pestilence, war, and death of charismatic personalities. For example, going back to Ancient Rome, blood exudation from the cult statue of *Iuno Sospita* in the sanctuary of Lanuvium is reported by Titus Livius among several prodigious phenomena presaging the second Punic war (1). Other more recent examples come from our own region of Abruzzo, Central Italy (3). In the village of Ripateatina near Chieti, a painting depicting the crucifixion exuded blood on the occasion of a raid of Turkish pirates in 1566. In 1728, in the same village, a cult statue of the Virgin exuded blood. A series of other episodes of the same type is documented up to the present in various villages of our region (3).

Recently, several episodes connected with the finding of blood stains or bloody tears exuding from statues and statuettes representing the Virgin Mary attracted wide mass-media coverage in Italy, eliciting manifestations of popular religious enthusiasm and possibly precipitating fraudulent or psychopathic emulation. In this respect, it should be noted that the abuse of popular credence is considered a crime according to Italian law.

We were recently involved in investigations concerning the nature of what appeared to be dried blood widely staining a statuette of the Virgin Mary. The problems that were brought to our attention concerned the identification of the origin of the blood and, if human, the determination of gender. Techniques based on Southern blot hybridization (4) or DNA fingerprinting (5) were inadequate because of the scarcity and partially degraded state of the genomic DNA recovered from the dried blood stains (6). This required the use of PCR-based analytical techniques.

The primate-specific *Alu* family of short interspersed repetitive elements is widely represented in the human genome (7). PCR-based methods targeting human *Alu* sequences may contribute to the evaluation of biological samples of suspected human origin. Thus, to test the human or animal origin of the dried statue blood, we analyzed, *Alu*-specific amplification products obtained from the unknown sample and from genomic DNAs of humans, various other mammals including a number of non-human primates, and a bird. Gender determination from blood samples often represents a crucial point in the context of forensic investigations. Presently,

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different PCR-based strategies, targeting either fixed sequence differences in X-Y homologous regions or distinct X- and Y-specific sequences, are available to identify the gender of human biologic samples (8–14). We addressed the problem of gender determination developing a multiplex nested PCR assay (15) targeting X- and Y-specific sequences (16–17).

Materials and Methods

Non-Human DNA Samples—Control primate tissue specimens had been collected by one of the authors (R.M-C) between 1984 and 1986, thanks to the kind cooperation of H.M. McClure and C. Allen, Yerkes Regional Primate Center, Atlanta, Ga.; M.K. Izard, P. Wright and M. Pereira, Duke University Center for the Study of Primate Biology and History, Durham, N.C.; M.W. Terry, Primate Information Center, Regional Primate Research Center, Seattle, Wash.; T.M. Butler, Southwest Foundation for Biomedical Research and Education, San Antonio, Tex., and A. Lock and E. Scherer, Division of Research Service, Veterinary Resources Branch, National Institute of Health, Bethesda, Md (18). These tissue samples were registered according to present CITES regulations. Samples of tissues from mouse, ox, pig, sheep, and chicken were commercially acquired. Genomic DNA from animal tissues was extracted using standard procedures (19).

DNA Extraction from Statue Blood and Human Control Blood—To minimize the risk of contamination with external DNA, operators utilized sterile and disposable gloves, sterilized and UV-irradiated scalpels, forceps, distilled water and buffers. Minute samples of dried blood were carefully scraped from different areas along the path of the blood flow. Dried blood (approximately 100 mg) was incubated at 37°C for 48 h in 10 volumes of lysis buffer consisting of 0.5 M EDTA (pH 8.5), 0.5% N-lauroylsarcosine and 100 μg/mL of proteinase K. The aqueous phase was recovered and DNA was precipitated adding 1 volume of isopropanol and 0.2 M sodium acetate. The pellet was purified using the QlAmp Blood Kit 50 (Qiagen Inc., Chatsworth, CA) according to manufacturer's specifications. This method yields 2–6 μg of DNA from 100 μL of blood. Purified DNAs were resuspended in 100 μL of 10 mM Tris-HCl (pH 9) and stored at 4°C, while 10 μL aliquots were monitored by electrophoretic fractionation on 2% agarose minigels, followed by ethidium bromide staining. Human DNAs, extracted using the same procedure from male and female clotted blood, were used as controls. DNA extractions and set-up of PCR reactions were performed in a dedicated laboratory, distinct from that in which amplified DNAs were manipulated.

PCR Amplifications for Alu, *DXZ4 and SRY Sequences*—PCR amplification for *Alu* primate-specific sequences was carried out following the protocol described by Tagle and Collins (20), with minor modifications. Briefly, PCR (30 cycles) was performed in 10 μl reactions containing 1 μL of resuspended DNA solution (approximately 20 ng of DNA), 250 ng of each primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 2 mM dithiothreitol, 200 μM dNTPs, 0.5 units Amplitaq polymerase (Perkin-Elmer-Cetus, Norwalk, CT). Primers used for amplification of *Alu* sequences were: 5′-GGATTACAGGCGTGAGCCAC-3′ (forward) and 5′-GATCGCGCCACTGCACTCC-3′ (reverse). PCR amplifications for DXZ4 and SRY sequences were performed in 10 μl of reaction buffer containing 1 μL of resuspended DNA solution, 200 μM of each dNTP, 6.7 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 6.7 mM MgCl2, 10 mM β-mercaptoethanol, 6.7 μM EDTA and 1.2 U of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT). PCR amplification protocols were based on the sequential use of external and internal sets of primers, designed to allow the simultaneous amplification of DXZ4 and SRY genes in a single reaction tube (multiplex nested PCR). The sizes of the final amplification products were selected to allow clear visibility following electrophoretic fractionation in an ethidium bromide-stained agarose gel. The male-specific external SRY primers were 5′-TAAAGGCAACGTCCAGGATAGAG-3′ (forward) and 5′-TAAGCATTTTCCACTGGTATCCCAG-3′ (reverse); the external DXZ4 primers were 5′-TGTCCTTGGCAGATGG-3′ (forward) and 5′-TCTCCTTCTCGTCCCTGCG-3′ (reverse). For external multiplex PCR, samples were denatured at 94°C for 5 minutes, followed by 26 cycles of amplification, comprising 90 sec at 59°C, 90 sec at 72°C, and 1 min at 94°C. The concentration of each primer was 0.25 mM. The internal multiplex PCR used one microliter (1 μL) of a 1:1000 dilution of external PCR product as a template. Primers employed for internal SRY amplification were: 5′-AACG-CATTCATCGTGTGG-3′ (forward) and 5′-TTGCTGATCTCT-GAGTTTCG-3′ (reverse). These primers yield a product of 83 bp. Internal DXZ4 primers were 5′-TTGGCAGATGGGACTCGG-3′ (forward) and 5′-ACCATCCTCCCATCCTAACG-3′ (reverse). These primers yield a product of 137 bp. The concentration of each internal primer was 0, 15 mM. Cycling conditions were as for external PCR, except that the annealing temperature was 60°C and, to exclude allelic drop-out due to stochastic variation, only 19 cycles were performed. To avoid non-specific annealing during the first PCR cycle, samples were placed in the DNA Thermal Cycler (model 480, Perkin-Elmer-Cetus, Norwalk, CT) only when the temperature reached 94°C. The products of the PCRs were fractionated by electrophoresis on agarose gels containing 3% Seakem (FMC Bioproducts; Rockland, ME) and visualized by ethidium bromide staining using *Hae* III digest of ΦX 174 (Boehringer Mannheim, Milano, Italy) as molecular-weight standard. Experiments were performed in duplicate using independent DNA extractions.

Results and Discussion

In order to test the primate specificity of the dried blood sample scraped from the statue we analyzed PCR amplification products of *Alu* sequences obtained from the statue blood, from human genomic DNAs and from genomic DNAs of chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), mandrill baboon (*Papio sphynx*), long-tailed macaque (*Macaca fascicularis*), squirrel monkey (*Saimiri sciureus*), lesser bushbaby (*Galago senegalensis*), mouse (*Mus musculus*), ox (*Bos taurus*), pig (*Sus scropha*), sheep (*Ovis aries*) and chicken (*Gallus gallus*). Primers and PCR conditions were as described (20). The results are illustrated in Fig. 1. *Alu* amplification products were obtained using DNAs from the statue blood (lanes 14 and 15), human blood (lane 2), apes (*Pan troglodytes*, *Gorilla gorilla*, *Pongo pygmaeus*, lanes 3, 4 and 5), and Old World monkeys (*Papio sphynx* and *Macaca fascicularis*, lanes 6 and 7). No amplification products of *Alu* sequences were obtained using DNA from the New World monkey *Saimiri sciureus* (lane 7), from the prosimian *Galago senegalensis* (lane 8) and from mouse (*Mus musculus*, lane 9), cow (*Bos taurus*, lane 10), pig (*Sus scropha*, lane 11), sheep (*Ovis aries*, lane 12) and chicken (*Gallus gallus*, lane 13). From these results it can be concluded that the statue blood originated from humans or from a non-human catarrhine primate. Obviously, the possibility that the statute blood derived from an ape

FIG. 1—*PCR amplification of* Alu *sequences from the dried statue blood, from human genomic DNA and from genomic DNAs of non-human mammals and a bird. Lane labeled M contains molecular weight marker (*Hae *III digest of* ΦX *174); lanes 1 to 16 contain* Alu *amplification products from: 1, human (*Homo sapiens*)-2, chimpanzee (*Pan troglodytes*)-3, gorilla (*Gorilla gorilla*)-4, orangutan (*Pongo pygmaeus*)-5, mandrill baboon (*Papio sphynx*)-6, long-tailed macaque (*Macaca fascicularis*)-7 squirrel monkey (*Saimiri sciureus*)-8, an African prosimian (*Galago senegalensis*)-9, mouse (*Mus musculus*)-10, ox (*Bos taurus*)-11, pig (*Sus scropha*)-12, sheep (*Ovis aries*)-13, chicken (*Gallus gallus*)-14 and 15, different samples of dried statue blood-16, PCR reagents (primers included) without DNA.*

or an Old World monkey should be considered highly unlikely, because of the rarity of these exotic primates in Italy.

To determine the gender of the dried statue blood we developed a multiplex nested PCR assay targeting the X-specific macrosatellite VNTR DXZ4 and the Y-specific SRY gene in the same reaction tube. Under the PCR conditions described above, the yield of the internal Y-specific 83 bp and X-specific 137 bp amplification products was sufficient to consistently allow clear visibility following electrophoresis in an ethidium bromide-stained 3% agarose gel. The results of a nested DXZ4 and SRY PCR amplification from statue blood and control human blood are illustrated in Fig. 2. Lanes 1 and 2 show the amplification products obtained using DNA extracted from control clotted blood of two human males, whereas the results of the PCR amplification from DNA extracted from control clotted blood and two human females are illustrated in lanes 3 and 4. Two bands with molecular weights corresponding to the amplification products of the SRY (83 bp) and DXZ4 (137 bp) sequences were detected in DNA from control blood of human males (lanes 1 and 2). Only one band, with molecular weight corresponding to the DXZ4 amplification product, was detected in lanes 3 and 4, containing DNA from control blood of human

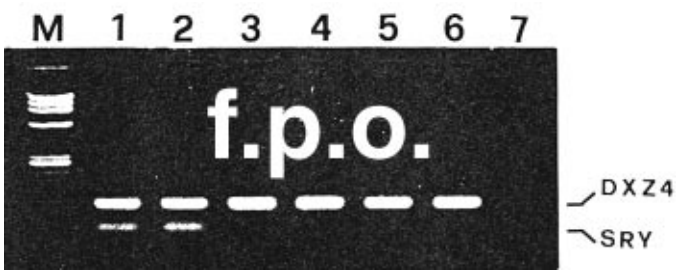

FIG. 2—*Gender determination of the dried statue blood and of male and female human control blood by multiplex PCR. Lane labeled M contains molecular weight marker (*Hae *III digest of* ΦX *174); lanes 1–7 illustrate the amplification products obtained from: control human genomic DNAs (2 males: lanes 1 and 2 and 2 females: lanes 3 and 4); DNAs extracted from the dried statue blood (lanes 5 and 6), PCR reagents (primers included) without DNA (lane 7).*

females. Lanes 5 and 6 show the amplification products obtained using DNA extracted from samples of dried blood scraped from the statue. A single band of the same size of the DXZ4 amplification product was detected in lanes 5 and 6, while no bands corresponding to the SRY amplification product could be observed. Lane 7, corresponding to the amplification control performed using all the PCR reagents without DNA template, contained only a band related to the PCR primers not utilized for amplification. Different PCRs, performed using DNA extracted from blood recovered from different areas of the statue, confirmed the above reported results (data not shown). While DXZ4 and SRY multiplex amplification may be used for gender determination, it cannot be employed to determine the human origin of the statue blood. In fact, in control experiments, we obtained amplification products in the range of the human DXZ4 and SRY bands from apes, old World monkeys, the prosimian *Galago senegalensis*, mouse, ox, pig and sheep.

In conclusion, after the analysis of *Alu* sequences and of X- and Y-specific sequences we were able to prove that the statue blood derived from an Old World simian primate of female gender, most probably a human female. Since the present study reports a single application of the above described techniques, additional validation would be needed for routine applications.

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