TECHNICAL NOTE

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Natural DNA Mixtures Generated in Fraternal Twins *in utero*

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ABSTRACT: Analysis of multiple genetic loci using short tandem repeats (STR) is widely used in human identity testing because the extensive polymorphism at these loci allows for a high degree of discrimination among individuals. We recently received a forensic case that included several pieces of evidence and reference blood samples. Upon initial testing, one of the suspects had a DNA profile that included three alleles at four of the nine loci tested (vWA, FGA, TH01, and D5S818). At each locus, two of the alleles appeared to be "major" alleles with a third "minor" allele present. The profile appeared to be a mixture of two people. Contamination of this first reference sample was suspected and a second, unopened blood specimen was requested from this individual. The DNA profile from this second reference specimen was identical to that of the original specimen at each locus. One of the evidence samples also displayed an identical mixed DNA profile matching that of the reference specimens mentioned above. The relative peak heights of the two "major" and one "minor" allele remained constant in all three samples. Additional background information revealed that the suspect had not received a bone marrow transplant or blood transfusion. However, it was disclosed that this individual is a fraternal (dizygotic) twin. We hypothesize that an exchange of blood cells between the fetuses occurred *in utero* and that the additional alleles present in these reference samples are derived from cells contributed by his twin sibling. No additional specimens from the suspect or his twin could be obtained for confirmation, and our hypothesis remains untested. Forensic scientists should be aware of this possibility when faced with a DNA profile in which extra alleles at multiple loci are detected.

KEYWORDS: forensic science, short tandem repeat, STR, polymerase chain reaction, DNA, identity testing, fraternal twins, dizygotic

Microsatellites or short tandem repeats (STR) have been mapped throughout the human genome (1,2). Because of their polymorphism and well-characterized genetic inheritance patterns, STR loci have become widely used for identity testing (1–10). Individuals inherit a single genetic copy from each parent and the presence of more than two alleles is suggestive of a mixture. There have been rare instances where a third allele has been identified at a particular locus, most frequently TPOX (11). Such cases may be attributed to genetic duplication of a small chromosomal region containing the STR locus, or the improper segregation of an entire chromosome, (e.g., trisomy). Such events usually are restricted to a single chromosome, resulting in atypical results at a single STR locus.

Dizygotic (fraternal) twins, present a unique situation. The rate for such events in the United States and Europe is estimated to be between 7–11 per 1000 births, while the rate in African blacks (Nigeria) is 45–50 per 1000 births. Dizygotic twins, as the name implies, arise from the independent fertilization of two ova by separate sperm cells. Each zygote will develop it's own placenta, but varying degrees of placental fusion have been observed. In a subset of cases, such twins can have fusion of their chorionic membranes, with the subsequent development of anastomoses between the vasculature of the twins. In such instances, there can be an exchange of hematopoietic cells between these two genetically distinct individuals. Since this exchange occurs *in utero* while the twins are immunologically tolerant, engraftment of the blood forming stem cells can occur. This naturally occurring mixture, referred to as chimerism, has been described infrequently. Documentation of such chimerism has been done through cytogenetic studies, red blood cell grouping, or DNA typing of VNTR loci (12–18). In one case, chimerism was detected in a healthy woman's peripheral blood lymphocytes by demonstrating a *male* karyotype using cytogenetic methods (18). Chimerism has also been observed in certain clinical settings such as following bone marrow or solid organ transplantation (19–21). In these circumstances the mixture is iatrogenic and relates to the medical procedures used to treat the patient.

This phenomenon of hematopoietic chimerism is not unique to humans. Cotton-top Tamarin monkeys are a naturally occurring chimeric species that are usually born as dizygotic twins. Due to placental vascular anastomoses, they frequently share bone marrow elements with their siblings (22).

Recently while analyzing specimens for a forensic case, we identified an evidentiary specimen that appeared to be an unbalanced mixture from two sources. The minor profile alleles were detected at four of the nine STR loci tested. Analysis of the reference specimens also revealed unbalanced mixtures identical to that of the evidence. We hypothesize that an exchange of hematopoietic components occurred between the twin fetuses *in utero.* The minor components most likely arose from hematopoietic components that originated from the suspect's twin.

Materials and Methods

Specimens

Two reference blood specimens from the suspect were received and tested. One of these reference specimens was from an EDTA tube collected and subsequently spotted onto gauze, dried, and sub-

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mitted for testing by the referring agency. The other reference sample consisted of an unopened EDTA tube. The evidence specimen was a scraping of apparent blood from under the fingernail of the suspect.

DNA Extraction

The specimens were extracted with Chelex according to Walsh et al. (23). Briefly, 1 mL of sterile water is added to 3–10 μL of whole blood or 3–5 mm^2 of bloodstain. This mixture is incubated for 30 min at room temperature to lyse the red cells. Following centrifugation, the cell pellet is treated with 200 μL of 5% Chelex solution (Bio-Rad, Chicago, IL) and incubated at 56°C for 60 min. The specimen is then vortex mixed for 10 s, incubated at 100°C for 8 min and then centrifuged for 3 min at 15 000 rpm. The supernatant is then suitable for amplification using polymerase chain reaction (PCR) as outlined in the next section.

PCR Amplification

Specimens were analyzed by PCR amplification of polymorphic markers composed of short tandem repeats (STR). Nine STR markers including D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317, D7S820 and the gender marker, Amelogenin, were amplified (AmpF1STR Profiler PCR Amplification Kit; Perkin Elmer-Applied Biosystems, Foster City, CA). The specimen DNA was amplified for 28 cycles (94°C for 1 min, 59°C for 1 min, 72°C for 1 min) based on the manufacturer's recommendations. The yield of amplified product was visualized and estimated from a 2.5% agarose gel prior to analysis by capillary electrophoresis on an ABI Prism™ 310 Genetic Analyzer (Perkin Elmer-Applied Biosystems, Foster City, CA). This analysis was carried out using a 47 cm capillary filled with POP-4 polymer and genetic analyzer (GA) buffer. The PCR products were denatured at 95–100°C for 3 min, prior to injection. The amplified fragments were analyzed and assigned specific allele designations using Genotyper™ software, version 2.5 (Perkin Elmer-Applied Biosystems, Foster City, CA). The evidence specimen (A2) was extracted and amplified twice with different concentrations of DNA (≈ 1 ng and ≈ 4 ng). The two reference specimens from the suspect were individually extracted and each was amplified twice using approximately the same DNA concentration as the evidence specimen.

Results/Discussion

STR analysis has become a powerful method for determining the source of biological specimens. Typically, results obtained from evidentiary specimens must be compared to results obtained from one or more reference specimens. The results either exclude or include a reference as a possible contributor. If the reference individual is not excluded as a potential source, statistical calculations are performed to determine the likelihood of a randomly selected individual matching the DNA profile observed.

Occasionally, an unusual situation arises when an individual exceeds the expected number of alleles at a STR locus. Usually individuals inherit a single genetic copy from each parent, and the presence of more than two alleles is indicative of a mixture. There have been some instances in which a third allele has been identified at a particular locus and can be attributed to genetic duplication of a chromosomal segment or the improper segregation of an entire chromosome, e.g., trisomy (11). The vast majority of such genetic alterations would affect only a single chromosome. Since most markers used in forensics are located on separate, independently assorting chromosomes, only a single STR locus is affected.

Dizygotic or fraternal twins present a unique situation. In dizygotic twins, those having a fused dichorionic membrane may exchange blood components via vascular anastomoses. These naturally occurring chimeras (individuals whose cells derive from two genetically distinct sources) have been described infrequently. The paucity of documented cases in the literature may be due to the fact that assay systems used in the past were not extremely sensitive or that chimerism was not specifically studied. There have been a limited number of cases described over the years in which blood components were shown to have been exchanged between twins *in utero.* In some instances there was a 50/50 mixture from the two sources while in other circumstances there was a mixture consisting of only a few percent of the other twins' cells (12–18). In such cases, the exchange of hematologic progenitor cells occurs early during the course of development. During this timeframe, the immune system is in the process of defining "self." If these foreign cells are presented during this critical stage of immune system development, the individual perceives these cells as belonging to "self" and a state of immunologic tolerance is achieved. This concept of neonatal induction of immune tolerance has been recognized for over 50 years and has been the subject of recent reviews (24).

The results of STR analysis on reference and evidence specimens are shown in Table 1. The nine STR loci gave identical results when the evidence and reference profiles were compared. In this case, four of the nine loci studied demonstrated an additional allele indicating a potential mixture. The ratio of major to minor contributor in this apparent mixture remained constant in all samples that displayed this profile. The four loci with apparent mixtures were vWA, FGA, THO1, and D5S818 (Fig. 1; Fig. 2*A–D*

TABLE 1—*Results of STR analysis on specimens. Data are reported as* phenotypes.

Specimen	Genetic Locus									
	D3S1358	vWA	FGA	Amel.	TH01	TPOX	CSF1P0	D5S818	D13S317	D7S820
Evidence 98A2										
Major contributor	14, 18	17, 19	19, 21,	X Y	8, 9,	8	11, 12	10, 14	8, 11	10
Minor contributor		16	23		9.3			11		
Reference 98A6 (tube #1)										
Major contributor	14, 18	17, 19	19, 21,	X Y	8, 9,	8	11, 12	10, 14	8, 11	10
Minor contributor		16	23		9.3			11		
Reference (tube #2)										
Major contributor	14, 18	17, 19	19, 21,	X Y	8, 9,	8	11, 12	10, 14	8, 11	10
Minor contributor		16	23		9.3			11		

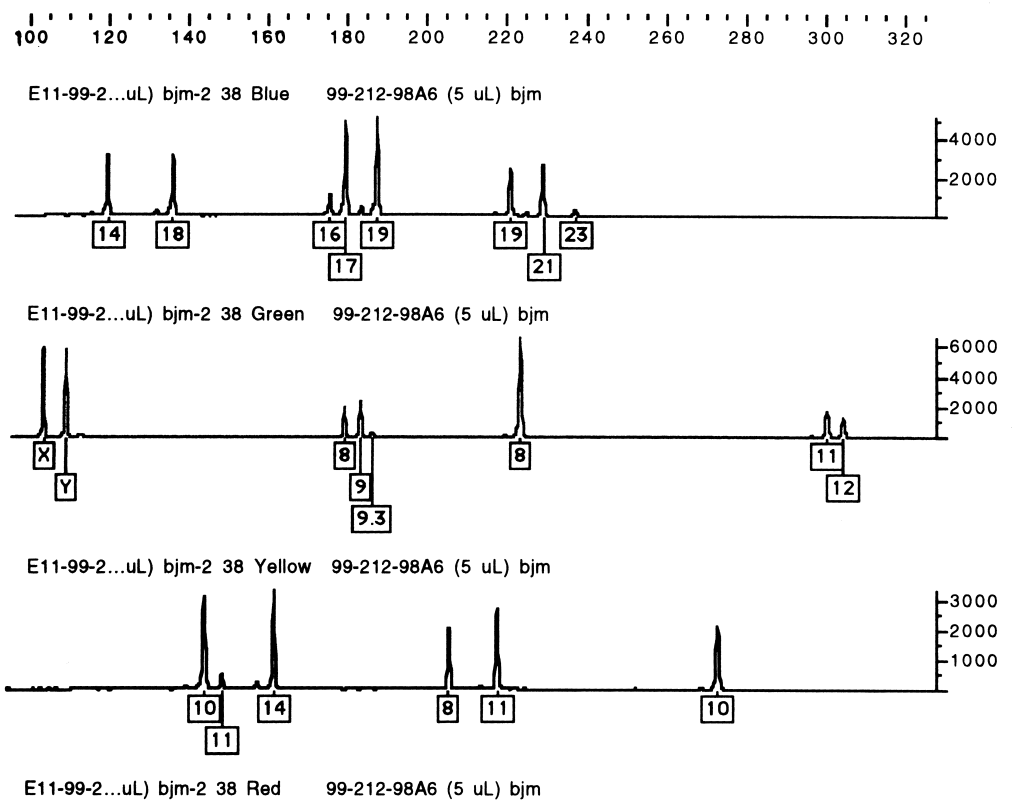

FIG. 1—*Electropherogram of the STR results obtained from the evidence specimen (98A6). The vertical scale is the fluorescence intensity in relative fluorescent units (RFU) and the horizontal scale is the length of the amplified fragments in nucleotide bases. The numbers under the peaks refer to the allelic designation at the individual loci. The genetic loci represented are from left to right, Top row; D3S135, vWA, FGA. Row 2; Amelogenin, TH01, TPOX, CSF1PO. Row 3; D5S818, D13S317, D5S820. Row 4; ROX size standard.*

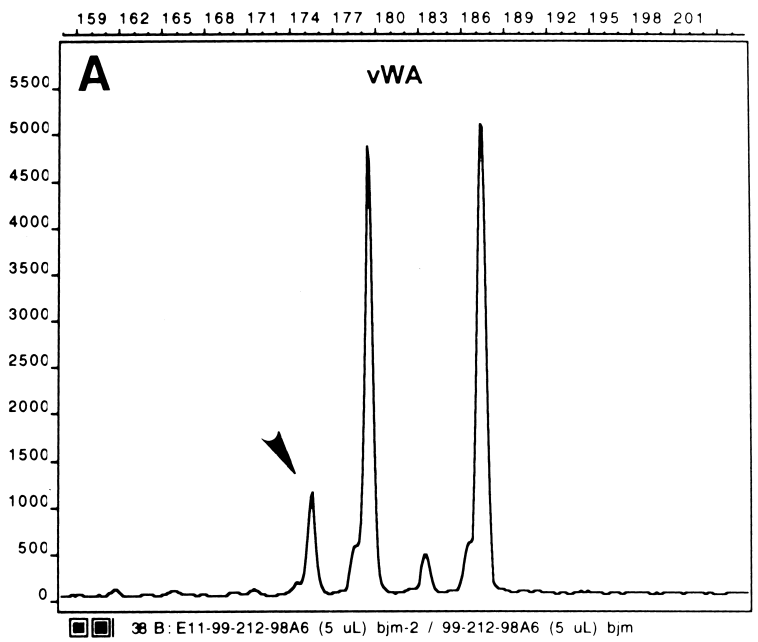

FIG. 2—*Electropherograms demonstrating the STR results at the vWA (panel A), FGA (panel B), TH01 (panel C), and D5S818 (panel D) loci obtained from the evidence specimen (98A6). The vertical scale represents relative fluorescent units (RFU) and the horizontal scale is the length of the amplified fragments in nucleotide bases.*

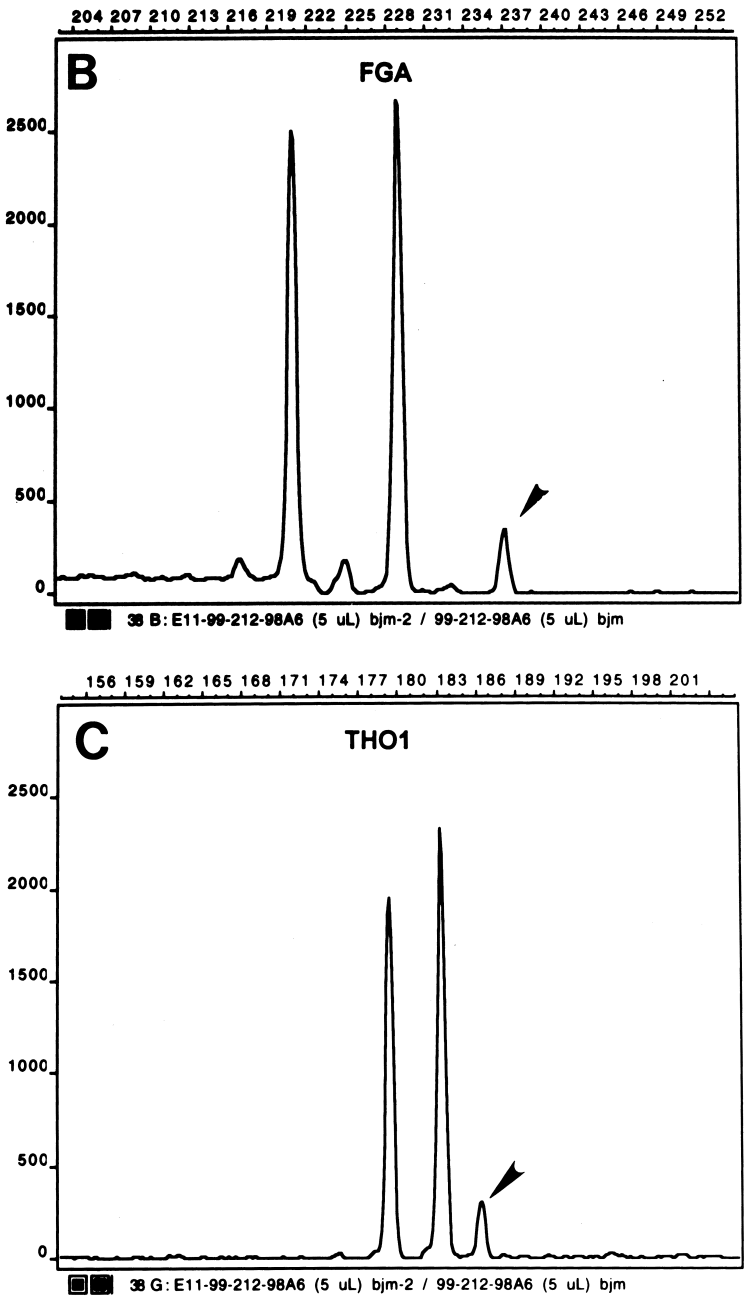

FIG. 2 *(Continued.)*

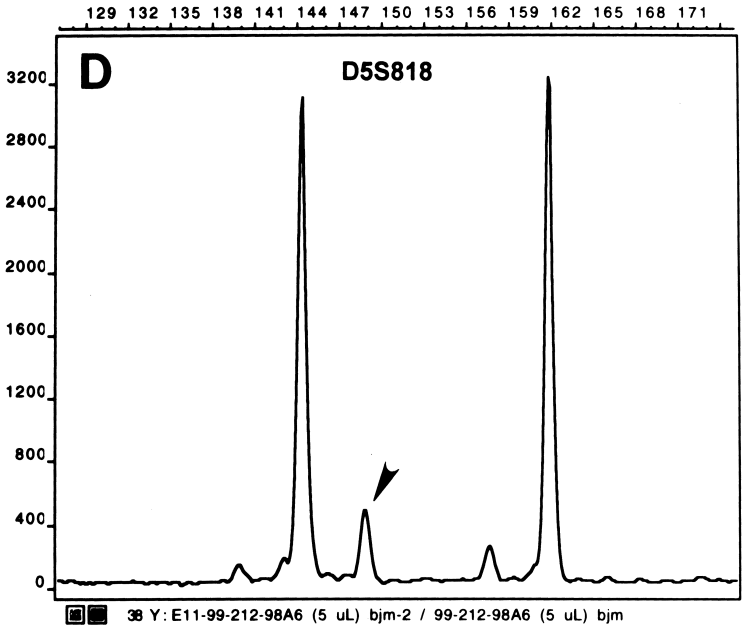

FIG. 2 *(Continued.)*

with minor alleles denoted by arrows). In the case of the vWA locus, the extra allele was located in a stutter position (Fig. 2*A*). However, the peak height was greater than 10% of the main peak, consistent with the presence of an extra allele. In all other cases, the extra alleles were in non-stutter positions (Fig. 2*B–D*). These four STR loci are found on separate chromosomes (chromosome 12, 4, 11, and 5). This confirms that this finding is not due to a single gene segment duplication or chromosomal trisomy. The minor component in the mixture was consistently found to be approximately 10% of the major profile at the affected loci. Based upon the high rate of heterozygosity for these STR loci (70–80%) we would expect approximately 6–7 heterozygous genotypes at these 9 STR loci in unrelated parents. Therefore, approximately 50% of these heterozygous alleles should be shared among offspring. This would be roughly 3–4 loci which closely approximates the observed frequency of 4 found in this case.

Further investigation revealed that the suspect was a fraternal (dizygotic) twin, and had not received a transplant or received a blood transfusion. Therefore, we hypothesize that the source of the minor component is his fraternal twin. Confirmation requires additional specimens from the suspect's family members. Unfortunately, as of this writing, no other family members have been tested. The probability of an unrelated individual matching the nine-locus profile from these specimens is very rare and if the multiple allele components is factored into the calculation, it is essentially unique. In forensic casework, such an unusual genetic condition would greatly increase the certainty as to the source of the genetic material.

In conclusion, it is important that the forensic community is aware of the existence of individuals having a natural mixture of DNA profiles in their blood samples. If one was to compare the profile obtained from the skin or semen of such an individual with their blood profile one would have to explain why additional alleles were present in the reference blood specimen and not in these other tissues. One solution would be to collect a hair-root, semen, or buccal swab as a reference source. Such samples should contain the normal complement of STR alleles, as they are derived from non-hematopoietic sources. Similar caution should be used if references are obtained from patients who have undergone allogeneic hematopoietic stem cell transplantation. In these transplant patients, situations may arise in which a reference blood specimen may falsely exclude an individual as being the source of an evidentiary specimen. Somatic tissue, such as hair and semen, would be genetically dissimilar to the reference blood profile of such a transplant recipient. In these circumstances it would be prudent to acquire ancillary reference specimens, such as buccal cells, in addition to a blood specimen.

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