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Establishing Paternity Using Minisatellite DNA Probes When the Putative Father is Unavailable for Testing

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ABSTRACT: A paternity case involving a putative father who had died a few years earlier in an automobile accident was referred to the laboratory for testing. The child and his mother, the deceased's parents, and nine of the deceased's siblings were available for analysis. As previously reported, paternity testing using red blood cell groups, human leukocyte antigens (HLA), red blood cell enzymes, serum proteins, and immunoglobulin allotypes gave a cumulative paternity index of 43 300 and a combined probability of paternity equal to 99.998%. RFLP analysis using *Hinf* I and *Sau* 3A single digests and the minisatellite deoxyribonucleic acid (DNA) probes 15.1.11.4 and 6.3 showed no exclusion of paternity and gave nearly conclusive evidence that the putative father was the biological father of the child.

KEYWORDS: pathology and biology, paternity, deoxyribonucleic acid (DNA), restriction fragment length polymorphism (RFLP)

Wyman and White [*1*] were the first to report the discovery of a hypervariable locus identified with an arbitrary probe. Since this initial discovery, several hypervariable loci have been reported, including the insulin gene [*2*], the zeta-globin pseudogene [*3*], the Harvey-*ras* oncogene [*4,5*], and the myoglobin gene [*6*]. The polymorphic nature of these loci is due to a variable number of tandemly repeated (VNTR) oligonucleotide sequences and accordingly have been called VNTR loci [*7*]. However, the full potential of these hypervariable loci in forensic biology was not recognized until Jeffreys et al. [*8*] published their results using minisatellite deoxyribonucleic acid (DNA) probes which hybridized to multiple VNTR loci, therefore allowing practical individualization of very small amounts of DNA from peripheral blood. These same authors also noted that the bands arising from a restriction fragment

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length polymorphism (RFLP) analysis were stably inherited and segregated in a Mendelian fashion [6]. Therefore, minisatellite DNA polymorphisms would be theoretically valuable in disputed parentage investigations. This was subsequently demonstrated in one such case [9]. Likewise, Balazs et al. [10] employed probes that hybridized to single VNTR loci to investigate cases involving disputed paternity.

This paper reports a case in which minisatellite DNA probes were used to establish the paternity of a man who had died and therefore was not available for testing. These probes were employed for research purposes only, and the results were not used in the report to the court. Results from conventional paternity testing were given to the appropriate legal authorities and have been reported elsewhere [11].

Case Report

A paternity case involving a putative father who had died in an automobile accident was referred to the Family Blood Grouping and Immunogenetics Laboratory at the Medical College of Virginia, Richmond, Virginia. The deceased's parents and nine of his siblings (five brothers and four sisters) were available for testing in addition to the child and his mother. Two siblings of the deceased (one brother and one sister) were unavailable for testing. The pedigree of the family is shown in Fig. 1. All individuals were cooperative, for they had all accepted the child as part of their family; however, the state needed to have evidence of the relationship before Social Security benefits could be dispersed.

Conventional paternity testing was performed using the red blood cell groups ABO, Rh, MNSs, Duffy, Kidd, and Kell; the red blood cell enzymes phosphoglucomutase-1 (PGM_1), esterase D (ESD), glyoxylase I (GLO_1), acid phosphatase (ACP_1), adenylate kinase (AK), and adenine deaminase (ADA); the serum proteins group specific component (Gc), haptoglobin (Hp), and factor B (Bf); and the immunoglobulin allotypes Gm, Am, and Km, and human leukocyte antigens (HLA). The resulting combined paternity index was 43 300 and the probability of paternity was 99.998% [11].

Two minisatellite DNA probes (15.1.11.4 and 6.3) obtained from Dr. Alec J. Jeffreys, Leicester University, Leicester, England, were used in this current study.

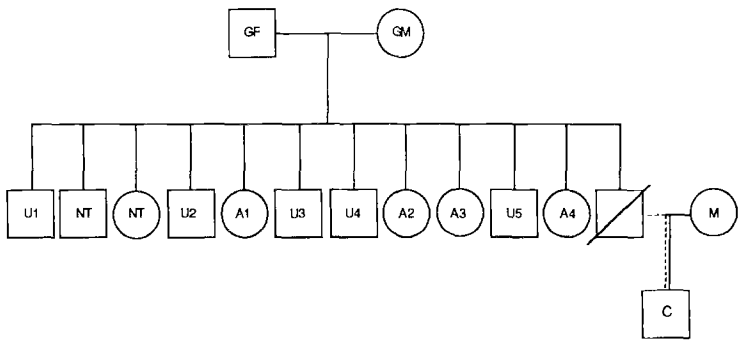

FIG. 1—*Alleged pedigree of the family.* GF = *alleged paternal grandfather;* GM = *alleged paternal grandmother;* U1, U2, U3, . . . = *alleged paternal uncle 1, 2, 3, . . .;* A1, A2, A3, . . . = *alleged paternal aunt 1, 2, 3, . . .;* M = *mother;* C = *child;* NT = *not tested;* ——————— = *alleged relationship.* ——————— = *assumed relationship.*

Materials and Methods

DNA Extraction from Whole Blood

Twenty millilitres of venous blood was collected from each individual in ethylenediaminetetraacetate (EDTA), and mononuclear cells were separated on Lymphocyte Separation Medium (LSM) (Organon Teknika Corp.). Cells were washed once in barbital buffer and were lysed by suspension in STE buffer (0.1*M* sodium chloride [NaCl], 0.01*M* Tris, 0.01*M* EDTA, pH 8.0) containing 1% w/v sodium dodecyl sulfate (SDS) and 200 μg/mL of proteinase K. Samples were incubated at 60°C for 30 min followed by another incubation at 37°C for a minimum of 45 min. The lysate was extracted once with an equal volume of PCI-9 (100 g of phenol, 100 mL of chloroform, 1 mL of isoamyl alcohol, 10 mL of 50m*M* Tris [pH 9.0], 10 mL of water [H_2O]), followed by another extraction with an equal volume of SEVAG (24 parts chloroform: 1 part isoamyl alcohol). The DNA was precipitated by the addition of two volumes of 95% ethanol. Following air-drying, the DNA was dissolved in 200 μL of deionized water and the amount was quantitated by recording absorbances at 260 and 280 nm using a Perkin-Elmer Lambda 3 spectrophotometer.

Electrophoretic Separation of Restriction Endonuclease Digested DNA Fragments

DNA from each individual (10 μg [35 μL]) was digested with either 20 units of *Hinf* I or 16 units of *Sau* 3A at 37°C for at least 3 h under the conditions recommended by the manufacturer (Bethesda Research Laboratories) and in the presense of 2.4m*M* spermidine trihydrochloride. The resulting DNA fragments were electrophoretically separated on a 20- by 18- by 0.8-cm 0.6% horizontal agarose slab gel using a tris-borate-EDTA (TBE) tank buffer (0.089*M* Tris, 0.089*M* boric acid, 0.002*M* EDTA). Electrophoresis was carried out at 45 V for 22 h. The following molecular weight standards were used for later comparison: Lambda DNA/*Hin*d III Fragments and 1-kb Ladder DNA markers (Bethesda Research Laboratories). Following electrophoresis, the gel was stained with ethidium bromide and photographed using an MP-4 Polaroid Land Camera and a Spectroline TS-302 ultraviolet transilluminator as a light source.

Southern Transfer of DNA Fragments

Southern transfer of the DNA fragments from the gel to a nylon membrane (Zeta-Probe, Bio-Rad) was done under alkaline conditions. The gel was first placed in 0.25*M* hydrochloric acid (HCl) for 5 to 10 min to partially depurinate the DNA, thus breaking the larger fragments into smaller pieces and allowing for more complete transfer to occur. It was rinsed in deionized water, and a downward Southern transfer was carried out overnight using 0.4*M* sodium hydroxide (NaOH). Following transfer, the membrane was briefly rinsed in 2× SSC (0.3*M* NaCl, 0.03*M* trisodium citrate), blotted between filter papers, and allowed to air-dry for a few minutes.

Preparation of the Probes

Purified M13 phage DNA containing insert (15.1.11.4 or 6.3) obtained by transfection of *E. coli* JM101 cells with M13 recombinants was used as template DNA in producing the radiolabelled probes. The probes were prepared using a modification of the procedure reported by Jeffreys et al. [6]. Approximately 400 ng of M13 template DNA was annealed with 2 ng of 17-mer sequencing primer No. 1 (Bio-Rad) in 11 μL of 9m*M* magnesium chloride ($MgCl_2$), 9m*M* Tris (pH 7.5) at 60°C for 30 min, followed by 15 min at room temperature. Extension of the primer was accomplished by adding 16 μL of a 78μ*M* deoxyadenosine tri-

phosphate (dATP), 78μM deoxyguanosine triphosphate (dGTP), 78μM deoxythymidine triphosphate (dTTP), 10mM Tris (pH 7.5), 0.25mM EDTA mix, 3 μL of α-^{32}P-dCTP (3000 Ci mmol^{-1}), and 5 units of Klenow polymerase (Bio-Rad) and incubating at 37°C for 15 min. To complete extension, 2.5 μL of 0.5mM chase deoxycytidine triphosphate (dCTP) was added, and the mixture was allowed to incubate another 15 min at 37°C. The DNA was cut at the 3′ end of the radiolabelled insert using 20 units of either *Bam* HI (insert 15.1.11.4) or *Eco* RI (insert 6.3) and denatured by adding 5.3 μL of 1.5M NaOH, 0.1M EDTA. The single-strand radiolabelled probe was separated by electrophoresis through a 1.2% low melting point agarose gel under alkaline conditions [*12*] at 20 V for 4 h. Visualization of the probe was accomplished by autoradiography. The segment of the gel containing the probe was excised and melted in 500 μL of H_2O at 100°C for 10 min.

Prehybridization, Hybridization, and Autoradiography

Each Southern blot was prehybridized overnight with gentle shaking at 37°C in 75 mL of a solution containing 50% formamide, 5× SSC (0.75M NaCl, 0.075M trisodium citrate), 50mM sodium phosphate, monobasic (NaH_2PO_4) (pH 6.5), 5× Denhardt's solution (0.1% w/v bovine serum albumin, 0.1% w/v ficoll 400, 0.1% w/v polyvinylpyrrolidone), 0.1% SDS, and 250 μg/mL of yeast ribonucleic acid (RNA). The hybridization solution was made by combining the prepared probe with 75 mL of the above prehybridization mixture. Each blot was hybridized for 36 h with gentle shaking at 37°C. Four room temperature washes in 2× SSC, 0.2% SDS over a 30-min period were followed by a more stringent wash at 60°C in 1× SSC, 0.2% SDS for 60 min. Autoradiography was performed at −70°C in a DuPont X-ray Cassette with Quanta III Intensifying Screens using Kodak XAR 5 X-ray film.

Results

Conventional paternity indices and probabilities of paternity cannot be calculated when using probes that hybridize to multiple VNTR loci because alleles cannot be assigned to specific loci, and thus allele frequencies cannot be ascertained. Therefore, an alternate method based upon the probability of band sharing is used in the statistical analyses of this case. Three probabilities are calculated: (1) the probability that an unrelated couple, that is, unrelated grandparents, share the obligatory paternal fragments with the child; (2) the probability that a random man shares these fragments; and (3) the probability that the deceased inherited these fragments and thus shared them with the child.

All fragments present in the child's pattern but absent in the mother's are obligatory paternal bands, and thus must be carried by at least one of the alleged paternal grandparents if the deceased is the biological father of the child. Using this criterion, all four autoradiograms resulting from the two single digests with *Hin*f I and *Sau* 3A and hybridization with probes 15.1.11.4 and 6.3 indicate that the deceased is not excluded as being the father of the boy. Figures 2 and 3 show the autoradiograms obtained by hybridizing probe 15.1.11.4 to *Hin*f I and *Sau* 3A digested DNA blots. Only paternal bands longer than 4 kb are indicated with a "square"; however, all bands of known paternal origin including those less than 4 kb are used in the following calculations. Two other autoradiograms obtained by hybridizing probe 6.3 to *Hin*f I and *Sau* 3A digested DNA blots are not shown but are used to calculate the following probabilities.

Assuming that probes 15.1.11.4 and 6.3 hybridize independently of each other (previous results indicate they are nearly independent [*8*]) and that the probability of band sharing for each fragment is 0.26 (a conservative estimate) [*9*], the probability that the child shares the paternal bands with an unrelated couple can be calculated. Twenty-three bands of known paternal origin were detected in the child from blots of DNA that had been digested with *Hin*f I and hybridized with probes 15.1.11.4 and 6.3. All of these fragments were present in

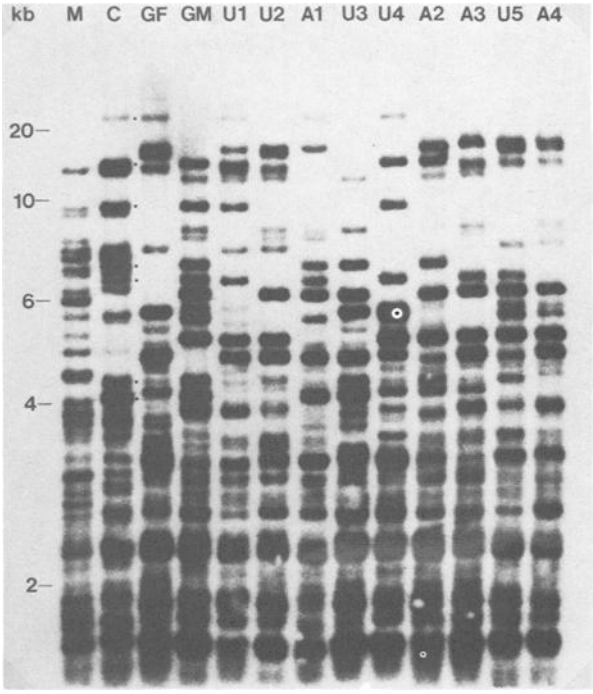

FIG. 2—*Autoradiogram of* Hinf *I digests using probe 15.1.11.4.* M = *mother;* C = *child;* GF = *alleged paternal grandfather;* GM = *alleged paternal grandmother;* U1, U2, U3, . . . = *alleged paternal uncle 1, 2, 3, . . .;* A1, A2, A3, . . . = *alleged paternal aunt 1, 2, 3, . . .;* ■ = *fragments of known paternal origin which are longer than 4 kb. Note: fragments less than 4 kb were also used in the calculations but are not indicated in the photograph.*

either the alleged paternal grandfather or grandmother. The chance that at least one of two unrelated grandparents would carry one of the necessary fragments is given by the binomial $1 - \binom{2}{0}(0.26)^0(0.74)^2 = 0.45$. Therefore, the probability that at least one of two unrelated grandparents would carry each of the twenty-three fragments is $(0.45)^{23} = 1.1 \times 10^{-8}$. Likewise, eighteen bands of known paternal origin were found in the child using *Sau* 3A digested DNA, and these bands were all present in at least one of the alleged paternal grandparents. The probability of unrelated grandparents sharing these fragments with the child is $(0.45)^{18} = 5.7 \times 10^{-7}$.

The probability that a random man would share the 23 *Hinf* I fragments with the child and thus be a possible father is $(0.26)^{23} = 3.5 \times 10^{-14}$. This same probability for the *Sau* 3A fragments is $(0.26)^{18} = 2.9 \times 10^{-11}$.

The probability that the putative father shared the necessary fragments with the child can be calculated as follows. The chance that the alleged paternal grandparents passed a heterozygous band which they did not share with one another to the deceased is 0.5, while the chance they passed a shared heterozygous band is 0.75. In instances where zygosity cannot be determined as a result of sharing of bands between alleged paternal grandparents and all siblings of the deceased, the following calculation is made. The chance that both alleged paternal grandparents are heterozygous in these instances is $\binom{9}{9}(0.75)^9(0.25)^0 = 0.075$. Thus, the chance that at least one of the alleged paternal grandparents is homozygous for the fragment is $(1 - 0.075) = 0.925$. If at least one grandparent is homozygous, the deceased must have inherited that fragment; however, the chance both grandparents are heterozygous

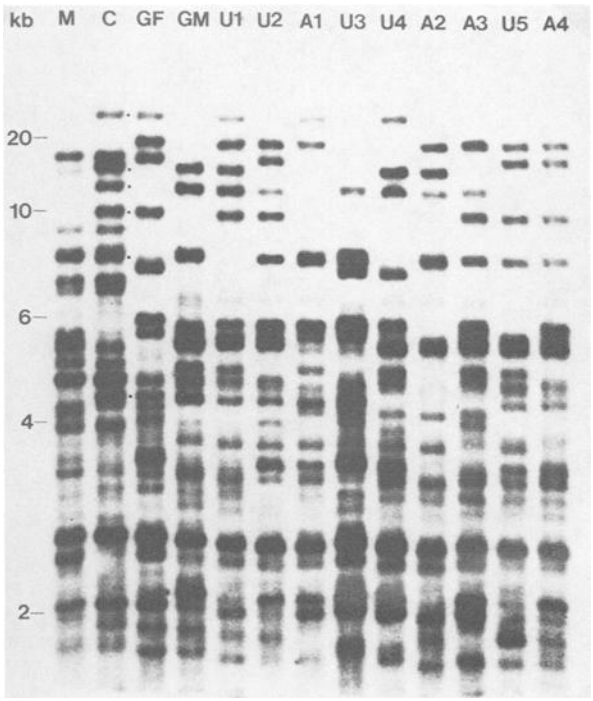

FIG. 3—*Autoradiogram of* Sau *3A digests using probe 15.1.11.4.* M = *mother;* C = *child;* GF = *alleged paternal grandfather;* GM = *alleged paternal grandmother;* U1, U2, U3, . . . = *alleged paternal uncle 1, 2, 3, . . .;* A1, A2, A3, . . . = *alleged paternal aunt 1, 2, 3, . . .;* ■ = *fragments of known paternal origin which are greater than 4 kb. Note: fragments less than 4 kb were also used in the calculations but are not indicated in the photograph.*

and the deceased inherited this band is $(0.75)(0.075) = 0.056$. The combined chance that the deceased inherited this fragment is $(0.925 + 0.056) = 0.981$. Of the 23 necessary bands for *Hinf* I digests, 20 are unshared heterozygous bands in the alleged paternal grandparents, 1 is a shared heterozygous band, and 2 are of unknown zygosity. Thus, the probability that the deceased had inherited all 23 necessary fragments from his parents and shares these bands with the child is $(0.5)^{20}(0.75)(0.981)^2 = 6.9 \times 10^{-7}$. Likewise, this same probability for *Sau* 3A digests is $(0.5)^{15}(0.75)^3 = 1.3 \times 10^{-5}$.

Although these latter probabilities are low, they must be compared to those obtained for the random man. It can be seen that with *Hinf* I digests, the deceased was 2×10^7 times more likely to share the necessary fragments with the child than is a random man. In a similar manner, *Sau* 3A digests indicated the deceased was 4.5×10^5 times more likely to share these fragments with the child than is a random man.

All five of the alleged paternal uncles that were tested are excluded as being the father of the child. However, one alleged uncle was not available for testing and thus cannot be excluded on the basis of genetic evidence.

Discussion and Conclusions

For reasons noted in the previous section, conventional paternity indices and probabilities of paternity cannot be calculated when using minisatellite DNA probes. However, if approximate band frequencies and the degree of heterozygosity were known within a population,

estimates of paternity indices and probabilities of paternity could be calculated without knowing true allele frequencies.

Alternatively, several probes that recognize a single highly polymorphic VNTR locus have been developed [7], and allele frequencies can be determined. Employing a battery of these probes would allow a complete statistical analysis to be done, including the conventional calculations for the paternity index and probability of paternity, while not diminishing the individualizing potential of this powerful technique. Baird et al. [13] used a similar approach by employing two probes that recognized hypervariable loci; however, more probes would be necessary to achieve the degree of individualization obtained by using minisatellite DNA probes.

In conclusion, minisatellite DNA probes appear to be a very powerful and useful tool in paternity investigations. RFLP analysis using these probes can identify with virtual certainty one particular man as the father of a child. However, statistical analyses are very difficult and are subject to several assumptions. For this reason, a battery of probes each recognizing a different single VNTR locus may be a more practical approach to investigations involving disputed parentage, since they would allow calculations of a paternity index and a probability of paternity using standard techniques.

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