Justine N. Ivey,[1] *B.Sc.; Bentley A. Atchison,*[1] *Ph.D.; and Anna M. Georgalis,*[1] *B.Sc.*

Assessment of PCR of the D17S30 Locus for Forensic Identification

REFERENCE: Ivey, J. N., Atchison, B. A., and Georgalis, A. M., "**Assessment of PCR of the D17S30 Locus for Forensic Identification,**" *Journal of Forensic Sciences*, JFSCA, Vol. 39, No. 1, January 1994, pp. 52–63.

ABSTRACT: PCR analysis of the VNTR locus D17S30 was assessed for its potential use in forensic identification analysis. "Allelic drop-out," the inefficient amplification of some alleles, complicates the interpretation of DNA typing at this locus.

PCR conditions were varied in an effort to improve amplification of the alleles at this locus. Such changes included the use of denaturants, formamide and DMSO, to overcome any incomplete denaturation of template strands due to GC content or allele size. Lowering the annealing temperature during the PCR cycle enhanced the amplification of a larger fragment, but this was not related to the D17S30 locus. It appears that the structure of the genome of some individuals rendered PCR amplification inefficient at this locus.

KEYWORDS: pathology and biology, polymerase chain reaction (PCR), deoxyribonucleic acid (DNA), variable number of tandem repeat (VNTR), D17S30, allelic drop-out

The D17S30 VNTR locus is a minisatellite region located on chromosome 17 and detected by the probe pYNZ22. PCR amplification of tandem repeat sequences at the D17S30 locus has been reported as being suitable for identification purposes in forensic science [*1–3*] due to the fact that the alleles are sufficiently different in size to be easily distinguished by simple electrophoresis techniques.

This paper describes anomalous results observed in using a published procedure for the amplification of the D17S30 VNTR locus by PCR.

Materials and Methods

Biological Samples

Blood Samples—Whole blood samples were collected by venipuncture under sterile conditions in 10 mL ethylenediaminetetraacetic acid (EDTA) and stored at 4°C until extraction.

Received for publication 1 Feb. 1993; revised manuscript received 18 June 1993; accepted for publication 21 June 1993.

[1]Scientist-Class I, Molecular Haematology; Scientist in Charge-Clinical Sciences; and Chief Scientist-Molecular Haematology, respectively, Victorian Institute of Forensic Pathology, Department of Forensic Medicine, Monash University, South Melbourne, Australia.

Oral Swabs—Buccal cell samples were collected by rubbing the cotton tip of a sterile swab on the inside of both cheeks. Swabs were air dried and stored in individual plastic containers at 4°C until extraction.

DNA Extraction

DNA Extraction from Whole Blood

DNA was extracted from whole blood samples by a salting-out procedure described previously [*4*]. The quantity of DNA extracted from each sample was determined from the absorbance at 260 nm.

DNA Extraction from Oral Swabs

The "Chelex" extraction procedure used was an amended version from Walsh et al. [*5*]. The entire cotton tip of the oral swab was suspended in 1 mL of distilled water in a 1.5 mL eppendorf tube and incubated at room temperature for 30 min. The swab was then agitated with a pipette tip for 2 min to dislodge cells off the substrate. The swab and pipette tip were discarded and the sample centrifuged in a Beckman microfuge for 2 min at 13 000 rpm. Without disturbing the cell pellet, all but approximately 50 μL of the supernatant was removed and discarded. Five percent "Chelex" (Bio-Rad) in water was added to a final volume of 200 μL and the cell pellet resuspended by vortexing. The sample was then incubated at 56°C for 30 min, vortexed at high speed for 10 s and boiled in a boiling water bath for 8 min. The sample was then vortexed again followed by centrifugation for 3 min at 13 000 rpm. Aliquots (40 μL) of the supernatant were used in each PCR amplification.

PCR

Amplification of the D17S30 VNTR locus was initially carried out according to the method described by Horn et al. [*1*] using the primers, 5′-CACAGTCTTTATTCTT-CAGCG-3′ and 5′-CGAAGAGTGAAGTGCACAGG-3′. PCR was carried out with 100 ng of human DNA in 100 μL reaction volumes: similar results were obtained with a range of DNA concentrations from 5 ng to 1 μg. All PCR experiments were conducted under stringent conditions designed to eliminate contamination (separate laboratories for DNA purification, PCR set-up and product analysis). Negative controls were included in experiments to detect any spurious contamination. Amplification was performed in a Perkin-Elmer/Cetus Instruments thermal cycler 480.

Visualization of PCR Products

Agarose

Electrophoretic analysis of the PCR products was performed in 2% agarose/0.75% NuSieve gels in 0.5 × TBE (45 mM Tris base, 45 mM Boric acid, 1.1 mM disodium EDTA). PCR product (20 μL) was added to 2 μL of loading buffer (100 mM Tris-HCL, pH 8.0, 200 mM EDTA, 2% sarcosyl, 15% Ficoll-400, 0.1% Bromophenol blue, 0.1% Xylene cyanol) and then 20 μl of this loaded onto the gel. DNA bands were visualized by staining with ethidium bromide and photographed on a UV transilluminator.

Polyacrylamide

Ultrathin Layer Polyacrylamide Gel Electrophoresis was used as an alternative system in some experiments. Gels were cast onto gel bond (FMC) using a modified version of the Flap technique described by Allen et al. [6]. In general, 3 μL aliquots of PCR product were loaded onto the gel and electrophoresed for 2 to 4 h. Bands were visualized with silver staining described by Allen et al. [6].

Bst N1 Digestion of D17S30 PCR Products

Bst N1 digestion of PCR products was performed with an excess of enzyme using buffer supplied by the manufacturer (Stratagene).

Results

Family Studies

The DNA from four families was amplified according to the conditions described by Horn et al. [1]. Figure 1 shows an agarose gel, stained with ethidium bromide, containing the amplified DNA from families "A" and "B." Lanes 1, 2 and 3 contain DNA from father A, mother A and child A, respectively. Child A did not show the maternal allele even though parentage of this child was confirmed by RFLP and blood group typings.

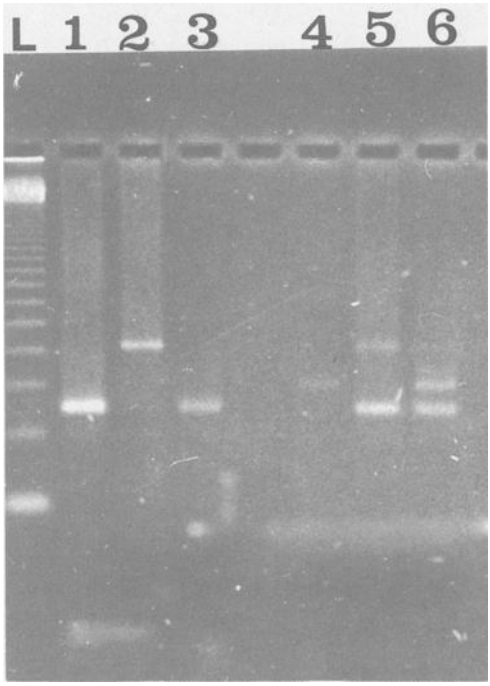

FIG. 1—*Ethidium bromide-stained agarose gel showing DNA amplified from two families at the D17S30 locus according to Horn et al.* [1]. *L: 123 bp ladder. 1–3: Family A (father, mother, child, amplified from 100 ng salt-extracted DNA). 4–6: Family B (father, mother, child, amplified from 100 ng salt-extracted DNA).*

Lanes 4, 5, and 6 contain DNA from father B, mother B and child B respectively. In this case, the child inherited two alleles, one from each parent, in a classical Mendelian fashion.

The results of testing families "C" and "D" are shown in Fig. 2. In family "C" (lanes 1 to 5) the third child (lane 5) did not appear to have a paternal allele. The other children (lanes 3 and 4) appeared to inherit one allele from each parent, although in lane 4 the band at 450 bp was weakly amplified. Of particular note is that four of the individuals in this family possessed a band at 450 bp, which was also seen in family "D."

The results of testing family "D" are shown in lanes 6 to 9 of Fig. 2. In lane 6, the father showed an allele at 310 bp that was intense, while a band at 450 bp was barely visible. An additional band at 590 bp was also apparent. Similarly, in the mother (lane 7), an intense band at 240 bp was present as well as two other faint bands at 450 bp and 730 bp.

It appears that the upper faint bands present in the parents (590 to 730 bp) were true alleles since they were inherited by the two children in which the alleles were poorly amplified by PCR. However, the question then arises as to which of the remaining bands seen in the parents are alleles of the D17S30 locus.

Random Population

Profiles such as those described in the family studies were also observed in a random selection of individuals (Fig. 3). Of particular note is the profile in lane 3 which showed

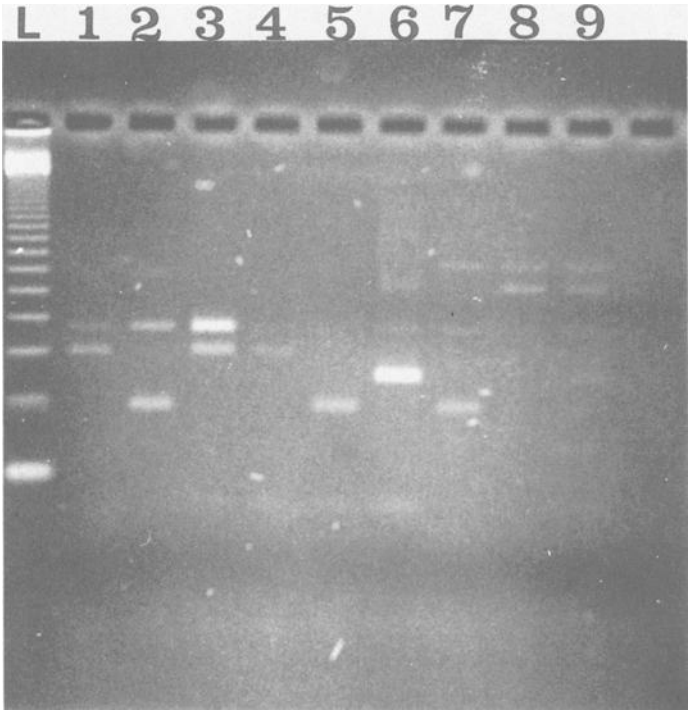

FIG. 2—*Ethidium bromide-stained agarose gel showing DNA amplified from two families at the D17S30 locus according to Horn et al.* [1]. *L: 123 bp ladder. 1–5: Family C (father, mother, children 1–3). 6–9: Family D (father, mother, child 1, 2).*

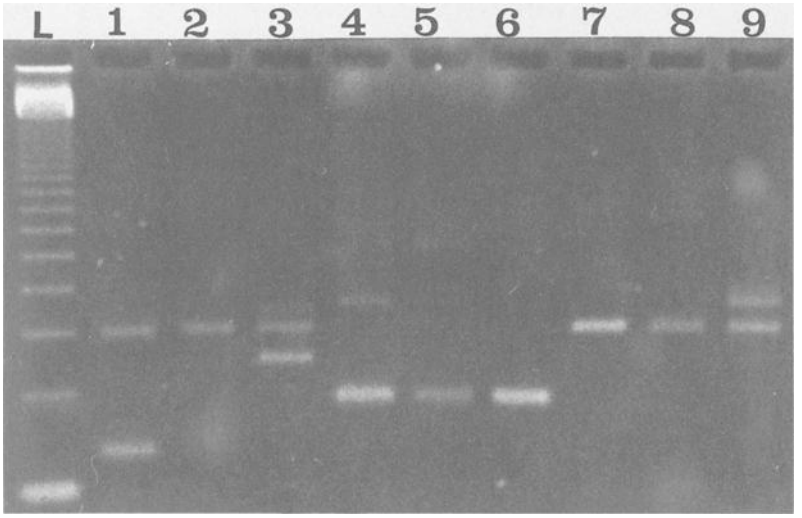

FIG. 3—*Ethidium bromide-stained agarose gel showing DNA amplified from a number of individuals chosen at random. Note the faint bands in lanes 3, 4, 5, and 8.*

three bands, the larger "allele" coinciding with the band observed in other individuals at 450 bp. In lanes 4 and 5 the larger allele was only weakly amplified. Clearly results such as these are not acceptable since the individuals in lanes 4 and 5 could easily be mistyped as homozygotes.

On the basis of the results obtained in the family studies and also critical examination of the random selection of samples it was necessary to optimize the PCR conditions in order to produce efficient and specific amplification of all D17S30 alleles.

Increased Denaturation of DNA

Incomplete denaturation of template strands due to high GC content can interfere with the amplification of alleles [*7*]. Since the D17S30 locus has a 65% GC content (estimated from the sequence data available from [*8*]) and has alleles which are reasonably large (up to 970 bp), a number of approaches were taken in an effort to overcome the problem of "allelic dropout."

(i) Initial Boiling of Template Strands—The reaction mixtures plus DNA templates were boiled for 8 minutes in a boiling water bath prior to addition of Taq polymerase and subsequent PCR amplification. This step failed to improve the amplification of alleles at the D17S30 locus (as there was no improvement over the results shown in Figs. 1 to 3, photographs of this and subsequent variations to the PCR are not shown).

(ii) Dimethylsulphoxide (DMSO)—the use of dimethylsulphoxide (DMSO) has been described by Saiki [*9*] as a possible solution to incomplete denaturation of template strands. In an effort to enhance amplification, 10% DMSO was included in the reaction mix. However, no PCR products were obtained, even with DNA which had previously amplified. DMSO may have inhibited the Taq enzyme, a phenomenon which has been described previously [*10*].

(iii) Formamide—Budowle et al. [*7*] have described the use of 5% formamide in PCR to facilitate denaturation. However, no PCR products were obtained when 5% formamide

was added to the PCR. This effect was possibly due to the inhibition of Taq enzyme by the formamide or changes to annealing conditions as a result of the lowered melting temperatures.

Increasing the Synthesis of Larger Alleles

A number of attempts were made to increase the intensity of the larger alleles in order to ensure accurate genotyping since faint bands can sometimes be mistaken for non-specific priming.

(i) Increased Extension Time—Extension time was increased from 8 min per cycle to 10 min per cycle to allow time for the larger alleles to be effectively synthesized. However, this step did not improve the synthesis of larger alleles. Extension time was also increased by adding one extra cycle, with 10 min extension, at the end of the normal 32 cycles. However, this failed to improve the typing results.

(ii) 7-Deaza-Deoxyguanosine Triphosphate (C^7 dGTP)—The use of 150 μM C^7 dGTP per reaction has been described by Innis, [*11*] to destabilize double stranded DNA so that efficient annealing and extension of alleles can proceed. However, the addition of 150 μM C^7 dGTP failed to improve results. In fact, the typing results produced were significantly worse than previously, with the larger allele in some individuals not amplified at all, while the smaller alleles were only just visible.

(iii) T4 Gene 32 Protein—Schwarz et al. [*12*] described the use of 0.5 to 1.0 nmole of gene 32 protein to improve the yield of long amplified fragments by at least 10 fold. However, the use of T4 gene 32 protein did not reduce the problem of unequal amplification of the alleles. The smaller allele was more intense in all cases and there did not appear to be any effect of using between 0.1 nmole and 1.0 nmole of T4 gene 32 protein.

Enzyme Concentration

The full PCR cycle for the D17S30 locus was lengthy (>9 hours) compared with other synthesis cycles like Apo B [*13*] and D1S80 [*14*]. Hence, the Taq enzyme may have lost activity and was not functioning efficiently. Three approaches were taken in an effort to address this problem.

(i) Reduced Denaturation Time—Reducing the denaturation time from 7 min to 3 min per cycle to help preserve enzyme activity did not improve the results.

(ii) Increased Enzyme with Reduced Denaturation Time—DNA samples were amplified using 1.5, 2.0 and 2.5 units of Taq polymerase per reaction with 3 min denaturation time. The results obtained using these three enzyme concentrations were not significantly different and all alleles were not amplified with equal efficiency.

(iii) Adding Enzyme—The addition of fresh Taq enzyme (1.5 units/reaction) after the first 20 cycles followed by a further 20 cycles did not improve the efficiency of allele amplification.

Variation of $MgCl_2$ Concentration

The $MgCl_2$ concentration was varied from 1.0 mM to 10.0 mM per reaction to determine the optimal concentration for peak enzyme activity. The optimal concentration for those bands amplified appeared to be 1.5 mM $MgCl_2$. However, variation of $MgCl_2$ concentration did not improve amplification of larger alleles or improve "allele drop-out" at this locus.

Variation of Primer Concentration

In order to enhance the amplification of larger alleles, primer concentration was varied. DNA samples were amplified using 100 ng, 200 ng and 300 ng of each primer. However, increasing the primer concentration did not improve amplification of the larger alleles.

Decreased Annealing Temperature

The presence of alleles that are not efficiently amplified may be due to a mutation in the primer binding site preventing primer annealing and hence efficient synthesis of some alleles. This was tested by lowering the annealing temperature to allow for less specific priming and therefore amplification of these alleles.

Figure 4 shows the results of testing family "A" as discussed previously. Lanes 2 to 4 show the results of amplification with 45°C annealing and lanes 5 to 7 show PCR product obtained using an annealing temperature of 55°C.

As shown previously, the child did not display a maternal allele when 55°C annealing was used but at 45°C the family showed classical Mendelian inheritance. Lowering the annealing temperature to 35°C increased the synthesis of the larger allele (lane 1). Thus, lowering the annealing temperature appeared to increase the efficiency of synthesis of D17S30 alleles. However, lowered annealing temperatures are likely to produce spurious bands and it was necessary to ensure that the band found at 35°C annealing was a true D17S30 allele.

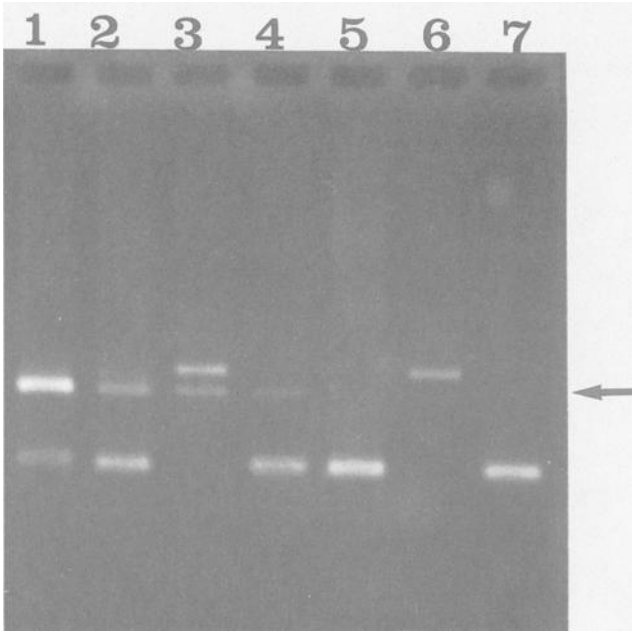

FIG. 4—*The effect of altering the PCR annealing temperature on the amplification of 100 ng salt-extracted DNA per reaction in family "A." 1: Child A - 35°C; 2: Father A - 45°C; 3: Mother A - 45°C; 4: Child A - 45°C; 5: Father A - 55°C; 6: Mother A - 55°C; 7: Child A - 55°C. The arrow shows the 450 bp band.*

Bst N1 Digestion

It was evident from the D17S30 sequence data available [*8*] that each 70 bp repeat unit contained one Bst N1 restriction enzyme site. Digestion of any allele from this locus should therefore yield a 70 bp fragment corresponding to digested repeat units, a 121 bp fragment corresponding to the last repeat unit plus the flanking and primer region, and a 45 bp fragment corresponding to the primer and flanking region before the first repeat.

In order to determine which bands were D17S30 alleles, a Bst N1 digest was performed on DNA from individuals giving anomalous profiles. Figure 5 shows the DNA from child "A" amplified using the three annealing temperatures, 35°C, 45°C and 55°C. The products of a Bst N1 digestion of the DNA amplified at these temperatures are also shown. The intense band at 450 bp produced by an annealing at 35°C did not digest with Bst N1. In contrast, the lower band at 310 bp, which is most intense at 55°C annealing, digested into the expected fragment sizes of 45 bp, 70 bp, and 121 bp. (The sizes of these restriction digestion fragments were confirmed by electrophoresis on a 10% polyacrylamide gel which was able to resolve these small fragments.) Figure 5 shows that the 310 bp band was completely digested with Bst N1.

Bst N1 digests were also performed on the DNA from the parents of this child. It was found that only the father's band at 310 bp digested into the correct size fragments while the 450 bp band produced at 35°C and 45°C annealing did not digest. For the mother, only the 590 bp band digested.

Thus, the band at 450 bp that appeared using annealing temperatures of 35°C and 45°C is not a specific D17S30 allele. It should be noted that this band was in the same

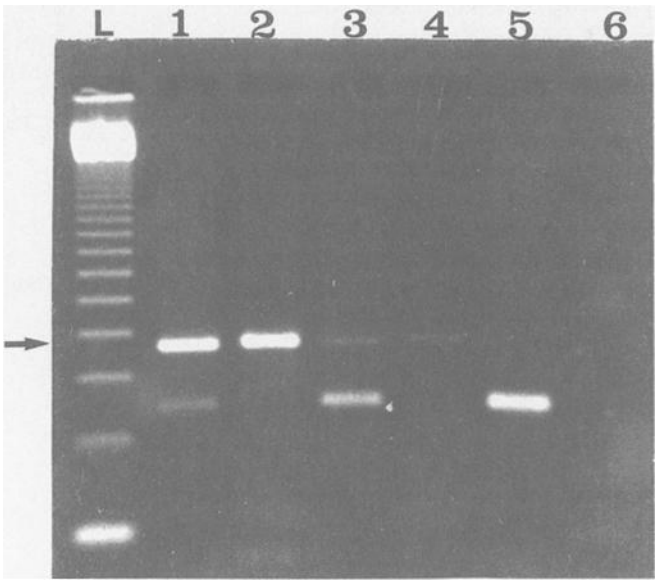

FIG. 5—*Bst N1 digestion of amplified DNA from child A using PCR at three annealing temperatures. L: 123 bp ladder 1: 35°C annealing: not digested with restriction enzyme 2: 35°C annealing: digested with Bst N1 3: 45°C annealing: not digested with restriction enzyme 4: 45°C annealing: digested with Bst N1 5: 55°C annealing: not digested with restriction enzyme 6: 55°C annealing: digested with Bst N1 The arrow shows the 450 bp band.*

position as one of the bands observed in families "C" and "D" under 55°C annealing (Fig. 2).

The DNA samples amplified from the parents of family "D" at the three annealing temperatures were also subjected to Bst N1 digestion. In this case, the band at 450 bp which both parents have in common was not digested, whereas the lower alleles, 310 bp in the father and 240 bp in the mother, did digest (results not shown). The large alleles at 590 bp and 730 bp shown in Fig. 2, were not efficiently amplified and therefore an assessment of whether or not they were digested by the Bst N1 enzyme could not be made.

Increased Detection Sensitivity

In order to ascertain whether child "A" in fact, had a weak maternal allele, not detected in ethidium bromide-stained gels, the detection sensitivity was increased by the use of polyacrylamide gels stained with silver. PCR products amplified at 55°C annealing temperature for family "A" were electrophoresed on a 7.5% polyacrylamide gel and stained with silver. Aliquots of 3 μL and 20 μL were both loaded onto the gel (Fig. 6). Even with the approximately 50-fold increased sensitivity afforded by the polyacrylamide system and the overloading of the sample with 20 μL aliquots, no maternal allele was detected in the child.

Discussion

There appears to be considerable variation in the efficiency of PCR amplification at the D17S30 locus. Some samples failed to amplify at all, some produced two extremely faint bands, some showed one intense band and one faint band, and others produced a "normal" heterozygous pattern of two intense bands.

Such "allelic drop-out" can also be observed by the examination of the data presented in Horn et al. [*1*]. An example is given in which DNA from two parents and three children from the same family has been amplified. One parent has two intense alleles but the other parent has one intense allele and one large allele which is barely visible.

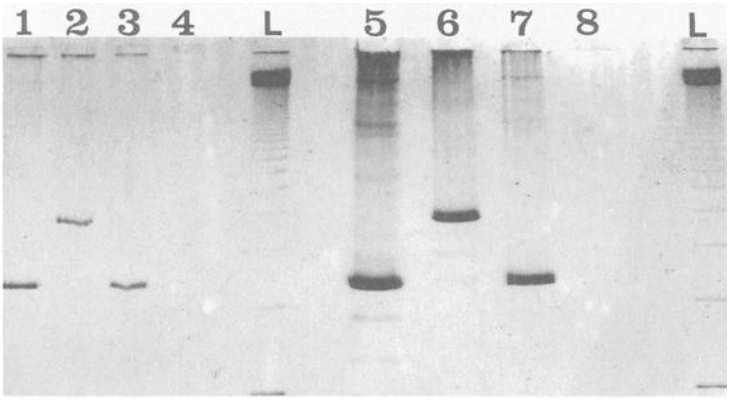

FIG. 6—*Polyacrylamide gel stained with silver showing DNA from family A amplified using an annealing temperature of 55°C. 1: Father A (3 μL loaded) 2: Mother A (3 μL loaded) 3: Child A (3 μL loaded) 4: Blank L: 123 bp ladder 5: Father A (20 μL loaded) 6: Mother A (20 μL loaded) 7: Child A (20 μL loaded) 8: Blank L: 123 bp ladder.*

Two of the children have inherited this faint band from this parent. Mistyping of these individuals as homozygotes for the intense allele could easily occur since the upper faint band may be disregarded as non-specific priming, or not even identified. If a family study had not been performed these weak bands may not have been included as part of the D17S30 locus. Clearly, the procedures for typing the D17S30 system are unsuitable for paternity testing. Interpretation of DNA typing in criminal cases would be complicated, especially when an apparent "homozygote" occurs. Accurate calculation of population frequencies would require estimates of the frequencies of "null" alleles or modification of the standard Hardy-Weinberg multiplication method of calculation (for example, using 2 p rather than 2 pq values as in RFLP studies) [*16*].

There are several theoretical reasons for the preferential amplification of PCR products and corresponding suggestions to overcome this problem.

GC Content

GC content has been reported as a possible cause of "allelic drop-out." However, the use of a range of denaturing agents (DMSO, formamide and C^7 d GTP) as well as boiling of the template prior to amplification, all failed to improve results. Since each allele in an AMPFLP system is similar in GC content and internal GC-rich sequences, it would seem unlikely that there is preferential amplification of an allele on the basis of GC content.

Allele Size

Preferential amplification of the smaller alleles may cause "allelic drop-out" of larger alleles. In an attempt to allow for the amplification of larger alleles, the extension time was increased and T4 gene 32 protein was included in the PCR. However, both these modifications failed to amplify "missing" alleles. Similarly, the children of family "D" (Fig. 2) were both heterozygous for larger alleles and, therefore, did not possess smaller alleles which may preferentially amplify. However, amplification of the alleles in these samples was inefficient. Examination of figures presented by Sullivan et al. [*2*] shows that faint bands are not always larger DNA fragments. PCR of some alleles containing large numbers of repeat units (12 repeat units corresponding to 940 bp) were amplified successfully, but very poor amplification was observed in some of the smaller alleles. Therefore, preferential amplification of a small allele does not appear to be relevant to the problem of allelic drop out.

Primer Sequence Variation

The failure of the above procedures to improve the PCR of the D17S30 system suggested that there was something inherent in the genome that rendered PCR amplification inefficient in some samples. A possible explanation appeared to be that there was sequence variation at the primer sites. Base pair mismatching, especially at the 3′ end of the primer, could reduce the efficiency of primer annealing and the amplification efficiency. Such sequence differences could lead to a "null" allele situation where heterozygous individuals who have inherited alleles differing in primer binding regions may appear as homozygotes due to the "null" allele that has not amplified. However, primer sequence variation cannot explain the presence of null alleles in all situations. For example, the child of family "C" (Fig. 2) should have displayed the paternal allele. Presumably, the primer sequences are the same in the father and child and the failure to amplify the allele in the child could be the result of PCR variation. The results shown in Fig. 2 were consistently obtained with different samples of DNA.

A further problem of PCR at this locus can be illustrated by family "D" in which the parents both appear to have three bands (also lane 3, Fig. 3). The middle band at 450 bp, which is shared by both parents in family "D" was not digestible with Bst N1. This suggests that this band is not part of the D17S30 locus because every repeat unit contains a Bst N1 site. However, to complicate interpretation further, the band at 450 bp has been observed in other samples and family studies appear to confirm it as being part of the D17S30 locus. It therefore appears that a monomorphic band at 450 bp is present in many individuals which may not be part of the D17S30 locus: in other individuals a band at this same position corresponds to a D17S30 allele with five repeat units.

In summary, the problems with this system are three-fold. Firstly, mistyping of individuals could occur due to the failure of some alleles to amplify at all. Secondly, mistyping could occur when the monomorphic band at 450 bp amplifies—it would not be possible to determine if this is a "real" D17S30 allele by an ethidium bromide-stained gel. Thirdly, the allele frequencies calculated for the D17S30 locus could be distorted by the difficulties encountered in achieving correct genotyping.

In this study we have used primers recommended by Horn et al. [*1*]. Results from other laboratories using two different sets of primers have been published [*3,15*], but these primers appear to produce the same problem of allelic drop out.

Overloading a gel may detect poorly amplified alleles [*15*] but the production of spurious PCR products would make the interpretation of such bands extremely difficult. In some cases (family A) increasing the detection sensitivity does not overcome the problem of allelic drop out.

Probing with internal repeat sequence probes would remove the problem of the spurious band at 450 bp and probing is used in some laboratories [for example, *2*]. However, in some samples the alleles are only barely amplified, if at all. Probing may detect poorly amplified alleles, but the intensity of a faint band relative to the strong band would not be changed. Other systems, less susceptible to allelic drop out do not require a time consuming probing step [*17*] and are, therefore, more suitable than D17S30.

Whatever the cause of the anomalous results, we do not recommend the D17S30 system for routine analysis at this stage. Incorrect genotyping could result in a number of cases either due to the non-amplification of an allele or due to the presence of the 450 bp band which may or may not be a D17S30 allele. Our tests have been conducted with "ideal" samples. The degradation of DNA in criminal case samples is likely to exacerbate the problem of allelic drop out and the interpretation of apparent homozygotes would be difficult. Similar problems are found in the interpretation of apparent homozygotes when using RFLP techniques. However, there are now a number of PCR systems available which produce small [*17*] DNA fragments. These systems appear to be less susceptible to allelic drop out, and since only small fragments are amplified, are less likely to be affected by degradation of DNA.

References

[*1*] Horn, G. T., Richards, B., and Klinger, K. W., "Amplification of a Highly Polymorphic VNTR Segment by the Polymerase Chain Reaction," *Nucleic Acids Research*, Vol. 17, No. 5, 1989, p. 2140.

[*2*] Sullivan, K. M., Tully, G., and Gill, P., "Potential Use of Polymerase Chain Reaction (PCR) in Forensic Analysis," Personal communication: Central Research and Support Establishment Report No. 717, Britain, 1990.

[*3*] Rand, S., Puers, C., Skowasch, K., Wiegand, P., Budowle, B., and Brinkmann, B., "Population Genetics and Forensic Efficiency Data of 4 AMPFLP's," *International Journal of Legal Medicine*, Vol. 104, 1992, pp. 329–333.

[*4*] Douglas, A. M., Georgalis, A. M., Benton, L. R., Canavan, K. L., and Atchison, B. A.,

"Purification of Human Leucocyte DNA: Proteinase K Is Not Necessary," *Analytical Biochemistry*, Vol. 201, 1992, pp. 362–365.

[5] Walsh, P. S., Metzger, D. A., and Higuchi, R., "Chelex 100 As a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material," *Biotechniques*, Vol. 10, No. 4, 1991, pp. 506–513.

[6] Allen, R. C., Graves, G., and Budowle, B., "PCR Amplification Products Separated on Rehydratable Polyacrylamide Gels and Stained with Silver," *Biotechniques*, Vol. 7, No. 7, 1989, pp. 736–744.

[7] Budowle, B., Coney, C. T., Jung, J. M., and Guista, A. M., "Research Regarding the Polymerase Chain Reaction for Forensic Utility — HLA-DQα Locus and AMP-FLPs," *FBI Procedure Manual*, Forensic Science Research and Training Center, VA, 1990.

[8] Wolff, R. K., Nakamura, Y., and White, R., "Molecular Characterization of a Spontaneously Generated New Allele at a VNTR Locus: No Exchange of Flanking DNA Sequence," *Genomics*, Vol. 3, 1988, pp. 347–351.

[9] Saiki, R. K., "The Design and Optimization of the PCR," H. A. Erlich, Ed., *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, New York, 1989, pp. 7–16.

[10] Innis, M. A. and Gelfand, D. H., "Optimization of PCRs," *PCR Protocols: A Guide to Methods and Applications*, Academic Press, 1990, pp. 3–12.

[11] Innis, M. A., "PCR with C^7 dGTP," *PCR Protocols: A Guide to Methods and Applications*, Academic Press, 1990, pp. 54–59.

[12] Schwarz, K., Hansen-Hagge, T., and Bartram, C., "Improved Yields of Long PCR Products using Gene 32 Protein," *Nucleic Acids Research*, Vol. 18, No. 4, 1990, p. 1079.

[13] Boerwinkle, E., Xiong, W., Fourest, E., and Chan, L., "Rapid Typing of Tandemly Repeated Hyper Variable Loci by the PCR: Application to the Apo B 3′ Hyper Variable Region," *Proceedings of the National Academy of Science*, Vol. 86, 1989, pp. 212–216.

[14] Kasai, K., Nakamura, Y., and White, R., "Amplification of VNTR Locus (pMCT118) by the PCR and its Application to Forensic Science," *Journal of Forensic Sciences*, Vol. 35, No. 5, September 1990, pp. 1196–1200.

[15] Walsh, P. S., Erlich, H. A., and Higuchi R., "Preferential PCR Amplification of Alleles: Mechanisms and Solutions," *PCR Methods and Applications*, Vol. 1, No. 4, 1992, pp. 241–250.

[16] Committee on DNA Technology in Forensic Science, *DNA Technology in Forensic Science*, National Academy Press, Washington D.C., 1992.

[17] Brinkmann, B., Rand, S., and Bajanowski, T., "Forensic Identification of Urine Samples," *International Journal of Legal Medicine*, Vol. 105, 1992, pp. 59–61.

Address requests for reprints or additional information to
Bentley A. Atchison, Ph.D.
Victorian Institute of Forensic Pathology
57-83 Kavanagh St.
South Melbourne
Australia 3205