CASE REPORT

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Prenatal Exclusion of Paternity by PCR-FLP Analysis of VNTR

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ABSTRACT: A prenatal paternity test was requested by a 30-year-old woman. Variable number of tandem repeat (VNTR) systems were used for DNA analysis by means of amplification and electrophoresis followed by ethidium bromide staining or Southern blotting and oligonucleotide hybridization. Exclusion of paternity could be established on the basis of the great polymorphism and heterozygosity indexes of these genetic systems. This rapid method presents several advantages in contrast with other recombinant DNA techniques such as HLA class II oligotyping or RFLP.

KEYWORDS: pathology and biology, paternity testing, VNTR, prenatal diagnosis, DNA amplification

The analysis of hypervariable sequences of human genome has shown VNTR (variable number of tandem repeat) regions as a powerful tool for paternity testing and forensic medicine [*1,2*]. These sequences consist of a tandem repeat of a common core unit and its polymorphism depends on the number of repeats. The collecting of great amounts of these highly polymorphic sequences can be approached by directed DNA amplification and fragment length analysis by electrophoresis (PCR-FLP), which show highly variable patterns for individuals according to codominant mendelian inheritance [*3*]. There are several well described VNTR systems developed by PCR methods using amplimers targeted to their conserved flanking regions: 33.6 [*1*], H-ras [*4*], pMCT118 [*5*], 3'ApoB [*6*], 3'IL-6 [*7*] and pYNZ-22 [*8*]. The allelism found in these genetic systems was high as well as their heterozygosity indexes, presenting an enormous value for individual identification [*2*].

This study reports the PCR-FLP analysis of six VNTR systems for paternity testing

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on fetal chorionic villi obtained during the 16th week of gestation. Eventually, a definitive exclusion of paternity was achieved.

Case History

A paternity test was requested by a 30-year-old woman who had extramarital sexual relationships. Since her pregnancy, she was under significant psychological distress because of doubts about the identification of paternity.

Material and Methods

Peripheral blood samples were collected from the mother and her extramarital partner. The husband's venopuncture could not be performed because of personal reasons. Chorionic villi were obtained by transabdominal fine needle aspiration with a yield of 30 mg [*9*] of which half was processed for DNA analysis. DNA was isolated by standard phenol-chloroform extraction [*10*].

We synthesized primers by the phosphoramidite method designed to flank the repetitive units of the following VNTR loci: 33.6 [*1*], H-ras [*4*], pMCT118 [*5*], 3'ApoB [*6*], 3'IL-6 [*7*] and pYNZ-22 [*8*] and locus-specific oligonucleotide probes (LSO) for 33.6 and H-ras. Sequences of both amplimers and LSO are shown in Table 1.

PCR reactions were performed in a volume of 100 μL (50 μL for pMCT118) according to the conditions shown in Table 2. The standard buffer contained a final concentration of 50 mM KCl, 10 mM Tris- HCl pH 8.3 and 0.2 mM each dATP, dCTP, dGTP and dTTP.

33.6 and H-ras amplification products required Southern blotting transference [*11*] onto nylon membrane (Zeta-Probe, Bio-Rad, USA) and hybridization after electrophoresis in 1% agarose gels. The membrane was prehybridized at 65°C in 5X SSC (1X SSC = 0.15 M NaCl, 0.015 M Na-citrate), 20 mM Na-phosphate pH 7.0, 10X Denhardt's (1X Denhardt's = 0.02% Ficoll, 0.02% Polyvinylpyrrolidone, 0.02% BSA), 5% SDS, 100 μg/mL salmon sperm DNA (sonicated and denatured) for 1 hour and subsequently hybridized with 1×10^6 cpm/mL of 5'-end ^{32}P-labeled locus- specific oligonucleotide probe overnight at 65° C with the same prehybridization solution. The membrane was washed twice with 6X SSC for 30 min at room temperature and autoradiographed with intensifying screens (Lightning Plus, Dupont, USA) for 1 to 3 h at −80°C and X- OMAT XAR-5 Kodak films.

PCR products of the other systems were electrophoresed in 10% Polyacrylamide (pMCT118) or 3% agarose gels (pYNZ-22, 3'ApoB, 3'IL-6). Polyacrylamide electrophoresis was performed in 89 mM/l Tris-HCl, 89 mM/l borate, 2 mM/l EDTA (pH = 8.0) TBE buffer for 1800 Vh, and agarose electrophoresis 40 mM/l Tris-HCl, 40 mM/l Acetic acid, 2 mM/l EDTA (pH = 8.0) TAE buffer at 5 V/cm for 3 h. Gels were subsequently stained with ethidium bromide (0.5 μg/mL) for 20 to 25 min, destained in water for 15 min to hours and photographed by ultraviolet (UV) transillumination.

Results and Discussion

Four out of the six genotyped VNTR systems produced paternity exclusion of the extramarital partner. This fact would make it unlikely that mutations could be the cause for exclusion.

Autoradiographs from H-ras and 33.6 systems showed exclusion only for H-ras (Fig. 1*A*). pMTC118 also showed exclusion (Fig. 1*B*) as well as pYNZ-22 and 3'IL-6 systems did (Fig. 1*C*). 3'ApoB VNTR, like 33.6, did not reject paternity. In the four exclusion cases there were fetal alleles not present in either the alleged father or in the mother

TABLE 1—*Sequence of synthetic oligonucleotides for VNTR analysis by PCR-FLP.*

Locus	Map	LSO (5′-3′)	5′ Primer (5′-3′)	3′ Primer (5′-3′)	Reference
D1S111/33.6	1q	CCTCCAGCCCTCCTCCAGCCCT	TGTGAGTAGAGGAGACCTCAC	AAAGACCACAGAGTGAGGAGC	[1]
HRAS/H-ras	11p15.5	CACTCCCCCTTCTCTCCAGGGGACGCCA	TTGGGGGAGAGCTAGCAGGG	CCTCCTGCACAGGGTCACCT	[4]
pMCT118	1p	No probe	GAAACTGGCCTCCAAACACTGCCCGCCG	GTCTTGTTGGAGATGCACGTGCCCCTTGC	[5]
3′-ApoB	2	No probe	ATGGAAACGGAGAAATTATG	CCTTCTCACTTGGCAAATAC	[6]
3′-IL6	5q23.q31	No probe	GCAACTTTGAGTGTGTCACG	GACGTGATGGATGCAACAC	[7]
D17S5/pYNZ-22	17p13.3	No probe	CGAAGAGTGAAGTGCACAGG	CACAGTCTTTATTCTTCAGCG	[9]

TABLE 2—*DNA amplification conditions.*

System	Denaturation (°C/sec.)	Annealing (°C/sec.)	Extension (°C/sec.)	DNA[a] μg	Cl_2Mg[a] mM	Amplimers[a] pmol.	Taq-Pol.[a] units	Cycles
33.6	95/60	64/120	72/360	0.25	1.5	25	3	20
H-ras	95/60	64/120	72/360	0.25	1.5	25	3	20
pMCT118[b]	95/60	65/60	70/540	0.10	1.5	50	2.5	25
3′-ApoB	94/60	63/60	72/120	0.10	2.5	50	3	26
3′-IL6	94/15	55/30	72/60	0.10	1.5	25	2.5	28
pYNZ-22[c]	95/25	58/30	72/390	0.10	1.5	25	2.5	30

[a]Final concentration on the mix reaction.
[b]Amplification reaction in a final volume of 50 μL. Others in 100 μL.
[c]Perkin-Elmer 9600 DNA Thermal Cycler. Others on Perkin-Elmer 4800 DNA Thermal Cycler (Perkin-Elmer, USA).

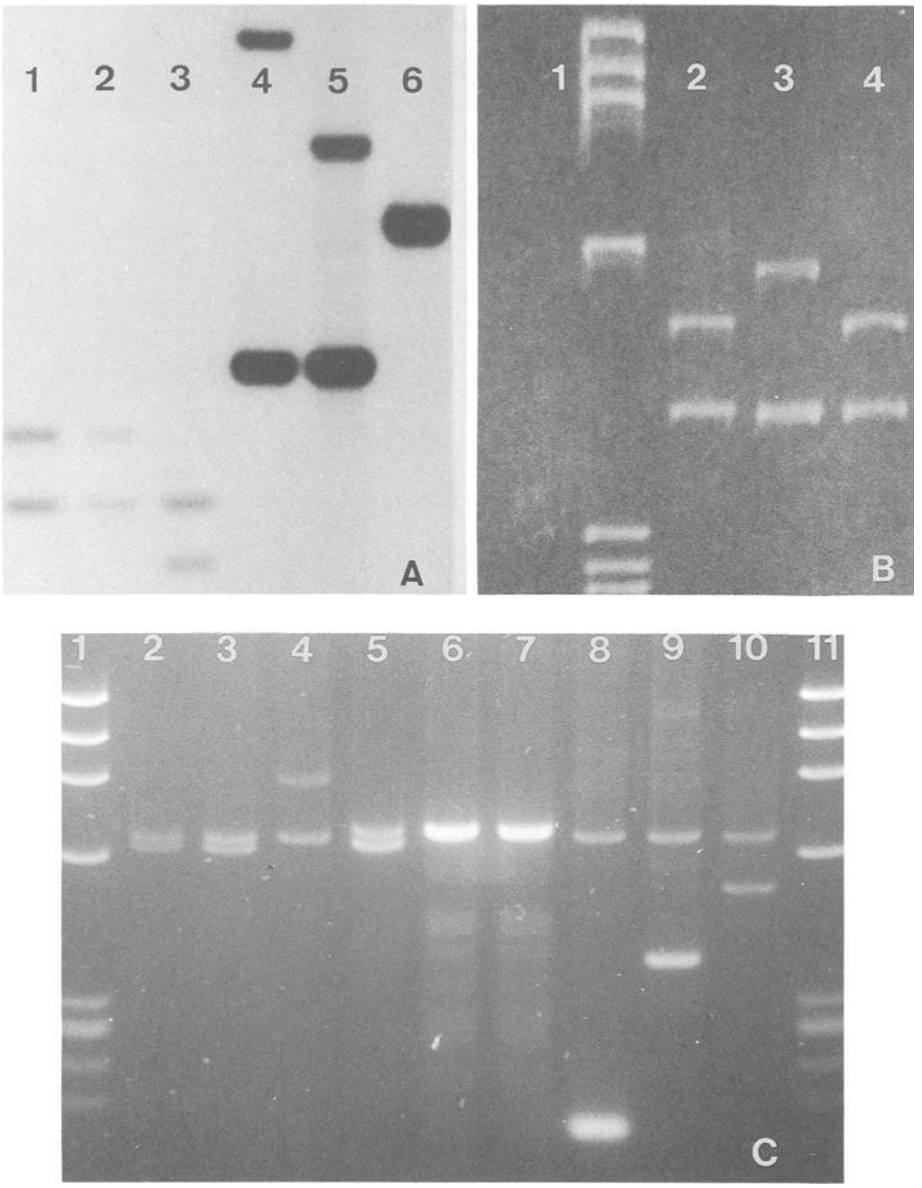

FIG. 1—A. *Alleles seen by PCR-FLP (autoradiography) for 33.6 (lanes 1, 2, 3) and H-ras (lanes 4, 5, 6) VNTR. Lanes 1 and 4 correspond to the mother; lanes 2, 5 are from the chorionic villi sample; and lanes 3, 6 from the alleged father. Exclusion of paternity could be only obtained for H-ras system.* B. *D1S80 typing of amplified DNA from alleged father (lane 2), chorionic villi (lane 3) and mother (lane 4) after PAGE and ethidium bromide staining. Lane 1 corresponds to a molecular weight marker (ΦX-174 DNA digested by Hae III). Exclusion of paternity can be clearly seen.* C. *Typing for 3'ApoB (lanes 2, 3, 4), 3'IL-6 (lanes 5, 6, 7) and pYNZ-22 (lanes 8, 9, 10) on agarose electrophoresis. Lanes 2, 5, 8 correspond to the chorionic villi DNA; lanes 3, 6, 9 to the mother; lanes 4, 7, 10 to the alleged father; and lanes 1, 11 to ΦX-174 digested by Hae III. Exclusion of paternity was only seen for pYNZ-22 and 3'IL-6 VNTR systems.*

genotype. No extra bands were observed on any amplified VNTR loci from fetal DNA so that the presence of maternal cells within the chorionic villi sample can be excluded.

Amplification procedures on VNTR systems must be standardized not only for Mg2 + /dNTP concentrations, and pH of the reaction mix, but that template DNA concentration should also be considered. In this respect, we have found that the amount of DNA may be critical in order to get successful results, where low quantities (50 to 100 ng) improved them and an excess (0.5 to 1 μg) would give no amplification (data not shown).

In conclusion, this case supports VNTR analysis as a powerful tool for prenatal paternity testing [*12*] because definitive and rapid exclusion could be achieved. At the same time, this method presents the advantage that DNA amplification allows testing with lower amounts of DNA obtained from almost any biological specimen, including non fresh samples in which DNA could be highly fragmented. Furthermore, it also has a higher level of resolution between alleles than any RFLP technique. In the future it will be possible to design multiplexed PCR in order to carry out a number of VNTR systems together, and the use of much less DNA (1 to 10 ng) in the PCR reaction.

For prenatal paternity testing an approach through HLA class II oligotyping can also be nicely tried. Nevertheless, the use of a high number of probes when a fine specificity is desired, apart from crossreactivity in certain cases or the presence of new alleles, establishes some degree of disadvantages.

Acknowledgments

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References

[*1*] Jeffreys, A. J., Willson, V., Thein, S. L., "Hypervariable 'Minisatellite' Regions in Human DNA," *Nature*, Vol. 314, 1985, pp. 67–73.

[*2*] Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, J., Kumlin, E., White, R., "Variable Number of Tandem Repeat (VNRT) Markers for Human Gene Mapping," *Science*, Vol. 235, 1987, pp. 1616–1622.

[*3*] Jeffreys, A. J., Wilson, V., Thein, S. L., Weatherall, D. J., Ponder, B. A. J., "DNA 'Fingerprints' and Segregation Analysis of Multiple Markers in Human Pedigrees," *American Journal of Human Genetics*, Vol. 39, 1986, pp. 11–24.

[*4*] Capon, D. J., Chen, E. Y., Levinson, A. D., Seeburg, P. H., Goeddel, D. V., "Complete Nucleotide Sequences of the T24 Human Bladder Carcinoma Oncogene and Its Normal Homologue," *Nature*, Vol. 302, 1983, pp. 33–37.

[*5*] Kasai, K., Nakamura, Y., White R., "Amplification of a Variable Number of Tandem Repeats (VNTR) Locus (pMCT118) by the Polymerase Chain Reaction (PCR) and its Aplication to Forensic Science," *Journal of Forensic Science*, Vol. 35, No. 5, September 1990, pp. 1196–1200.

[*6*] Boerwinkle, E., Xiong, W. J., Fourest, E., Chan L., "Rapid Typing of Tandemly Repeated Hypervariable Loci by the Polymerase Chain Reaction: Application to the Apolipoprotein B 3' Hypervariable Region," *Proceedings of the National Academy of Science USA*, Vol. 86, 1989, pp. 212–216.

[*7*] Bowcock, A. M., Rey, A., Erlich, H., Sehgal, P. B., "Rapid Detection and Sequencing of Alleles in the 3' Flanking Region of the Interleukin-6 Gene," *Nucleic Acids Research*, Vol. 17, 1989, pp. 6855–6864.

[*8*] Batanian, J. R., Ledbetter, S. A., Wolff, R. K., Nakamura, Y., White, R., Dobyns, W., Ledbetter, D., "Rapid Diagnosis of Miller-Dieker Syndrome and Isolated Lissencephaly Sequence by the Polymerase Chain Reaction," *Human Genetics*, Vol. 85, 1990, pp. 555–559.

[*9*] Brambati, B., Oldrini, A., Lanzani, A., "Transabdominal Chorionic Villus Sampling: A Freehand Ultrasound-Guided Technique," *American Journal of Obstetricy and Gynecology*, Vol. 157, 1987, pp. 134–137.

[*10*] Vicario, J. L., Martínez-Laso, J., Gómez Reino, J. J., Gómez Reino, F. J., Regueiro J. R., Corell A., Segurado O. G., Arnaiz-Villena A., "Both HLA Class II and Class III DNA Poly-

morphisms Are Linked to Juvenile Rheumatoid Arthritis Susceptibility," *Clinical Immunology and Immunopathology*, Vol. 56, No. 1, 1990, pp. 22–28.
[*11*] Southern, E., "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis," *Journal of Molecular Biology*, Vol. 95, 1975, pp. 503–508.
[*12*] Lobbiani, A., Nocco, A., Vedrietti, P., Brambati, B., Colucci, G., "Prenatal Paternity Testing by DNA Analysis," *Prenatal Diagnosis*, Vol. 11, No. 5, 1991, pp. 343–346.

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