## CASE REPORT

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# Removal of a PCR Inhibitor and Resolution of DNA STR Types in Mixed Human-Canine Stains from a Five Year Old Case

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**ABSTRACT:** The analysis of biological trace evidence from a reopened investigation into a 1991 murder from Vernon, B.C. revealed mixed human and dog bloodstains on blue jean pants that contained a PCR inhibitory substance. The presence of the inhibitory substance was detected by the inhibition caused from adding a small aliquot of the test DNA extract into a PCR reaction designed to produce a known standard product. The removal of the PCR inhibitory substance was accomplished by treating the extracted DNA with Thiopropyl Sepharose 6B beads. DNA profiles from two human contributors and a canine were obtained using species specific polymorphic STR markers. The two human DNA profiles obtained from blue jean pants were resolved, one matched the suspect and the other matched the victim. The DNA profile from the canine component matched that obtained from the known sample of the victim's dog who was also slain during the assault. This evidence along with other DNA typing evidence was critical in obtaining a resolution of the case.

**KEYWORDS:** forensic science, polymerase chain reaction, short tandem repeat, inhibitor, blood stain, DNA typing, human, canine, case

The polymerase chain reaction (PCR) amplification of polymorphic short tandem repeat (STR) loci is becoming the prevalent technology in the forensic DNA typing of human biological trace evidence (1–5) and for the genetic testing of animals both domestic (6) and wild (7). These multi-allelic markers are widely dispersed in most genomes and have been extensively used in genetic mapping (8,9). Amplified products can be resolved by polyacrylamide gel electrophoresis (PAG) into bands which can be sized and/or designated as discrete alleles. Bands can be detected directly by silver staining or through incorporation of fluorescent dye labeled PCR primers and a laser enhanced detection system using a DNA sequencing instrument. By incorporating a set of fluorescent dye tags it is possible to simultaneously amplify more than one STR locus at a time in a process termed multiplex PCR analysis. Since STRs are abundant, multi-allelic and amenable to PCR analysis, they appear to be well suited for identity testing in both humans and animals.

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### *Case History*

In 1991, a 68-year-old man was found beaten to death at his residence in Vernon, British Columbia. The victim's pet, a terrier dog, was also found dead inside a backyard shed. Both the man and his dog had fatal wounds to the head. Known blood samples were recovered from the victim and his dog for future comparisons using DNA analysis. Among the numerous exhibits collected in the investigation and submitted to the RCMP's Vancouver Forensic Laboratory was a pair of blue jean pants and a sweater reportedly from the suspect. Small quantities of blood were identified on the sweater and on four areas of the jeans; area A: left leg front thigh, area B: left knee cap, area C: 2 stained areas combined from near knee cap on both legs, and area D: combined stains from left and right pant leg.

In 1992, after organic extraction, the amount of DNA was determined based on hybridization to the D17Z1 probe and analysis by ethidium bromide stained agarose gel electrophoresis. The presence of human DNA on the sweater and both human and non-human DNA on the jeans, presumably canine, was identified. Since there was an insufficient quantity of DNA to proceed with RFLP-VNTR analysis, the DNA extracts were stored until technological advances became available for a more sensitive DNA analytical process.

In 1996, the case was reopened and PCR-STR analysis attempted by RCMP Forensic Biologists. The DNA profiles obtained from the blood on the suspect's sweater at 6 human STR loci matched those of the known sample from the deceased, however, no profile was obtained from the blood on the jeans. Since this evidence was key, it was decided to further strengthen the crown's case by attempting DNA analysis of the non-human, putative canine, DNA from the blue jeans. An attempt to PCR amplify canine STR loci by scientists at PE AgGen (Davis, California) was unsuccessful. Next we employed a strategy which involved confirming the presence of an inhibitor in the jean extract by spiking an amplification reaction of a plasmid sequence with a small amount of DNA extracted from one of the forensic samples and observing that no product was produced. This was followed by the successful re-

moval of PCR inhibitors from forensic samples using Thiopropyl Sepharose 6B beads. DNA analysis at 4 human and 11 canine STR loci was then performed on the remaining DNA.

## Methods

### *DNA Isolation and Quantitation*

Genomic DNA was extracted from forensic samples using a standard proteinase K and phenol/chloroform extraction protocol (10), followed by concentration on Microcon 100 filters (Amicon/Millipore Corp., Bedford, Massachusetts). The human DNA was quantified by slot blotting and hybridization with either $P^{32}$-dCTP labeled or alkaline phosphate conjugated D17Z1 (11,12). Total DNA was quantified by agarose gel electrophoresis with a series of known DNA concentrations followed by ethidium bromide staining and optical density spectrophotometry.

### *Human STR-PCR Amplification*

Amplification of STRs HUMvWA31, HUMTHO1, HUMF-13A1, and HUMfes/fps with fluorescent dye labeled primers was done simultaneously (multiplex STR 2) using the conditions described by Kimpton et al. (2,3) except for the use of 1X Taq® DNA Polymerase buffer (Perkin Elmer, Montreal) and 0.16 μg/μL of bovine serum albumin (Sigma, Mississauga, Ontario). Amplification of amelogenin for gender determination and STRs HUMD21S11 and HUMFGA with fluorescent dye labeled primers was done simultaneously (multiplex STR 1) using conditions described by Fregeau et al. (4,13). All amplifications were completed in a GeneAmp®PCR System Perkin and Elmer 9600 thermocycler (PE Applied Biosystems, Foster City, California).

An aliquot of 1 to 4 μL from each amplification reaction was mixed with 4 fmoles of ROX labeled ABD 2500 internal lane size standard in loading buffer and heat denatured prior to loading. This freshly prepared loading buffer consisted of 8M urea, 1X TBE, and 5 mg/mL blue dextran (13). The gels used were either 12 cm from well to read (STR2) or 24 cm from well to read (STR1) and consisted of 8M urea, 6% (19:1) acrylamide: bisacrylamide (BioRad, Mississauga, Ontario and GIBCO BRL Life Technologies Ultra Pure, Burlington, Ontario, respectively) in 1X TBE. After a pre-run to a temperature exceeding 42°C, the samples were loaded and run on a ABD 373A sequencing instrument (PE Applied Biosystems, Foster City, California) for approximately 3 h set at 1250 v, 85 mA, 85 W for STR2 or 5 h set at 1800 v, 85 mA, 85 W for STR1. Allele sizes were estimated using the local Southern method from GeneScan™ Analysis v.1.2.1 and Genotyper™ Analysis v.2.0 software (PE Applied Biosystems, Foster City, California).

### *Canine STR-PCR Amplification*

Both 1 μL and 5 μL from each sample (or dilution) were amplified in two multiplex reactions employing fluorescent dye labeled primers; the first amplifying at 7 STR loci ($CATA_1$, PEZ6, PEZ 8, PEZ 12, PEZ 18, FHC 2010, FHC 2054) and the second at 4 STR loci (PEZ 3, PEZ 5, PEZ 11, FHC 2079). Primer sequences for PEZ loci are listed in Table 1, $CATA_1$ and FHC loci have been previously published (14,15).

An aliquot of 0.2-2.0 μL from each amplification reaction was mixed with 2 μL loading solution consisting of: 6 volumes formamide, 1 volume ABD loading buffer, 1 volume of ROX labeled ABD internal lane size standard. The samples in loading solution were heat denatured at 95°C for 5 min, before loading. The gels used were 36 cm from well to read and consisted of 5% polyacrylamide (Syngel™ from FMC, Rockland, Maine) in 1X TBE. After a pre-run for 5 min at 1000v, 35 mA, 50 W, the samples were loaded and run on a ABD 377 sequencing instrument (PE Applied Biosystems, Foster City, California) for approximately 2 h, set at 3000 v, 60 mA, 200 W, while maintaining temperature at 51°C. DNA fragment analysis was performed with GeneScan™ Analysis and Genotyper™ Analysis v.2.0 software.

TABLE 1—*Primer sequences for canine specific PEZ loci.*

| STR Loci | Forward Primer | Reverse Primer |
|---|---|---|
| PEZ 3 | CACTTCTCATACCCAGACTC | CAATATGTCAACTATACTTC |
| PEZ 5 | GCTATCTTGTTTCCCACAGC | TCACTGTATACAACATTGTC |
| PEZ 6 | ATGAGCACTGGGTGTTATAC | ACACAATTGCATTGTCAAAC |
| PEZ 8 | TATCGACTTTATCACTGTGG | ATGGAGCCTCATGTCTCATC |
| PEZ 11 | ATTCTCTGCCTCTCCCTTTG | TGTGGATAATCTCTTCTGTC |
| PEZ 12 | GTAGATTAGATCTCAGGCAG | TAGGTCCTGGTAGGGTGTGG |
| PEZ 18 | GAGAAGATAAAGCAATTCTC | AAGTCATTAATCTCTCCTCG |

### *Testing for PCR Inhibition*

A 1 μL aliquot of the DNA extracts (25 μL original volume) from 2 areas of the blood stained blue jeans was added into PCR amplification reactions designed to produce PEZ 290, a 290 base ROX-labeled sizing standard from a plasmid (PE Applied Biosystems, Foster City, California). A sample of one of the forensic control human DNA samples was used as control for the organic extraction process.

### *Removal of Inhibitor*

The DNA extracts were treated with Thiopropyl Sepharose 6B (Sigma, Mississauga, Ontario) as described in Williamson et al. (16). Recovered DNA was concentrated on a Microcon 100 filter. The amount of DNA remaining was then determined using slot blot hybridization with D17Z1 alkaline phosphate conjugated probe (ACES™ $2.0^+$ Human DNA Quantitation System BRL GIBCO Life Technologies, Burlington, Ontario).

## Results and Discussion

### *Assessment of DNA Yield*

Since less than 40 ng of DNA was recovered from the forensic samples, RFLP analysis was not attempted in 1992. The blood stain on the sweater yielded about 12 ng of human DNA while the yield of human DNA recovered from the 4 areas on the blue jeans ranged from about 12 ng to 23 ng. The integrity agarose gel result showed little high molecular weight staining and showed a slightly different variation in signal intensity than would be expected from the slot blot hybridization results for the blue jean samples (presumably due to the presence of non-human DNA).

### *Initial STR Analysis*

The DNA profiles obtained indicated a mixed sample was collected from blood on the sweater. The major component DNA profile of the two person mixture matched that from the known sample of the deceased at 5 human STR loci (Table 2). Median values for all putative matches (consisting of known origin and questioned origin samples) were determined and a match window of ±0.75 base was used as the criteria to declare a match. The estimated frequency of occurrence for this DNA profile from the RCMP Canadian Caucasian population database using a floating bin method

TABLE 2—*Allele size estimates in bases for STR-DNA profiles from blood stained sweater and known samples from deceased and suspect.*

| STR Locus | Known Sample from Victim | Known Sample from Suspect | Blood Stained Sweater |
|---|---|---|---|
| D21S11 | 1) 218.03 2) 226.60 | 1) 232.88 2) 239.88 | No product detected |
| FGA | 1) 280.20 2) 286.60 | 1) 290.74 2) 290.74 | 1) 280.08 2) 286.49 |
| vWA | 1) 142.77 2) 150.98 | 1) 146.78 2) 150.89 | 1) 142.79 2) 150.80 |
| THO1 | 1) 157.98 2) 162.09 | 1) 166.16 2) 170.54 | 1) 157.85 2) 161.99 Major<br>3) 166.14 4) 170.29 Minor |
| F13A1 | 1) 195.11 2) 195.11 | 1) 191.45 2) 195.32 | 1) 195.21 2) 195.21 |
| fes/fps | 1) 217.78 2) 226.31 | 1) 210.21 2) 222.00 | 1) 218.01 2) 226.41 Major<br>3) 210.29 Minor* |

* The anticipated approximate 222 base allele for the minor component could not be clearly distinguished from an n-4 product of the 226.41 base allele from the major component of the amplicon.

TABLE 3—*Allele size estimates in bases for STR-DNA profiles from suspect's blood stained blue jeans.*

| STR Locus | Area #1 | Area #2 | Area #3 |
|---|---|---|---|
| vWA | 1) 146.75 2) 150.75 | 1) 146.81 2) 150.78* Major<br>3) 142.65 Minor | 1) 146.45 2) 150.82 |
| THO1 | 1) 166.09 2) 170.37 Major<br>3) 157.79 Minor<br>4) 173.61 Trace | 1) 166.17 2) 170.19 Major<br>3) 157.75 4) 161.95 Minor<br>5) 173.38 Trace | 1) 166.10 2) 170.17 Major<br>3) 157.63 4) 161.86 Minor |
| F13A1 | no product detected | 1) 191.23 2) 195.30† | |
| fes/fps | no product detected | 1) 210.16 2) 222.42 Major<br>3) 218.44 4) 226.79 Minor | |

* Approximate 2 fold difference in peak height relative to the peak at 146.81 bases.
† Approximate 2 fold difference in peak height relative to the peak at 191.23 bases.

with a 1.5 base window was 1 in 4.9 million. Only two STR loci yielded a profile from the minor component of the mixed blood stain (Table 2). No DNA typing profile was obtained from the blood stained blue jeans from either human or canine STR loci.

*Detection and Removal of PCR Inhibiting Substance*

The failure to obtain a DNA profile at both human and canine STR loci from the blood stained blue jeans and the presence of adequate quantities of DNA for analysis raised suspicions that a PCR inhibitory substance had co-extracted with the DNA. A PCR inhibitory substance had been encountered in another homicide investigation recently reported (18). This suspicion was confirmed when 1 μL of one of the DNA extracts from the jeans was found to prevent the PCR amplification of a 290 base ROX-labeled sizing standard in a test PCR reaction.

The remaining DNA was treated with Thiopropyl Sepharose 6B resin, the inhibitor allowed to bind before recovering the DNA from the bead resin. A second slot blot quantitation using the more sensitive chemiluminescent detection approach showed human DNA of sufficient quantity for PCR analysis to be attempted for areas A, B and C, but not D. Area D was to be used for canine STR PCR analysis.

*Final STR Analysis*

DNA analysis of the blue jean bloodstains with human multiplex STR2 loci yielded DNA typing profiles from a mixed sample. The major component DNA profile matched that of the known sample from the suspect at 4 STR loci while the minor component matched that of the known sample from the deceased at 4 STR loci (Table 3). The estimated frequency of occurrence for the DNA profiles from the RCMP Canadian Caucasian population database was determined to be 1 in 66,000 and 1 in 13,000 respectively for the major (endogenous) and minor (foreign) components.

TABLE 4—*Genotype designations for canine STR-DNA profiles from suspect's blood stained blue jeans.*

| STR Locus | Known Sample from Deceased Terrier | Area #4 |
|---|---|---|
| PEZ 1 | DD | DD |
| PEZ 18 | DF | DF |
| UCB 2054 | FF | FF |
| UCB 2010 | BD | BD |
| PEZ 12 | EH | EH |
| PEZ 6 | DH+ | DH+ |
| PEZ 8 | DE | DE |
| PEZ 3 | EE | EE |
| PEZ 5 | AD | AD |
| PEZ 11 | FH | FH |
| UCB 2079 | BB | BB |

Analysis of the remaining DNA with two canine multiplex STRs yielded a DNA profile matching that of the known sample from the deceased terrier at 11 STR loci (Table 4). In the general dog population the matching probability of unrelated individuals for the 11 STR loci, using frequencies for designated alleles, was estimated at 1 in 3 billion. The population database for the dog population was based on STR results from groups (18–35 dogs/breed) of unrelated individuals from 15 pure breeds and an "all breed" panel composed of 96 purebred individuals of many different breeds. A total number of 516 dogs were tested for dog STR markers in the generation of a dog population database.

## Conclusion

Advances of new technology, such as PCR-STR DNA typing, can provide the opportunity to reinvestigate cases that could not be solved or brought to closure with the technology of the day. Conversely, there are challenges in the application of new technology to the diverse environment characteristic of forensic samples. The case report of the 1991 Vernon, B.C. homicide is a reopened investigation where there was insufficient DNA suitable for RFLP DNA analysis. The application of PCR-STR DNA analysis provided evidence that the blood on the sweater reportedly worn by the suspect originated from the deceased. The other key piece of evidence, blood stains on jeans from the suspect, initially did not yield a DNA profile. It was later deduced that the DNA from the blue jeans contained both human and nonhuman DNA along with an inhibitor of the PCR reaction.

The PCR inhibition was confirmed by spiking an amplification reaction of a plasmid sequence with a small amount of DNA extracted from one of the forensic samples and observing that no amplified product was produced. The removal of PCR inhibitors from these forensic samples was accomplished by using Thiopropyl Sepharose 6B beads. The Microcon 100 filter and BSA are thought to remove PCR amplification inhibition that could be caused by Heme from blood stains (17). The Sepharose beads are thought to remove dyes like that found in blue jean material (16) which can be inhibitory to the PCR amplification reaction. Following treatment for these inhibitors, DNA typing profiles from two humans and a canine contributor were obtained from the treated DNA.

The DNA profile obtained from the canine DNA matched that of the known sample from the deceased's terrier. The DNA profiles obtained from the human DNA could be resolved into two contributors: one matching the deceased and the other the suspect. The combination of this evidence and the DNA profile from the sweater were key factors in the resolution of this case by the B.C. Supreme Court.

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## References

1. Gill P, Kimpton CP, Urquhart A, Oldroyd N, Millican E, Watson S, et al. Automated short tandem repeat (STR) analysis in forensic casework—a strategy for the future. Electrophoresis 1995;16:1543–52.
2. Kimpton CP, Gill P, Walton A, Urquhart A, Millican ES, Adams M. Automated DNA profiling employing multiplex amplification of short tandem repeat loci. PCR Methods and Applications 1993;3:13–22.
3. Kimpton CP, Fisher D, Watson S, Adams M, Urquhart A, Lygo J, et al. Evaluation of an automated DNA profiling system employing multiplex amplifications of four tetrameric STR loci. Int J Leg Med 1994;106: 302–11.
4. Fregeau CJ, Fourney RM. DNA typing with fluorescently tagged short tandem repeats: A sensitive and accurate approach to human identification. Biotechniques 1993;15:100–19.
5. Hochmeister MN, Budowle B, Eisenberg A, Borer UV, Dirnhofer R. Using multiplex PCR amplification and typing kits for the analysis of DNA evidence in a serial killer case. J Forensic Sci 1996;41:155–62.
6. Menotti-Raymond M, David VA, Stephens JC, Lyons LA, O'Brien SJ. Genetic individualization of domestic cats using feline STR loci for forensic applications. J Forensic Sci 1997;42(6):1039–51.
7. Craighead L, Paetkau D, Reynolds HV, Vyse ER, Strobeck C. Microsatellite analysis of paternity and reproduction in arctic grizzly bears. J Hered 1995;86:255–61.
8. Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. Am J Hum Genet 1991;49:746–56.
9. Murray JC, Buetow KH, Weber JL, Ludwigsen S, Scherpbier-Heddema T, Manion F, et al. A comprehensive human linkage map with centimorgan density. Science 1994;265:2049–54.
10. Royal Canadian Mounted Police. Biology section methods guide, Chapter III.1, Isolation of DNA from body fluid stains. Revised 92, October 16.
11. Waye J, Presley L, Budowle B, Shutler G, Fourney R. A simple and sensitive method for quantifying human genomic DNA in forensic specimen extracts. BioTechniques 1989;7(8):852–5.
12. Royal Canadian Mounted Police. Biology section methods guide, Chapter III.1, Slot blot quantitation of human genomic DNA. Revised 96, March 14.
13. Royal Canadian Mounted Police. Biology section methods guide, Chapter III.3, PCR typing: Fluorescent multiplex amplification of short tandem repeat (STR) loci. Revised 95, March 24.
14. Primmer CR, Matthews ME. Canine tetranucleotide repeat polymorphism at the VIAS-D10 locus. Animal Genetics 1993;24(4):332.
15. Francisco LV, Langston AA, Mellersh CS, Neal CL, Ostrander EA. A class of highly polymorphic tetranucleotide repeats for canine genetic mapping. Mammalian Genome 1996;7:359–62.
16. Williamson JM, Waye JS, Newall P, Bing DH, Blake E. The use of a comprehensive approach for neutralization of PCR inhibitors found in forensic samples and its use in a homicide/sexual assault case. Proceeding from the Sixth International Symposium on Human Identification 1995. Promega Corporation, Madison, WI. 1996;186–7.
17. Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K. Identification of the heme compound copurified with DNA from bloodstains, a major inhibitor of PCR amplification. J Forensic Sci 1994;39:362–72.
18. Waye JS, Blake ET, Williamson JM, Bing DH. DNA evidence in the Christine Jessop homicide case. Can J Forensic Sci 1998;55–62.

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