

## CASE REPORT

### CRIMINALISTICS

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# Determining the Source of Equine Bloodstains by Dinucleotide Repeats\*

**ABSTRACT:** A novel multiplex of independent dinucleotide tandem repeat (DTR) loci was previously described that is capable of not only discriminating human and equine DNA, but of identifying a single equine source. We report a case in which a bloodstained syringe and two needles were found during inspection of a barn by inspectors of the Pennsylvania Racing Commissions. Using the multiplex and single-locus detection, all 21 equine DTR markers were detected in a suspect horse and two evidence samples, indicating the evidence samples came from the suspect animal. Only six markers were detected in the third evidence sample because the volume of blood was limited. Following whole-genome amplification and single-locus PCR, the third evidence sample detected a total of 17 markers and the likelihood of identity (probability from suspect horse/probability from a random pacer) was  $7.0 \times 10^6$ . The DTR multiplex has some technical limitations, but it is already practical for casework.

**KEYWORDS:** forensic science, racehorses, sample identification, bloodstains, short tandem repeats, multiplex polymerase chain reaction, whole-genome amplification

There are several ways to prevent doping of race horses, which adversely affects their welfare. These include testing of blood or urine postcompetition and the prohibition of needles and syringes from racetracks, except when they are used by practicing veterinarians. The PA Racing Commissions may require testing a biologic sample for presence of drugs whether it has been obtained from a horse or from a syringe or needle found after unannounced barn searches. A sample's chain-of-custody documentation may be challenged so that it is important to identify the source of the sample. The origin of a sample from a needle or syringe may be equine or human, and some blood samples may be limited in quantity or degraded. Therefore, it is important to examine the sample DNA for species and individual by robust methods. Recently, analysis of 21 equine dinucleotide tandem repeat (DTR) loci (1) and whole-genome amplification (WGA) (2) have permitted identifications with almost no ( $3.1 \times 10^{-13}$ ) error. In this article, we describe an actual case to demonstrate how dried blood from a needle and syringe was analyzed and shown to be from a suspect horse.

## Materials and Methods

### Samples

A suspect blood sample, one syringe and two needles containing bloodstains were received in the laboratory for identifying the source of blood. Evidence and suspect samples were collected during a routine barn search at a racetrack in PA.

### DNA Isolation and Quantification

Bloodstains were recovered from the syringe and needles by suspending in TE buffer. Total DNA was isolated by Genorise DNA Purification System (Genorise Scientific, PA) (1). DNA was verified on 0.8% agarose gel, and the quantity was estimated by TL100 software as well as OD<sub>260</sub> measurement (1,3). DNA quantity recovered from the suspect horse sample was 10.23 µg per 0.3 mL blood, 1.12 µg from syringe, 300 ng from needle #1, but no quantifiable DNA was recovered from needle #2.

### Whole-Genome Amplification

To recover quantifiably sufficient DNA for PCR analysis on needle #2, multiple displacement amplification (MDA) was conducted to amplify entire genomic DNA from the isolate using REPLi-g mini kit (Qiagen, CA) (2). A total of 1.2 µg DNA was obtained following WGA.

### PCR Amplification

A previously developed short tandem repeat (STR) typing method with minor modification was used in generating DTR profile for each sample by HotStar Taq DNA Polymerase Master Mix (Qiagen) (1). Briefly, A single tube of 15-µl reaction was

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TABLE 1—Equine and human (\*) short tandem repeat (STR) loci, dye assignment, sequences and concentrations (nM) of PCR primers, and PCR product size (bp).

STR	Dye	Primer 1	Primer 2	nM	Size
VHL20	D4	caagtcttacttgaagactag	aactcaggaggagaattctctcag	70	90–110
UMNe156	D2	agactagcttcaaatgcccc	cctatgcttgaaggagtgtg	90	102–124
HTG4	D3	ctatctcagttctgtgcaggac	ctccctccctccctctgttctc	40	128–140
AHT4	D2	aaccgcctgagcaaggaagt	tcccagagagtttaccctgg	90	146–162
HMS6	D3	gaagctgccagtattcaaccattg	ctccatctgtgaagtgttaactca	70	160–170
HMS7	D4	caggaaactcatgttgataccatc	tgttgtgaaacataccttgactgt	70	175–185
ASB9	D2	tttctcctccactacacac	tccttatcacaatagagcag	100	201–215
ASB2	D3	ccttcctgatgttaagctctg	cttccccagaagttagagcag	90	190–226
COR045	D4	taccgcaagtgaaccagttc	ttgtgggactgagcccttaac	70	217–233
HMS3	D2	ccaactcttgcacataacaaga	catcagtcagaagctgcgaacc	90	243–265
UMNe222	D3	accaagctatgagtcaggag	agcatctcatgtcctctgc	60	249–267
LEX074	D4	ccccctaaattcagaaagagagcc	ggaatttggagattatctgtgggc	15	273–291
TH01*	D2	ctgttctcccttatttccc	ggtacctggaaatgacactg	100	281–297
COR008	D3	aggacactgaaggctgaaag	tagatagcgtctggagggttc	90	297–321
HMS2	D2	acgggtggcaactccaagggaag	gatctctagctcagtaaatcacagg	100	305–325
UM015	Cy5	agtctggctgaggatactg	tttctgtcacattagaggg	40	330–346
COR018	D2	tgagcttctgtactcctgg	ccacatctgggagtactaga	90	343–368
UMNe116	D3	ctggctaaactcttattcc	acatgggagaaaatacacac	90	364–378
UMNe479	D4	gagatggatggaatagcttg	tgcccagcctgaaagatttc	60	377–385
HMS1	D2	cacttatcagagagccctcc	gtcaccactctatcagggg	90	399–409
TPOX*	D2	cacagcttgatctcctcatg	tgaactcctcaggtccaatc	100	425–445
UMNe191	D3	tgtcctcacttggcatgagtc	ccagatggtgaaacaaggggc	100	414–442
LEX073	D4	ttcagaacatcatcagcatcccc	ccaccactcaaagtactagggc	40	462–492
D18S51*	D3	actgcacttactctgagtg	cactttagccgacaaaaggc	100	470–494

assembled by 1× Master Mix, 3 mM MgCl<sub>2</sub>, 20–50 ng DNA template, 40–100 nM primers, 67 µg/mL BSA, and 667 µg/mL Triton X-100. PCR amplification was subjected to 95°C for 15 min, 30 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min with final extension at 72°C for 10 min in a TC-512 Thermal Cycler (Techne, NJ). The 24-plex PCR was conducted to detect 21 equine DTR loci (Table 1) and three human tetranucleotide tandem repeat (TTR) loci to detect contamination of equine sample by human DNA. To amplify each of 21 loci in the suspect and evidence samples, a single-locus PCR was conducted using the same conditions as in the multiplex method.

#### Detection of Amplified DTR Loci and Allele Designation

Amplified markers were measured for size using capillary electrophoresis (CE) by a CEQ8800 Genetic Analysis System (Beckman Coulter, Brea, CA) (1,3). Individually amplified STR loci for each sample were pooled in one well of a 96-well plate for fragment analysis. Allelic size was determined by comparing to an internal size standard-600 containing 33 fragments (60–640 nucleotides) with 10–20 nucleotides apart and by fragment analysis software (Beckman Coulter) (1). An allele was designated as number of repeat motif plus 0.1 for partial repeat (one nucleotide) (1,3,4).

#### DNA Sequencing

Allele 20 and stutter products at locus HMS1 were sequenced using GenomeLab™ Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter) as described previously (1). A single PCR product of allele or stutter was isolated from 6% polyacrylamide gel prior to sequencing.

#### Statistical Analysis

The random match probability (RMP) and likelihood ratio (LHR) were used to estimate the probability of sample matching (5).

RMP evaluates the probability of having a match under the following defense hypothesis: a random horse (not the suspect) is the contributor of the DNA sample, which is obtained as a product of genotype frequencies for Pacer Standardbred population. To compare two typical samples, a product of genotype frequencies of which independent loci have identical or distinct genotypes was calculated and LHR was obtained.

## Results and Discussion

### Multiplex PCR Revealed Detection of 20 Equine DTR Loci in the Suspect and Two Evidence Samples

Multiplex PCR indicated that all equine DTR markers except locus HMS1 were clearly detected in suspect horse, syringe, and needle #1 (data not shown). Results obtained from multiplexing were consistent with those from single-locus detection. Human TTR loci were not detected in the samples, suggesting that the source of the bloodstains was a horse not human.

A 24-plex STR method was developed as a novel approach to identify racehorse sample and contamination by human DNA (1). Although the method still uses DTR and internal size standard instead of allelic ladder, it had demonstrable advantages: higher power of identification, use of conventional allelic designation, and detection of contamination by human DNA. Multiplexing conditions were optimized according to DNA template, concentrations of magnesium and primer, and cycling parameters. We noted that it was helpful to include BSA and Triton in the multiplex assay to improve DNA amplification of challenging samples such as these case samples. These two reagents did not show any adverse effect on fragment sizes and thus, should not affect genotype results. Three human TTR markers in the 24-plex panel were human specific and did not recognize equine DNA (1). Based on the results at hand, it is clear that bloodstains in the syringe or needle #1 shared the same source with the suspect sample.

In the previous study, no deficiency of detection was observed for HMS1 locus in 261 samples. Difficulty in detecting HMS1

may suggest a rare mutation in the primer binding site in these case samples. It would be advisable to remove HMS1 from the panel if it continued to show deficiency in its detection.

#### Detection of 17 Equine DTR Loci in Needle #2

Only six markers were detected from needle #2 in 24-plex PCR (not shown). Following WGA of the DNA sample, eight more markers were detected while seven others showed weak or no signals by multiplexing. However, by single-locus PCR, DTR profiles for three additional markers (COR018, HMS1, and UMN191) were observed. Altogether, 17 markers were detected in needle #2 although sufficient DNA was generated following WGA. The incomplete DNA profile may be attributed to low quality or DNA damage.

DNA quantity contributes to the success of multiplexing and low DNA template such as 1 ng made some alleles undetectable (1). Needle #2 presented difficulty in the multiplex detection of equine DTR markers although the addition of BSA and Triton improved amplification. In addition, the result of this study indicated that WGA could improve DNA yield to cause some alleles to become detectable. WGA is a great approach for limited forensic samples that contain low level DNA (2). However, WGA may not recover or repair damaged DNA template, and thus has limitations in improving PCR assay if the template is damaged. Seven markers were heterozygotes in the suspect and two evidence samples, but were detected as homozygote in needle #2. Although it was not readily evident as to whether this high rate of homozygote was due to the limited detection of the difficult sample, allelic dropout in WGA treated sample may not necessarily indicate that WGA caused such effect. Evidence has shown that WGA did not eliminate but improved allelic detection of HMS1 when diluted DNA samples were subjected to WGA (not shown). Moreover,  $\phi$ 29 DNA polymerase used for MDA has an error rate of 1 in  $10^6$ – $10^7$  (6). If the absence of other alleles was because of the DNA damage or low quality, this sample may share common source with other three samples.

#### Locus HMS1 Was Detectable with Single-Locus PCR

Locus HMS1 was not detectable in multiplexing but was in single-locus PCR in all four samples (not shown). These four samples showed an identical product of allele 20 that was confirmed by DNA sequencing. Interestingly, the allele at locus HMS1 was not detected in the previous study in 171 Standardbred horses (1). Stutter peaks present in all samples were sequenced and were one repeat unit smaller. Needle #2 also showed two additional stutter products that were two and three repeat units smaller than the allelic product. Multiple stutter peaks in needle #2 may be related to low DNA quality.

While multiplex PCR showed difficulty in amplifying some markers in extreme circumstances such as degraded samples, a single-locus PCR may overcome this difficulty. It is necessary for multiplex method to reveal complete STR profile; however, this may not be always true especially in extremely difficult samples as presented in this case study. Although the robustness of the multiplex assay is in high demand in forensic science, this casework showed that single-locus detection is useful as an alternative approach when difficult DNA template is presented. However, single-locus amplification may not be needed if multiplexing can reveal complete STR profile.

#### Identification of Allelic and Stutter Peaks

All allelic peaks were identified by multiplex and single-locus PCR and by CE. They were identical between single and multiplex detections. Stutter peaks were observed in 17 of 21 markers. For instance, at locus ASB2, an identical peak pattern was observed between suspect (Fig. 1A) and syringe (Fig. 1C) samples. In both samples, the highest peak was designated allele 26. The third highest peak in the suspect sample and the second highest peak in the syringe were both three repeat units larger than the first allelic peak, and thus were designated as allele 29. Peak 216 (arrow) was one repeat unit smaller than the corresponding allelic peak and thus it was considered a stutter peak in both suspect and evidence

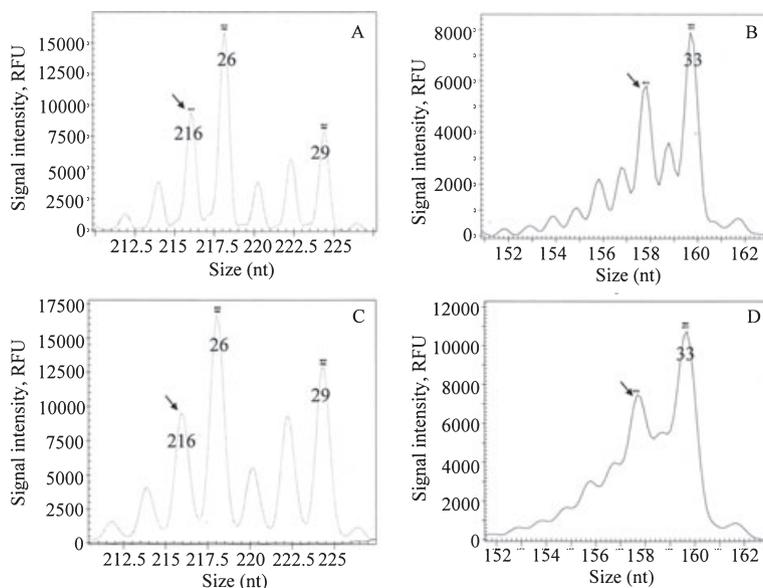


FIG. 1—Detection of stutter activity. In suspect (A, B) and evidence (C, D) samples, stutter peaks were detected at loci ASB2 (A, C) and AHT4 (B, D). Allelic peaks were labeled and designated by number of repeat unit determined by DNA sequencing. Typical stutter peaks are indicated by arrows and other minor peaks are characterized as non-allelic peaks (not labeled).

samples. The other minor peaks appeared as stutter or nonallelic products. Both samples (suspect and syringe) showed identical STR profile that was a heterozygote at locus ASB2. Locus AHT4 also showed an identical peak pattern between suspect (Fig. 1B) and syringe (Fig. 1D). In both samples, the highest peak was allele 33, the second highest peak that was one repeat unit smaller was considered a stutter product. The other minor peaks were smaller in size and one nucleotide apart and appeared as nonallelic products. Both samples (suspect and syringe) exhibited an identical genotype that was a homozygote at locus AHT4.

While DTR loci were employed to identify disputed samples, nonallelic peaks such as stutter peaks need to be clearly identified and interpreted. Stutter peak usually appear as one or more repeat unit smaller in size (7,8) and is defined as an artifact of PCR amplification (8). Multiple stutter peaks with partial repeat unit (single bp) apart were also observed (Fig. 1B). These irregular artifact peaks were much smaller than the regular stutter peaks with peak area ratio (PAR) of 0.28–0.44 to the allelic peaks (not shown). Stutter product was previously identified as shadow band by amplification of DTR loci, a nonallelic product probably from amplification slippage caused by a pause in DNA chain elongation (8). Occurrence of stutter activity depends on the nature of *Taq* DNA polymerase with processivity of approximately 40 (8). It was expected that a more processive thermostable DNA polymerase could reduce or eliminate stutter activity. In TTR locus, stutter activity mostly occurred as one repeat unit smaller than the corresponding allele (9). However, in DTR locus, nonspecific amplification often represents reduction by multiples of the repeat unit (8). Although stutter activity was very common, some markers (UMNe222, COR018, UMNe479, and UMNe191) showed minimal to no artifact activity. DNA templates from bloodstains and suspect sample showed no difference in stutter activity as a sign of the same nature of these samples.

With amplification of TTR loci, PAR of the stutter peak was usually less than 15% of allelic peak (7,9,10). In DTR loci, however, stutter peaks were often comparable to allelic peaks according to peak areas. This undesired activity may complicate interpretation of the results. However, in this case study, conclusion can be easily drawn because both evidence and suspect samples had identical peak patterns. Because of the disadvantages of DTR, it would be important to develop a method for detection of equine TTR for more effective forensic DNA testing. The drawback is that there are only few equine TTR sequences published with little information of its feasibility in forensic testing. Cloning and polymorphism characterization of more TTR sequences would definitely benefit the horse racing industry in sample identification and doping control.

*Comparisons of Genotypes between the Suspect and the Evidence*

The suspect and two evidence samples exhibited identical profiles (Table 2). Among 17 markers detected in needle #2, 79% alleles were identical to the other samples. Among 21 DTR markers, 14 loci accounting for 67% were heterozygote in the suspect and two evidence samples. In needle #2, only three markers showed distinct alleles that accounted for 28% heterozygote. The overall PAR was similar between the suspect and two evidence samples with an average of approximately 0.70.

*Random Match Probability and Likelihood Ratio*

Except for alleles at locus HMS1, alleles at all other loci were present at frequency of 0.01–0.63 (1). All samples presented a new

TABLE 2—Comparisons of short tandem repeat (STR) profiles between suspect and evidence.

	Suspect	Syringe	Needle #1	Needle #2
VHL20	18/23.1	18/23.1	18/23.1	18/23.1
UMNe156	21/21	21/21	21/21	21/21
HTG4	16/20	16/20	16/20	16/20
AHT4	33/33	33/33	33/33	33/33
HMS6	17/19	17/19	17/19	ND/ND
HMS7	20/20	20/20	20/20	20/20
ASB9	15/15	15/15	15/15	ND/ND
ASB2	26/29	26/29	26/29	29/29
COR045	21/22	21/22	21/22	21/22
HMS3	23/25.1	23/25.1	23/25.1	25.1/25.1
UMNe222	10/16	10/16	10/16	ND/ND
LEX074	19/20	19/20	19/20	19/19
COR008	19/24	19/24	19/24	19/19
HMS2	18/18	18/18	18/18	ND/ND
UM015	14/18	14/18	14/18	14/14
COR018	16/17	16/17	16/17	16/16
UMNe116	19/20	19/20	19/20	19/19
UMNe479	24/24	24/24	24/24	24/24
HMS1	20/20	20/20	20/20	20/20
UMNe191	13/17	13/17	13/17	13/17
LEX073	34.1/34.1	34.1/34.1	34.1/34.1	34.1/34.1

allele (20) at locus HMS1 with calculated allele frequency of 0.006 ( $n = 172$ ). With allele frequencies defined in the Standardbred Pacer racehorse (Table 3) (1), RMP was  $3.1 \times 10^{-13}$  between the suspect and two evidence samples, and  $1.5 \times 10^{-10}$  between evidence 3 and other samples, assuming a random horse is the contributor of the DNA sample (5).

To estimate match probability between suspect and evidence samples, LHR was also calculated. LHR for the match between suspect and two evidence samples was  $3.2 \times 10^{12}$ . LHR for the match between needle #2 and other samples was  $7.0 \times 10^6$  although such a match is less likely compared with the one between the suspect and remaining two evidence samples.

TABLE 3—Allele frequencies for alleles detected in the suspect and evidence samples ( $n = 172$ ).

STR	Allele	Frequency	Allele	Frequency
VHL20*	18	0.377	23.1	0.044
UMNe156	21	0.544	NA	NA
HTG4	16	0.436	20	0.099
AHT4*	33	0.474	NA	NA
HMS6*	17	0.152	19	0.447
HMS7*	20	0.377	NA	NA
ASB9	15	0.301	NA	NA
ASB2*	26	0.257	29	0.196
COR045	21	0.146	22	0.386
HMS3*	23	0.383	25.1	0.056
UMNe222	10	0.175	16	0.693
LEX074	19	0.023	20	0.281
COR008	19	0.091	24	0.477
HMS2*	18	0.386	NA	NA
UM015*	14	0.395	18	0.155
COR018*	16	0.187	17	0.594
UMNe116*	19	0.184	20	0.360
UMNe479*	24	0.711	NA	NA
HMS1	20	0.006	NA	NA
UMNe191*	13	0.237	17	0.243
LEX073*	34.1	0.126	NA	NA

\*Random match probability (RMP) and likelihood ratio (LHR) were calculated over 13 independent loci.  
STR, short tandem repeat; NA, not applicable.

Equine sample identification with proper interpretation of the result is rarely reported (11). Most alleles shown in these case samples were common in the Standardbred Pacer horses (Table 3). Among 42 alleles in 21 loci, six alleles appeared less than 10 in 100 horses in the limited population especially the alleles at locus HMS1 was detected for the first time. Although the RMP and LHR were calculated based on the allele frequency observed in the limited population, an accurate matching probability could be drawn if a larger population was observed.

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