J Forensic Sci, Jan. 2004, Vol. 49, No. 1
Paper ID JFS2003061_491
Available online at: www.astm.org

CASE REPORT

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Assignment of Paternity in a Judicial Dispute Between Two Neighbor Holstein Dairy Farmers

ABSTRACT: DNA profiling was used as evidence to assign paternity in a dispute between two neighbors in a judicial case of undue appropriation of cattle offspring from five alleged Holstein sires. Five offspring were genotyped using ten genetic markers (nine microsatellites and the BOLA-DRB3 locus). The computer program CERVUS was used to estimate the LOD score values and the confidence of paternity assignments. The results presented here show that three out of five paternity cases were assigned at 95% of confidence to a single sire with a LOD score ranging from 2.53 to 3.55. A fourth male was assigned using its Δ value. Finally, all alleged sires were excluded from the paternity of the fifth offspring, probably due to the existence of an non-sampled male in the studied population. We concluded that the likelihood-based approach, included into CERVUS program, was a powerful tool in cattle kinship analysis when dealing with judicial dispute particularly when the dam's genotype was absent, allowing the assignments of paternity at 95% level of confidence in situations usually used by dairy and beef cattle producers in Argentine (e.g., multi-sire pasture mating).

KEYWORDS: forensic science, paternity, DNA typing, microsatellites, BOLA-DRB3, Holstein

A number of DNA marker types are suitable for identification and kinship analysis in cattle populations. Within the last decade, short tandem repeat markers (microsatellites) and amplified fragment length polymorphism (AFLP) markers have been successfully used in bovine animal identification and parentage testing (1–6). More recently single nucleotide polymorphism (SNP) has been proposed as an alternative technique (7). These molecular markers have been satisfactorily used to resolve judicial cases like paternity and maternity disputes, specific assignments of unknown samples and cattle stealing cases in domestic animals (8–12).

Laboratories that resolve paternity cases in livestock animals usually genotype pedigree animals that are breed under intensive management. They apply the exclusion-based paternity method (13). However, Argentine cattle husbandry usually practices "multi-sire pasture mating." This makes established paternity inference inapplicable because it is common that more than one sire might remain non-excluded. In those cases, it is crucial to estimate the likelihood of each assignment of paternity and also to determine a criterion that permitted the assignment of paternity to the most likely male with a known level of statistical confidence. Marshall et al. (1998) reported a statistical confidence for likelihood-based inference in natural populations that could also be applied in bovine populations with multi-sire pasture mating.

In 2001, unusual rains caused flood of a large area of the Argentine's Pampas region. As a consequence, the barbed wire mesh that divided two neighbor Holstein dairy farmers was damaged, and their animals were mixed. Because of this, one of these breeders asserted his right over five offspring that were born in the neighbor ranch.

In this report we describe a judicial case of undue appropriation of cattle offspring where DNA profiling was used as evidence to assign paternity in a dispute between two neighbor Holstein dairy farmers.

Materials and Methods

DNA Extraction

Blood samples were obtained from 10 Holstein cattle corresponding to five alleged sires (P1, P2, P3, P9, P10) and five offspring (O4, O5, O6, O7, O8). In addition, 34 samples of unrelated Holstein cattle from 3 additional dairy farms were collected in order to estimate gene frequencies for the microsatellites used in the Argentine Holstein population. Reference sampled herds were chosen because they are located in the same geographical and environmental region and they have similar husbandry type in relation to the studied population. Genomic DNA was isolated from lymphocytes cells using the DNAzol® reagent (Invitrogen, Carlsbad, CA) following the manufacturer instructions.

Genetic Markers

DNA typing was performed by PCR using ten genetic markers (nine microsatellites and the class II gene BOLA-DRB3). ETH225, INRA023, BM1824, BM2113, SPS115, TGLA122, and TGLA227 microsatellites were suggested by the International Society of Animal Genetics to be used for the International Comparison Test, while the microsatellites MGTG7 and TGLA53 were included in the FAO list for biodiversity studies.

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Received 24 Feb. 2003; and in revised form 4 Sept. 2003; accepted 4 Sept. 2003; published 17 Dec. 2003.

PCR Amplification and Genetic Analysis of PCR Products

Microsatellites—PCR was carried out in a total volume of 25 μL, containing 20 mM Tris-HCl (pH = 8.4), 50 mM KCl, 0.75 to 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.75 U Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 to 0.8 μM of each primer, and 10 to 20 ng of DNA. The cycling conditions were: a denaturation step of 2 min at 94°C, followed by 10 cycles of 1 min at 92°C, 45 sec at 57 to 62°C, and 50 sec at 72°C, and followed by 25 cycles of 1 min at 90°C, 45 sec at 57 to 62°C, and 50 sec at 72°C with a final elongation step of 15 min at 72°C. Variants were detected on 5% (19:1) polyacrylamide denaturing gel by silver staining. Alleles were identified (bp size) by gel mobility comparison that corresponded to previously typed DNAs, which were included in the gel as standards.

BOLA-DRB3—Amplifications of the second exon of BOLA-DRB3 gene were performed by heminested-PCR, using the method described by van Eijk et al. (14). Twelve microlitres of the amplification products were digested at the temperature recommended by the suppliers with 2.5 U of *Rsa* I, *BstY* I and *Hae* III restriction enzymes. Restriction fragments were resolved in 6% polyacrylamide minigels at 170 volts for 45 min and stained with ethidium bromide (0.5 μg/mL).

Statistical Analysis

First, the allele frequencies and the expected heterozygosity were calculated using genotype data for the studied markers. The parentage exclusion probability for the same set of genetic markers in the Argentine Holstein population was estimated assuming (1) that the mother's, its offspring's and alleged father's genotypes were known and (2) when one of the parental genotype was unavailable (15,16). In both cases, single and double exclusion criteria were considered (15).

We used a likelihood-based approach for the assignment of paternity to most likely male. The LOD score for each paternity inference was estimated as the natural logarithm of the combined likelihood ratio obtained at each studied locus. Furthermore, the statistic Δ, defined as the difference in LOD scores between the most-likely male and the next most likely male, was calculated in order to discriminate among non-excluded alleged sires.

Using allele frequencies from the study population, a simulation program generated criteria for Δ that permitted the assignment of paternity to the most-likely male with a known level of statistical confidence. In this sense, distributions of Δ values were generated through 10,000 simulations of paternity inference. The following parameters were taken into account in the simulation model: number of candidates' rates, proportion of candidate male samples, proportion of loci typed and error rate (1%). The statistical analysis was performed using the Cervus© 2.0 program (15).

Results and Discussion

The computer program CERVUS is now a widely used tool by scientists to determine paternity in wild populations (15–18). In this report, we describe a judicial case of undue appropriation of cattle offspring where DNA profiling was used as evidence to assign paternity in a dispute between two neighbor Holstein dairy farmers. The confidences of paternity assignment were estimated using the previously named program.

Table 1 shows the genotypes observed at each locus for the disputed offspring and their putative sires. In total, 86 alleles were detected across the 10 studied loci for the Holstein samples. This gives a mean of alleles per locus of 8.60. The number of alleles per locus varied from 5 to 18, while the expected heterozygosity ranged from 0.669 ± 0.0439 to 0.937 ± 0.0134 (Table 1). The mean expected heterozygosity across the 10 studied loci was 0.788 ± 0.0829.

These data resulted in a total exclusionary power of 0.99989 for the single exclusion and 0.99794 for the double exclusion when mother, her offspring and putative father genotypes are known. However, these values decrease to 0.99616 and 0.96355 respectively, when one parental genotype is unavailable (Table 1).

Using the available paternal and offspring genetic data and the classical paternity inference approach, three sires could be assigned to their corresponding offspring. Previous studies showed that the success of paternity inference is influenced by factors such as the number of male candidates, the quality of the markers used, and the level of confidence required (15). Furthermore, the absence of dam's genotype significantly decreases the percentage of paternity cases that are assigned to a single sire for the respective populations. However, the results presented here show that three out of five of paternity cases (O4, O7 and O8) were assigned with 95% confidence to a single sire with a LOD score ranging from 2.53 to 3.55. In the case of O6, two males (P1 and P2) showed positive LOD score values but exhibited a single mismatch with its offspring (Table 2). This single mismatch was not conclusive evidence of exclusion of these males from paternity as they could be considered to be the consequence of laboratory typing error, null allele or mutation. Nevertheless, applying the double exclusion criteria and Δ ranking, the P1 sire instead of P2 was assigned to the paternity of O6 (Table 2). Finally, all alleged sires were excluded from the paternity of the calf O5. Offspring O5 exhibited more than two mismatches with each sire genotype. We suspected that this result was due to the existence of an unsampled male in the studied population.

TABLE 1—*Number of alleles (n_a), expected unbiased heterozygosities (h_e) and exclusion probability ($PrEx_1$ and $PrEx_2$) at nine microsatellites and at BoLA-DRB3 locus in the reference Argentine Holstein population.*

Genetic Marker	n_a	h_e	$PrEx_1^*$	$PrEx_2$†
ETH225 (D9S1)	7	0.730 ± 0.0355	0.323	0.502
INRA023 (D3S10)	6	0.788 ± 0.0249	0.377	0.555
BM1824 (D1S34)	6	0.768 ± 0.0228	0.351	0.528
BM2113 (D2S26)	8	0.773 ± 0.0335	0.376	0.557
SPS115	7	0.669 ± 0.0439	0.254	0.423
TGLA122 (D21S6)	14	0.851 ± 0.0205	0.515	0.682
TGLA227 (D18S1)	7	0.847 ± 0.0498	0.446	0.623
TGLA53 (D16S3)	8	0.863 ± 0.0486	0.477	0.650
MGTG7 (D23S5)	5	0.695 ± 0.0700	0.244	0.403
BoLA-DRB3	18	0.937 ± 0.0134	0.717	0.835
Total $P_{\text{single exclusion}}$			0.99616	0.99989
Total $P_{\text{double exclusion}}$			0.96355	0.99794

* Exclusionary power when the mother's and alleged father's genotypes are known.

† Exclusionary power when one parental genotype is unavailable.

TABLE 2—*Males assigned with 95% confidence, LOD score and delta values for each paternity.*

Calve	Assigned Sire	LOD Score	Delta Value
O4	P10	2.53	2.53*
O5	NA	NE	NE
O6	P1	6.11×10^{-1}	1.63×10^{-1}*
O7	P10	3.55	3.55*
O8	P9	3.51	3.51*

NA: not paternity assignment; NE: not estimated.

In summary, using ten genetic markers (nine microsatellites and the BOLA-DRB3 locus), we concluded that the likelihood-based approach (CERVUS program) was a powerful tool in cattle kinship analysis when dealing with judicial disputes, particularly when the dam's genotype was absent. The approach allowing the assignment of paternity at 95% confidence in situations usually used by dairy and beef cattle producers in Argentine (e.g., multi-sire pasture mating). In addition, genetic data and the CERVUS program could be used in production systems that utilize multiple-sire mating strategies, and where paternity testing is necessary to identify those sires producing calves with superior characteristics.

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