Physical, behavioral, endocrinologic, and cytogenetic evaluation of two Standardbred racehorses competing as mares with an intersex condition and high postrace serum testosterone concentrations

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Case Description—2 Standardbred racehorses that had been winning races while competing as mares underwent postrace drug testing and had serum testosterone concentrations above the acceptable limit for female racehorses.

Clinical Findings—Initial physical examinations by the referring veterinarian revealed ambiguous external genitalia and suspected intra-abdominally located testes leading to a preliminary diagnosis of male pseudohermaphroditism. Horses were referred for further evaluation of sex. Physical examination of the external genitalia confirmed the findings of the referring veterinarian. Transrectal palpation and ultrasonography revealed gonads with an ultrasonographic appearance of testes. On cytogenetic analysis, both horses were determined to have a 64,XY karyotype and 8 intact Y chromosome markers and 5 *SRY* gene markers, which were indicative of a genetic male and confirmed an intersex condition. Additionally, both horses had some male-type behavior and endocrinologic findings consistent with those of sexually intact males.

Treatment and Outcome—Taken together, these findings confirmed that both horses were male pseudohermaphrodites. Both horses returned to racing competition as males.

Clinical Relevance—As of October 1, 2008, the Pennsylvania Horse and Harness Racing Commissions implemented a postrace drug testing policy that included analysis of blood samples for anabolic and androgenic steroids and set maximum allowable concentrations of testosterone for racing geldings and females. Within 8 months of initiation of this drug testing policy, the 2 horses of this report were identified as having an intersex condition. This raises the possibility that intersex conditions may be more common in racing Standardbreds than was previously suspected. (*J Am Vet Med Assoc* 2011;238:751–754)

In December 2008, a 6-year-old Standardbred racehorse that had been winning races while competing as a mare underwent postrace drug testing and had a serum testosterone concentration above the acceptable limit for female racehorses (horse 1). The horse reportedly had no history of anabolic or androgenic steroid administration. The referring veterinarian arrived at a tentative diagnosis of male pseudohermaphroditism on the basis of ambiguous external genitalia and manually palpable internal structures consistent with testes. At the request of the racing commission, the horse was referred for further evaluation of sex.

Physical examination of the external genitalia revealed an ambiguous phallic structure approximately 4 × 3 cm (width × height) consistent with a poorly differentiated glans penis approximately 10 cm below

ABBREVIATION

hCG	Human chorionic gonadotropin

the level of the anus. Manual eversion of the structure revealed the presence of a urethral orifice enveloped within a small urethral fossa. The phallus appeared to suspend from a median perineal raphe that extended up toward the anus. Examination of the inguinal region revealed a small, undeveloped udder with 2 small, distinct teats.

Examination of the caudal portion of the abdomen by transrectal palpation revealed 2 soft, ovoid structures at the 5 and 7 o'clock positions just cranial to the brim of the pelvis. These 2 structures had limited mobility around the region of the inguinal rings. Transrectal ultrasonographic examination was performed by use of 5-MHz linear array and 7.5-MHz microconvex transducers (B-mode with color flow Doppler capabilities) and revealed that the structures comprised a homogeneous, echogenic parenchyma consistent with the appearance of an intra-abdominal testis. The left gonad measured 2.15 × 2.47 cm, and the right gonad measured 2.53 × 3.37 cm. On color flow Doppler ultra-

sonographic images, each contained vascular structures consistent with a central vein and pampiniform plexus attached to an associated tubular duct that coursed caudally and dorsomedially toward the apex of the urinary bladder similar to the ductus deferentia and ampullae. Tissue consistent with a bilobed prostate gland was also identified. Structures consistent with ovaries, a uterus, or a cervix were not identified.

To evaluate behavior, the horse was brought to an ovariectomized, estrogen-treated stimulus mare in a breeding shed situation. The horse approached the mare without any specific male-type behavior response.

Serum samples were collected and submitted for endocrinologic analysis. Serum testosterone concentration was 90.10 pg/mL. This was higher than the reference limit for a sexually intact female (< 50 pg/mL) and lower than expected for a sexually intact male (> 200 pg/mL) in the month of December. Serum estrogen and progesterone concentrations were 2.0 pg/mL and 0.10 ng/mL, respectively, which was the lower limit of the assay for both hormones. Serum inhibin concentration was 0.32 ng/mL and was within reference range for a nonpregnant mare (0.1 to 0.7 ng/mL) and below the expected serum concentration for a stallion (2.2 to 3.4 ng/mL).[a] An hCG (10,000 U, IV)[b] stimulation test was performed in an attempt to determine whether there was a source of endogenous testosterone production. The baseline serum concentration of testosterone was 85.8 pg/mL. A modest increase in serum testosterone concentration was detected at 1 and 2 hours after hCG administration (114 pg/mL at 1 hour and 110.6 pg/mL at 2 hours). These results were consistent with the presence of testicular tissue in the abdomen and were further evidence that the gonadal structures visualized on abdominal ultrasonographic examination were testes.

Hair follicles, heparinized blood samples, and skin biopsy specimens were submitted to the Molecular Cytogenetics Laboratory at Texas A&M University for analysis. Results from all cells evaluated consistently yielded a 64,XY karyotype, indicating a genetic male with no detected chimerism or mosaicism from > 50 skin fibroblasts and > 50 peripheral blood lymphocytes examined. Polymerase chain reaction analysis of genomic DNA extracted from hair follicles, peripheral blood lymphocytes, and skin fibroblasts was performed by use of oligonucleotide primer sets corresponding to specific Y chromosome markers of the *SRY* (sex-determining region of the Y chromosome) gene and other known markers of the Y chromosome for genetically normal male horses. Results indicated that the horse of this report had 8 intact Y chromosome markers and 5 *SRY* gene markers, which was indicative of a genetic male. By comparison, *SRY* gene deletions had previously been identified at the cytogenetics laboratory for other horses with a 64,XY intersex condition. Similarly, PCR analysis of genomic DNA by use of oligonucleotide primer sets spanning exon 2 of the known X chromosome–linked *AR* (androgen receptor) gene revealed the presence of the *AR* gene.[c] Taken together, these findings confirmed diagnosis by the referring veterinarian of male pseudohermaphroditism.

In May 2009, a 5-year-old Standardbred racehorse that had been winning races while competing as a mare underwent postrace drug testing and had a serum testosterone concentration above the acceptable limit for female racehorses (horse 2). Although this horse had no reported history of anabolic or androgenic steroid administration, an unknown dose of hCG had been administered to the horse approximately 24 hours before racing. The injection was given as an ovulation induction agent because the horse was perceived to be demonstrating undesirable behavior that was judged by the trainer to be related to estrus.

Examination of the external genitalia revealed findings similar to those of horse 1. Transrectal palpation and ultrasonographic examination of the caudal portion of the abdomen identified gonadal and tubular structures consistent with male reproductive organs. Distinct structures consistent with paired bulbourethral glands and a bilobed prostate were identified caudal to the bladder and dorsolateral to the pelvic urethra. Structures consistent with ovaries, a uterus, or a cervix were not identified.

When brought to an ovariectomized, estrogen-treated stimulus mare in a breeding shed situation, the horse had stallion-like behavior, including male-type approach, vocalization, teasing, mounting, and thrusting with erection of the phallus-like structure. The stimulus mare responded to the horse as a mare usually responds to a stallion. When turned into a paddock containing recently voided feces of other stallions, the horse had male-type elimination-marking behavior. On exposure to a stallion across a teasing rail, the behavior of the horse was resistant and guarded, resembling what could represent either submissive stallion-like behavior or that of a mare in diestrus.

Serum samples were collected and submitted for endocrinologic analysis. Baseline serum testosterone and progesterone concentrations were 701.8 pg/mL (reference limit, > 200 pg/mL) and 0.3 ng/mL, respectively. Both were within the reference range limit for a stallion during spring. Serum estrogen concentration of 2 pg/mL (lower limit of assay) was lower than the reference range for either stallions or mares. Following hCG challenge, serum testosterone concentration changed from the baseline of 701.8 pg/mL to 687.7 pg/mL at 2 hours and to 817.1 pg/mL at 4 hours. The 2-hour response was lower than expected. Changes in serum testosterone concentrations in response to hCG stimulation were lower than expected for a stallion with both testes within the scrotum, but within the range often seen in stallions with intra-abdominally located testes.

Hair follicles, heparinized blood samples, and skin biopsy specimens were submitted for cytogenetic analysis to the Molecular Cytogenetics Laboratory at Texas A&M University. As for horse 1, cytogenetic analysis of chromosome spreads from 30 peripheral blood lymphocytes and 20 skin fibroblasts consistently revealed a 64,XY karyotype. No chimerism or mosaicism was detected. Polymerase chain reaction analysis of genomic DNA from hair, peripheral blood lymphocytes, and skin fibroblasts as described for horse 1 revealed similar markers on the Y chromosome and *SRY* gene and the presence of exon 2 of the *AR* gene.[c] Taken together, these findings confirmed that this horse also was a male pseudohermaphrodite.

Discussion

On October 1, 2008, the Pennsylvania State Horse and Harness Racing Commissions implemented a mandatory postrace drug testing policy that included analysis of blood samples for the presence of anabolic steroids and androgenic steroids with the aim to eliminate administration of these types of drugs in competing racehorses. Criteria for negative and positive test results were defined for females and geldings and separately for sexually intact males.[1] The 2 horses reported here were evaluated by a referring veterinarian in the field when serum testosterone concentrations in postrace blood samples, which were obtained after winning, were higher than the concentrations permitted for females. These 2 horses were identified within 2 and 8 months of Pennsylvania's mandatory postrace drug testing policy. This raises the possibility that intersex conditions may be more prevalent in racing Standardbreds, or in horses in general, than was previously appreciated. The question may be answered as further data become available from Pennsylvania and from other states now testing for anabolic steroids and androgenic steroids.

The referring veterinarian identified intersex conditions in these horses on the basis of physical examination findings. The combination of ambiguous external genitalia, the apparent absence of a female reproductive tract on transrectal palpation of the abdomen, and the apparent presence of palpable internal testis-like structures led to the preliminary diagnosis of male pseudohermaphroditism in both horses. Because each of these horses had won considerable amounts of money during their careers as putative mares, the Pennsylvania State Harness Racing Commission requested state-of-the-art genetic evaluation to enable a more informed decision on sex designation for racing horses.

In each horse, transrectal ultrasonographic examination of the caudal portion of the abdomen revealed an internal reproductive tract including structures consistent with male accessory sex glands and intra-abdominally located testes with no evidence of female structures. Consistent with these physical findings, endocrine testing revealed steroid hormone concentrations most similar to those found in cryptorchid stallions. A previous report[2] on serum testosterone concentrations in horses with a 64,XY intersex condition revealed a range of concentrations from undetectable to 5.4 ng/mL; however, these affected horses were distinguished by phenotype rather than by gonadal type. Cytogenetic analysis consistently revealed a genetically normal male 64,XY karyotype in all cells examined. Although molecular cytogenetic analysis of intersex conditions of horses is limited, compared with that for humans, current genetic analyses in the horses of this report revealed the presence of an apparently normal SRY gene, a complete Y chromosome (on the basis of no deletions of the currently known Y chromosome and SRY gene markers), and an apparently normal exon 2 in the AR gene of somatic tissue specimens. Because the gonadal structures were not examined histologically, it could not be definitively determined whether the gonads were in fact testes. However, all evidence strongly suggested that to be the case. In reports[3–5] of similarly affected horses where gonads were removed and examined, histologic examination revealed seminiferous tubules with Sertoli cells and Leydig cells, but no evidence of spermatogenesis as is typical for intra-abdominally located testes.

Genetic control of testis differentiation is controlled by coordinated molecular events that initiate Sertoli cell differentiation of the embryologic, undifferentiated mesenchymal cells of the embryo's bipotential gonad. The expression of the SRY gene is recognized as the first step in initiating Sertoli cell differentiation in genetically male mammals.[6] Given the known importance of the SRY gene in sex determination, the SRY genes of the affected horses of this report were analyzed in an attempt to identify an underlying molecular cause of the intersex disorders. In both horses, no abnormalities of SRY gene were identified. Given the likely large number of downstream targets of the SRY gene, as well as other genes and proteins that are involved with phenotypic sex differentiation, genetic analysis in these horses was limited to current methods used to determine defects of the equine Y chromosome and SRY gene, which comprise a small facet of all the possible molecular causes of the observed disorders.

The SRY and AR genes in horses can be detected by PCR analysis; several mutations of the SRY gene have been defined in horses with a normal male karyotype (64,XY) and intersex condition based on mapping of the equine Y chromosome[7,c]; this work, detailing the physical genetic map of the equine Y chromosome, has provided substantial information regarding location of the SRY gene and sequences of other Y chromosome markers. The Y chromosomes in each of the horses of this report were compared with those of clinically normal males and also with horses with a 64,XY intersex condition evaluated at the cytogenetics laboratory. The results indicated that 5 of 5 markers of a portion of the SRY gene sequence were present in both horses of the present report, compared with 2 of 5 markers of the SRY gene sequence in another affected horse.[c]

Male pseudohermaphroditism has been reported previously in horses and has included 64,XX and 64,XY karyotypes and varied mosaic and chimeric karyotypes.[3,8–17] The phenotypic variations of horses with a 64,XY intersex condition have been documented and categorized previously.[2] However, it is reasonable to expect that many juvenile male pseudohermaphrodites may go unrecognized because without close inspection, the external genitalia may not be conspicuously different from that of a physically normal juvenile female. Analysis of DNA for parentage testing that is required for registration of horses does not include sex verification. The process of cytogenetic analyses in the affected horses of the present report required the collection of multiple tissue specimens to increase the probability of detecting cellular mosaicism or chimerism. Hair follicles, skin fibroblasts, and blood cells represent the embryological lines of the ectoderm (hair and skin) and mesoderm (blood cells). Ideally, to definitively rule out any gonadal mosaicism or chimerism, analyses of gonadal tissue is required.

In humans, there are many causes known to lead to abnormal sexual differentiation. Two well-described

conditions with genetically normal male karyotypes are androgen insensitivity syndrome and 5-α-reductase type II enzyme gene mutations. In humans, these conditions are known to be caused by specific mutations in the *AR* gene and the 5-α-reductase gene, respectively.[18,19] These specific molecular deficiencies have not yet been investigated in horses. Although conditions labeled as androgen insensitivity syndrome have been described in male pseudohermaphrodite horses,[20,21] these clinical reports included no analysis of either the *AR* or 5-α-reductase type II enzyme genes, thus raising the question of whether the terminology of androgen insensitivity syndrome was in fact correctly applied. In the present report, a limited analysis of the *AR* gene in 2 male pseudohermaphroditic horses is described. However, now that the equine genome is available, more complete analysis of the equine homologs of both the *AR* and the 5-α-reductase type II enzyme genes will be possible and conditions similar to androgen insensitivity syndrome and 5-α reductase enzyme deficiencies may yet be identified in horses with an intersex condition.

The heritability of 64,XY intersex conditions in horses was last evaluated more than 20 years ago. Pedigree analysis of 38 horses with a 64,XY intersex disorder from 6 separate pedigree lines (29 of which were from the Arabian breed) led to the proposal of 2 theories on the mode of inheritance of the intersex condition.[3] Five of the pedigree lines had an X chromosome–linked recessive or autosomal sex-limited dominant transmission of a sex-reversing gene by a carrier female, whereas the sixth pedigree line had transmission of a sex-limited dominant or Y chromosome–linked mutant gene by a carrier stallion. Heritability of the XX sex reversal condition in caprine, canine, and porcine species has been described.[22–24] In the present report, horses with the intersex condition shared common maternal and paternal pedigree lines within the 3 most recent generations. Further work would be necessary to evaluate heritability.

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c. Raudsepp T, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University, College Station, Tex: Personal communication, 2009.

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