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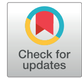
Original Research

Diagnosis of XX/XY Blood Cell Chimerism at a Low Percentage in Horses



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ABSTRACT

Disorders of sexual development (DSDs) are common in horses and cause economic loss in horse breeding. Thus, it is important to develop methods for unambiguous and fast identification of affected horses shortly after birth, as well as those that may propagate the condition to the next generation. Genetic causes of DSDs are multivarious and still little known, and thus development of diagnostic tests requires accumulating knowledge about individual cases and their etiologies. In particular, it is necessary to perform clinical, ultrasound, surgical, histological, cytogenetic, and genetic analyses with close attention in all the affected individuals. This report describes the case of a XX/XY chimeric horse with reproductive apparatus abnormalities and a very low percentage of XY cell in blood highlighting that to avoid undiagnosed case of cell chimeras, above all when studying DSD cases, it is essential to perform both genetic and cytogenetic analyses possibly on more than one tissue.

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1. Introduction

Reproduction and fertility are important concerns in horse breeding, and early identification of horses with congenital conditions that may lead to reproductive problems will bring a big benefit to horse industry.

Although cytogenetic and molecular tools have been developed for this purpose, most horses carrying disorders of sexual development (DSDs) are identified when they have already grown up, causing economic loss to the breeders, and in most cases, molecular causes remain unknown [1–4]. This is because of the limited knowledge about the molecular mechanisms regulating early development and sexual differentiation.

DSDs are among the main causes of horse subfertility or sterility. A variety of phenotypes are associated with this condition ranging from a phenotypically normal mare with gonadal dysgenesis to a horse with ambiguous external genitalia and internal male and female organs [5]. In horses, four types of DSDs have been diagnosed up to now: (1) sex chromosome abnormalities (63,X; 64,XX/64,XY; 65,XXX; 65,XXY; etc.,); (2) 64,XX *SRY*-negative with DSD; (3) 64,XY *SRY*-positive with DSD; (4) 64,XY *SRY*-negative. In horses, XX *SRY*-positive DSD has never been reported, probably because the *SRY* gene is located far from the pseudoautosomal region; thus, it is less susceptible to meiotic errors between the sex chromosomes compared to, for example, humans where *SRY* translocation to the X chromosome can occasionally occur [6].

XX/XY chimerism is classified as a chromosome abnormality, and it has been diagnosed in the main livestock species and in humans. It is caused either by the exchange of hematopoietic stem cells through placental circulation between dizygotic twins

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(blood chimerism) or by the fusion of two zygotes or embryos into a single individual at the very early stages of development (true chimerism) [7,8]. Phenotypic and physiological effects due to this condition are very variable and depend on both the causes and the affected species.

XX/XY chimerism has been rarely diagnosed in horses; this is either because twin pregnancy (the main origin of chimeras) causes serious economic loss as a result of a high rate of abortion and a tendency for poor postnatal development in the few foals that survive to term; thus, it is an unwanted condition normally terminated once detected [9,10]. However, large-scale DNA profiling or cytogenetic survey of horse populations [11,12] suggests that the available clinical data underestimate the actual prevalence of these cases.

From a scientific point of view, a procedure able to detect chimeras rapidly and early and to differentiate those caused by placental vascular anastomosis in a twin pregnancy rather than an early fusion of two zygotes or embryos would be very useful. In fact, the different phenotypes due to chimerism, and mainly those XX/XY, are a useful starting point for understanding the mechanism of sexual differentiation in mammals, but for this purpose, it is necessary to correctly identify affected animals as early as possible in their lifetime so that the development of the reproductive apparatus can be followed during all the growth phases allowing to accumulate new knowledge. Moreover, it is necessary to establish the cause of the chimerism; in twin pregnancy with placental anastomosis between the twins, one of them may miscarry without breeder's knowledge.

Vascular connections between placentas of heterosexual twins cause in ruminants the so-called free-martin syndrome [7,13] in which the female twin is sterile due to malformations of the reproductive apparatus, while in equine blood, chimeric heterosexual twins are both phenotypically and physiologically healthy and fertile [9,14]. This difference is probably due to the fact that placental vascular connections responsible for free-martin syndrome in ruminants and other species occur after the sexual differentiation of the equine [14].

A different condition is found when chimerism is due to the fusion of two zygotes or embryos. In this last case, the phenotype may be normal or ambiguous genitalia may be observed [15].

This report describes the diagnosis of the first case of a 64,XX/64,XY chimeric horse, showing a reproductive apparatus in which only male reproductive structures have been developed, with the aim to highlight the need of both cytogenetic and genetic analyses in all animals in which a correct genetic evaluation is required (clinical and DSDs cases, breeders).

2. Materials and Methods

2.1. Case

A 15-month-old Italian Saddlebred horse, registered as filly, was submitted to clinical evaluations due to abnormal conformation of external genitalia (Fig. 1) and stallion-like behavior. On physical examination, the horse showed a small penis of 11 cm in length in the ventral perineal region without scrotum and an underdeveloped mammary gland (Fig. 1). Urination occurred through a urethral fossa at the distal end of the penis. Transrectal ultrasonography did not allow to visualize internal genitalia. Castration (closed technique) (Supplementary Fig. 1) with primary wound closure was carried out using an inguinal approach. The horse was treated with an intramuscular dose of acepromazine (0.05 mg/kg) and, 20 minutes later, was intravenously administered detomidine (20 μg/kg) and butorphanol (0.02 mg/kg) mixed in the same syringe. Anesthesia was induced with intravenous administration of diazepam (0.05 mg/kg) and ketamine (2.2 mg/kg). After orotracheal intubation, anesthesia was maintained with isoflurane vaporized in oxygen and delivered via a large animal circle system. Two symmetrical hypoplastic testis-like structures were found in inguinal rings (Supplementary Fig. 2), removed, and processed for histological and genetic evaluation. Blood samples were collected to perform cytogenetic and genetic analyses.

2.2. Histopathologic Analyses

Pieces of testis-like structures of samples were fixed in buffered neutral formalin, embedded in paraffin, and sectioned at 3 μm for histopathology and immunohistochemistry. Serial sections were stained with hematoxylin and eosin. For immunohistochemical analysis, sections were mounted on SuperFrostUltraPlus slides, and an avidin—biotin—peroxidase-complex (ABC) technique with diaminobenzidine as the chromogen was performed to evaluate the expression of anti-Mullerian hormone (AMH) or Mullerian-inhibiting substance (MIS) using a monoclonal antibody (clone B-11, Santa Cruz Biotechnology, USA) specific for an epitope mapping between amino acids 535-560 at the C-terminus of MIS of human origin. Appropriate negative and positive controls included samples of adult normal horse testis, and sections pretreated with blocking peptide were used.

2.3. Cytogenetic Analyses

Blood lymphocytes were cultured in an RPMI medium with pokeweed for about 72 hours at 37.5°C. Two types of cultures, with and without 5-BrdU (20 μg/mL), were set up. 5-BrdU and H33258 (40 μg/mL) were added to the latter 3.5 hours before harvesting. Colcemid was added 1 hour before harvesting to all cultures and after a hypotonic treatment with 0.075M KCl, and three fixations with Carnoy's fixative cell suspensions were used to prepare slides that were allowed to dry and then stained for C- and R-banding or used for FISH mapping; 84, 400, and 30 metaphases were examined from slides with Giemsa staining, treated for C- and R-banding techniques, respectively. Karyotypes were arranged according to the Horse standard karyotype [16]. Probes used for FISH experiments were as follows: horse Y-specific BAC clone 147K8 from CHORI-241 library (https://bacpacresources.org/) and horse X-specific BACs 102C09 and 111A23 from INRA library [17]. BACs were grown overnight at 37°C in Luria Broth supplemented with chloramphenicol (12.5 μg/mL), and then BAC DNA was isolated according to standard protocols described by CHORI (http://bacpac.chori.org/). For each FISH experiment, about 250—300 ng of DNA was labeled with biotin by nick translation (Roche Diagnostic kit) or Cy3 (Amersham, Little Chalfont, UK). Biotin-labeled DNA was detected by use of FITC-conjugated avidin (Vector Laboratories, Burlingame, CA) as a green signal; direct Cy3 was detected as a red signal. The probes and the slides were co-denatured on a hot plate at 75°C for 4 minutes. Hybridization was performed in a moist chamber at 37°C overnight. The chromosomes were identified by means of simultaneous 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining. The digital images were obtained by use of a Leica DMR epifluorescence microscope (Leica Imaging Systems, Cambridge, UK) equipped with a CCD camera (Cohu, San Diego, CA, USA), and the FITC-avidin, Cy3, and DAPI fluorescence signals were detected with specific filters. The images were recorded, pseudocolored, and merged by use of QFISH software (Leica Imaging Systems). Moreover, 500 metaphases and nucleus were analyzed. Finally, chromosomes were counterstained with DAPI in Vectashield mounting medium (Vector Lab) antifade solution, and more than 500 metaphases and nucleus were analyzed using CytoVision (Leica Biosystems) software.

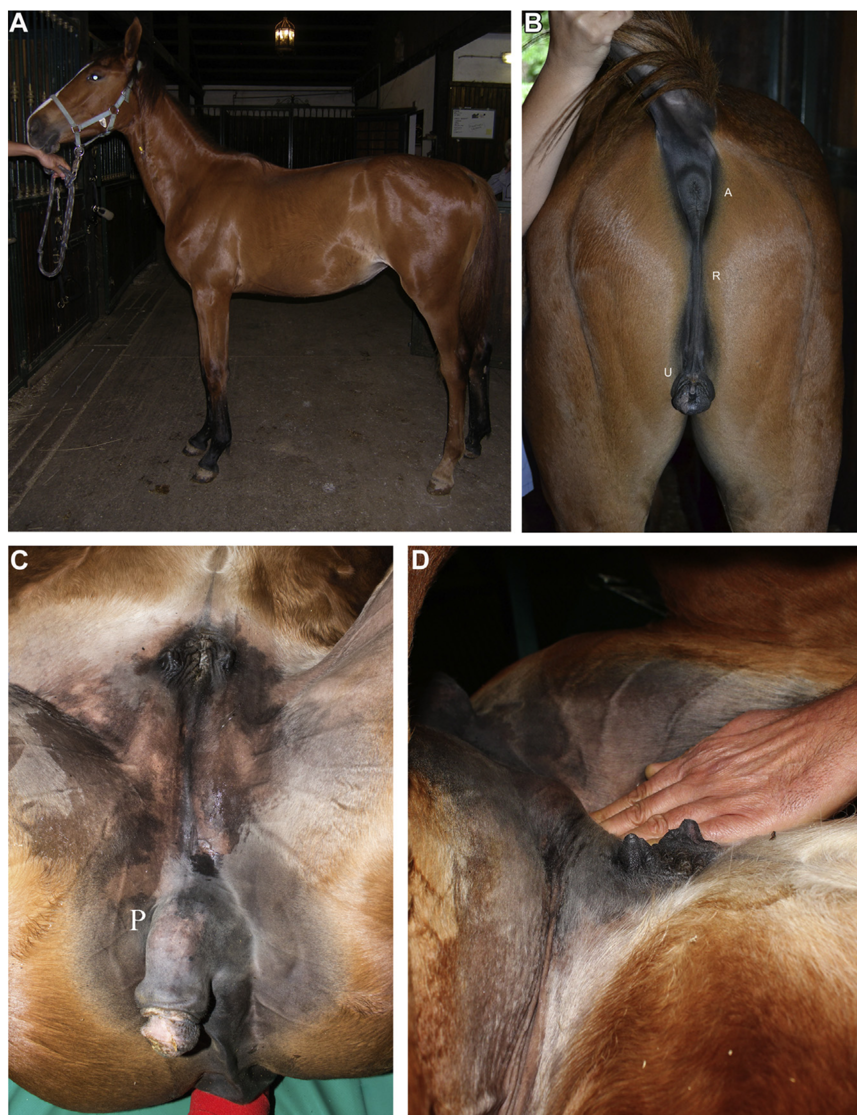


Fig. 1. (A) A 15-month-old Italian Saddlebred horse with DSDs. (B) Perineal region of the horse. A = anus; R = raphe, U = urethral opening. (C) Inguinal region of the horse in dorsal recumbency showing the penis (P) and (D) two well-developed testes and the subcutaneous position of the testes. DSDs, disorders of sexual development.

2.4. Molecular Analyses

DNA was extracted from whole blood with Wizard Genomic DNA Purification Kit (Promega), and from the testis-like structures with GenElute mammalian Genomic DNA Extraction Kit (Sigma). The DNA extracted from blood was tested by using qualitative polymerase chain reaction (PCR) using primers specific for *SRY*, *ZFY/ZFX*, and *EIF* (Table 1). Being all the primers specific for Y regions seem to work less in the investigated horse than in normal male control, PCRs with different number of amplification cycles (from

Table 1
Primers sequences, annealing temperatures, and product lengths of the examined genes.

Gene	Primer Name	Primer Sequence	Annealing	Length
SRY [18]	SRY-F	TGC TAT GTC CAG AGT ATC CAA CA	58	697bp
	SRY-R	TGA GAA AGT CCG GAG GGT AA		
ZFX/Y [18]	ZFX/Y-F	AAA TCA AAA CCT TCA TGC CAA T	58	Y 553bp; X 604bp
	ZFX/Y-R	TTC CGG TTT TCA ATT CCA TC		
EIF2s3Y [19]	EIF2s3Y_F	GAGCCATCTGTGTGATCGTC	58	223
	EIF2s3Y_R	TATTCCTGGCCCTAAGCACA		
ZFY [19]	ZFY_F	TGAGCTATGCTGACAAAAGGTG	58	186
	ZFY_R	TCTTTCCCTTGTCTTGCTTGA		
SRY	SRYQ-F	ACAGTCACAAACGGGAGGAG	58	149
	SRYQ-R	AAAGGGAACGTCTGCGTATG		
HPRT	HPRT-F	GAGGCCATCACATTGTAGCA	58	381
	HPRT-R	TCCCCACAGCAATTCTTACA		

25 to 35) were performed using the primers SRYQ and HPRT (as control) (see Table 1 for sequences). PCRs were performed as recommended by the Taq enzyme supplier (AmpliTaq Promega) using as start material DNA obtained from blood. The same primers were used to perform a Q-RT-PCR with SYBR Green (Invitrogen 11733-038) on DNA extracted from blood and from the testis-like structures to evaluate the percentage of XY cells in the clinical case and in a normal, fertile control stallion. The same DNA (from blood and testis-like tissue) was used for genotyping on a panel of 17 microsatellites according to International Society of Animal Genetics guidelines at the laboratory UnireLab srl to establish if the horse was a chimera or a mosaic.

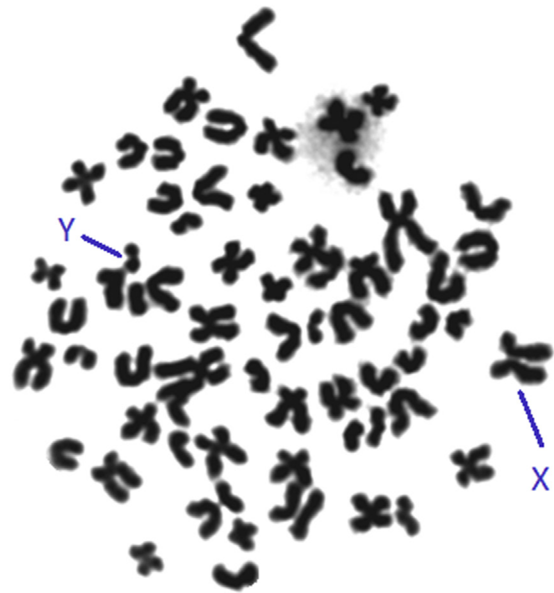
3. Results

3.1. Histopathologic Analyses

Both of the testes were composed of low number of small and hypocellular seminiferous tubules that lacked germ cells and spermatozoa and were lined by Sertoli cells, often with frothy, vacuolated apical cytoplasm (Fig. 2A). Sertoli cells extended from the undulating basement membrane and protruded into the lumen. The interstitial tissue, separating the tubules, was apparently increased because of the reduced number of tubules and was composed by well-developed fibrovascular stroma with embedded many plump oval fibroblast, various macrophages containing abundant, globular, intracytoplasmic, golden brown pigment (lipochrome), and few interstitial cells that had small round nuclei and eosinophilic, foamy cytoplasm. The histological findings observed were consistent with severe testicular hypoplasia and Leydig cell atrophy. Sertoli cells showed a diffuse and intense cytoplasmic immunolabeling for AMH (Fig. 2B).

3.2. Cytogenetic Findings and FISH Analyses

The analysis of 84 routinely Giemsa-stained karyotypes (without banding) showed only one male (XY) metaphase (1.19%) (Fig. 3). The analysis of 400 C-banded metaphases revealed only one XY metaphase (0.25%) (Fig. 4A) while no XY cells were detected among R-banded metaphases. Karyotyping of an R-banded XX metaphase did not show abnormalities (Fig. 4B); however, no information was obtained about the presence or absence of chromosome aberrations for R-banded XY cells. The presence of both the XX and XY cells in blood lymphocytes was further confirmed by FISH with horse Y-specific BAC 147K08 and X-specific BACs 102C09 and 111A23. Analysis of 450 interphase nuclei identified only 4 XY cells (0.8%), while no XY metaphases were observed in this analysis (Fig. 5).



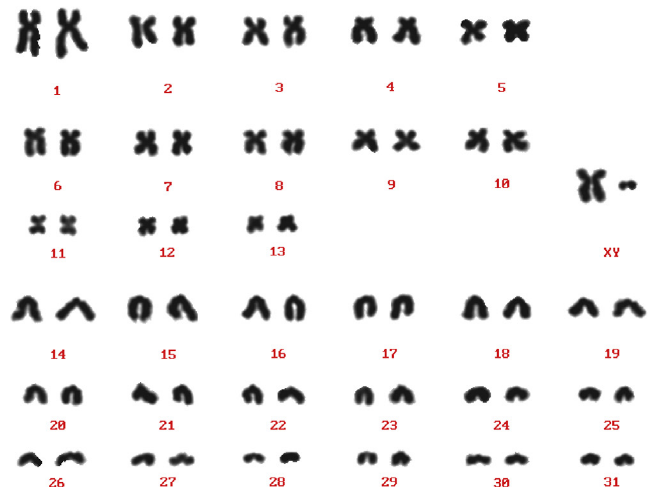


Fig. 3. A male metaphase and the corresponding karyogram of the Italian Saddlebred filly.

3.3. Molecular Analyses

Analysis by PCR with Y-specific markers confirmed the presence of the Y chromosome, though at a low percentage in the case compared to a normal male control. Fig. 6 illustrates qRT-PCR

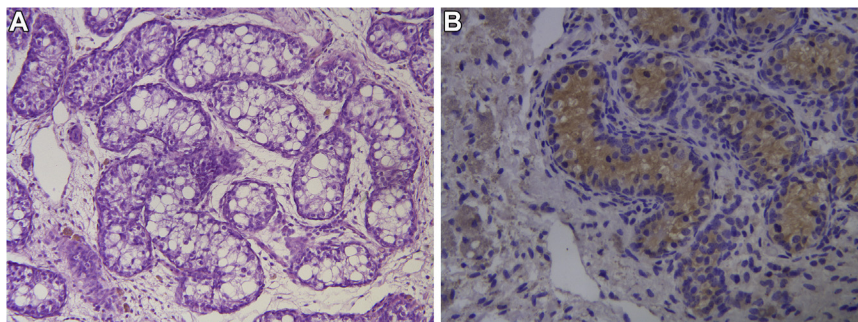


Fig. 2. (A) Section of the hypoplastic testicles showing small seminiferous tubules lined by a single layer of Sertoli cells (H.E. ×10). (B) Immunohistochemical stain showing diffuse intense anti-Mullerian hormone expression of Sertoli cells within seminiferous tubules (IHC, counterstaining with hematoxylin, ×20).

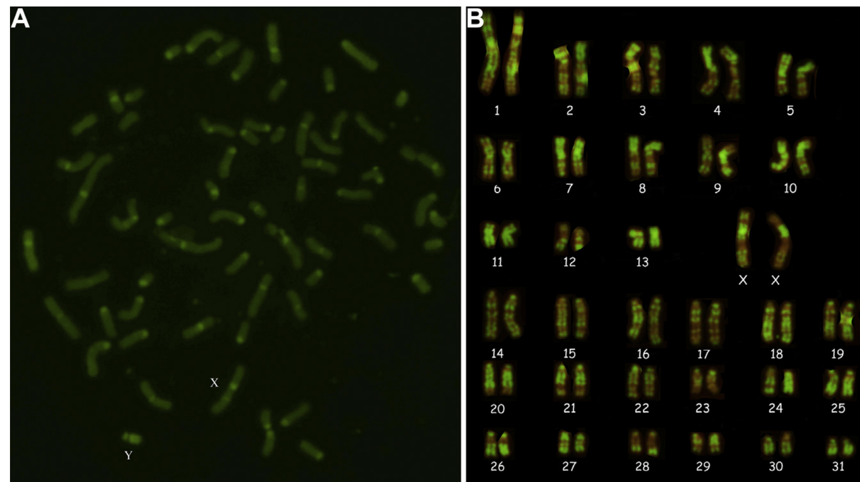


Fig. 4. (A) C-banded metaphase plate with 2n = 64;XY and, (B) R-banded karyotype with 2n = 64;XX of the Italian Saddlebred horse with ambiguous genitalia.

results for the SRY gene, in which amplification was analyzed at different cycles. In the clinical case, SRY amplification product becomes visible only at cycle 33, clearly indicating the low content of this gene in the case compared to the male control. Q-RT-PCR also allowed to quantify the amount of XY cells in the case. The Ct values for the *SRY* were as follows: 29.15 and 23.28 for the case and the control male, respectively, whereas the respective Ct values for the autosomal *HPRT* gene were 21.91 and 23.22. Using the delta-delta Ct method, we calculated the percent of XY cells as 0.68%. These results confirm the low level of blood XX/XY chimerism. The amplification profiles are shown in Supplementary Fig. 3. The same analyses were performed on testis-derived DNA and revealed 13% of XY cells thus almost 20 times more than that observed in blood.

Microsatellite genotyping in blood DNA showed the presence of one or two alleles per each marker. However, the same analysis in testis-derived revealed the presence of three alleles for the

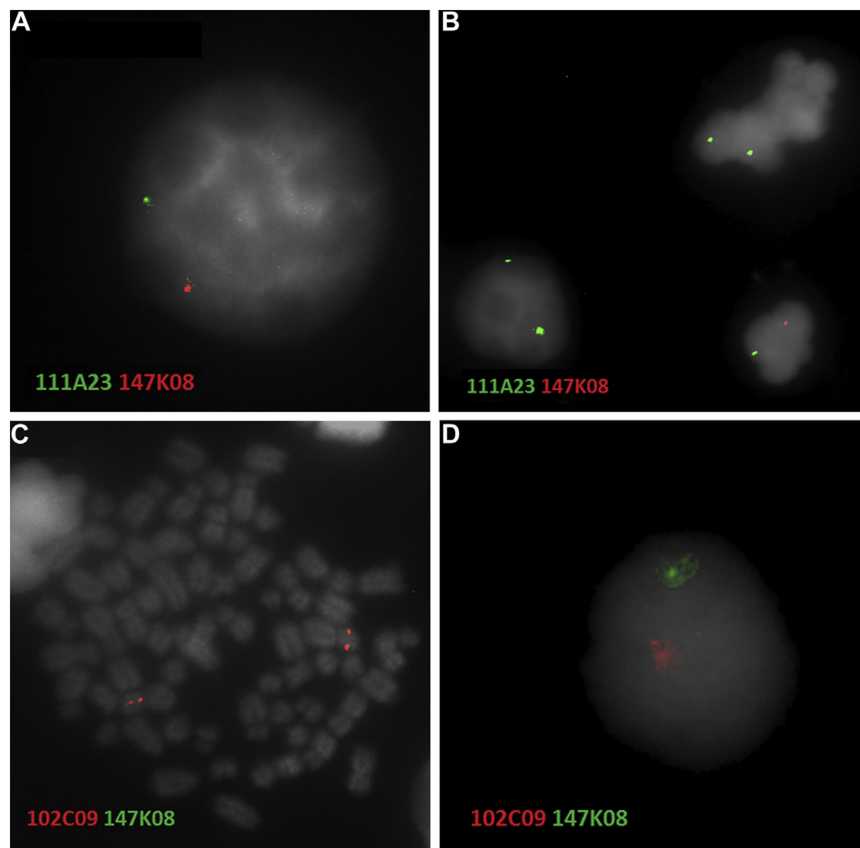


Fig. 5. FISH experiments on nuclei and metaphases of the filly. (A and B) XY and XX nuclei as revealed by FISH with Y-specific BAC 147K08 (red signal) and X-specific BAC 111A23 (green signal). (C) XX metaphase showing signals by X-specific BAC 102C09 (red signal). (D) XY nucleus showing signals by Y-BAC 147K08 (green signal) and X-BAC 102C09 (red signal).

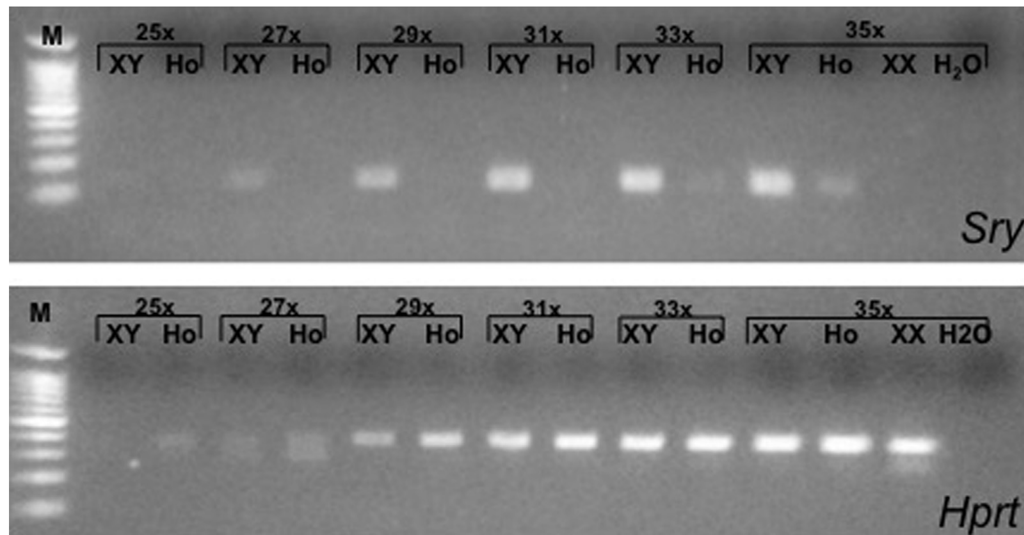


Fig. 6. PCR amplification of a portion of the *SRY* and *HPRT* genes at different cycles. M = 100 bp marker; XY = normal male; Ho = DSD Horse; XX = normal female; H_2O = water. The number reported the amplification cycles performed.

microsatellites ASB2, ASB23, CA425, HMS23, HMS6, HMS7, LEX003 indicating that the horse was a chimera, likely originating from the fusion of two zygotes or embryos (see Supplementary Fig. 4).

4. Discussion

Reproductive apparatus abnormalities observed in a 15-month-old horse led to deepen the clinical case by performing clinical, ultrasound, surgical, histological, cytogenetic, and genetic analyses with close attention.

Anatomical and histopathological findings of this horse indicate that during embryo development, the pathway of formation of the male genital apparatus has been correctly activated. This has led to testes formation and to their migration in inguinal canals. However, the genital tubercle has developed in the direction of male external genitalia without reaching a complete and proper conformation. The observed diffuse expression of AMH within Sertoli cells is similar to that of a previous study where a positive immunostaining of AMH was found in intersex gonad and cryptorchid testis [20] and comply with the absence of Mullerian derivatives. This can be due to postzygotic fusion of two distinct embryos rather than an early anastomosis between the vascular systems of twins (one of which has then be reabsorbed). In this latter case, in fact, typically no abnormalities of the reproductive organs are observed in either twins because when vascular anastomosis are formed, sexual differentiation is already undergone [5,9,21]. Conversely and in contrast with previously reported cases [11,22,23], the present case shows no derivatives from female reproductive organs while male organs are almost completely developed.

This phenotype may be due to the prevalence of XX cells over XY cells during critical stages of sex determination and sexual differentiation, so that although the Y chromosome initiates the SRY-pathway, the low amount of XY cells gene products may not be sufficient for proper and complete male development. On the other hand, the percentage of different cellular clones found in the blood of an animal does not allow to trace-back the growth trend of all different cellular clones during embryo development. FISH experiments on metaphases and interphase chromosome confirmed the chimeric condition at a very low level. To our knowledge, this is the first case of a chimeric horse where such a low percentage of XY cells in the blood (0.68%) is associated with the total absence of female structures. Genome-wide microsatellites genotyping performed on DNA from blood failed to reveal the presence of two cellular clones due to the low percentage of XY cells. Instead, the same analysis performed on DNA from gonadal tissue revealed the presence of more than 2 alleles for some markers suggesting that this 64,XX/64,XY horse is a chimera likely derived from postzygotic fusion of two distinct embryos (tetragametic chimera) [15]. This finding shows that when microsatellites genotyping is performed in a tissue with a very low percentage (<1%) of a particular cell clone chimerism may remain undiagnosed and eventually discovered only when the affected animal is old enough to show reproductive problems. Routinely, Giemsa-stained karyotypes (without banding) and CBA techniques seem to be more sensitive, thus indicating the need to always carry out them in a correct genetic evaluation of a livestock animal or of a clinical case. Moreover, early identification of individuals with cell chimerism will allow the improvement of the knowledge about reproductive organs development particularly molecular mechanism underlying this biological event.

5. Conclusion

SRY PCR positivity with 64,XX normal karyotype found in a 15-month-old horse with abnormalities of reproductive apparatus led to deepen Giemsa-stained (without banding) karyotyping and C-banding test allowing to diagnose XX/XY chimerism, and subsequently, microsatellite genotyping on DNA from gonadal tissue allowed to classify the case as a tetragametic chimera. To date, there are very few reports of XX/XY horse chimeras [11,22,23] with malformed genital apparatus, and this is the first one with a very low percentage (<1%) of XY cells in the blood and the complete regression of Mullerian ducts in favor of the development of male reproductive structures showing that also in horse, as already observed in other species [13,15], the proportion of XX/XY cells in the blood does not correlate with the development of reproductive organs.

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Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jevs.2018.06.016.

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