A patient with 60, XY and Disorders of Sex Development (DSD) in Gyr Breed Animal

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Abstract

An atypical case of sexual development disorder, where the presence of an intersex evidenced in the physical examination, is described in a twin male bull of the Gyr breed. Initially, it was believed that the animal analyzed corresponded to a case of freemartins, having a feminine appearance. In addition, the physical examination of the animal evidenced several abnormalities, had feminized phenotype, smaller size, did not develop male gonads and had a highly developed clitoris in the form of the penis. However, the results of cytogenetic and molecular analysis suggest that the sexual disorder presented in the female is not a case of freemartins and that the female is actually a male who presented a case of intersex. Although in the macroscopic examination of the organs were observed rudiments of what could be vulva, uterine horns and ovaries; the histological evaluation does not show the presence of female cells in these tissues, but if a histological structure of the epididymis is observed. This is an unusual situation of disorders of sex development with genital tract anomalies in cattle, probably associated to a mutation in a gene involved in the proper development of the male gonad.

1. Introduction

In mammals, the sex determinations of gonads play a pivotal role in the development of internal and external genitalia, secondary sexual characteristics and sexual behavior. Gonads arise in undifferentiated state during the fetal period, as both

Sertoli cells in the testes and granulose cells in the ovaries develop from a common supporting cell precursor. A sex chromosome genotype of XY leads to develop testes while genotype XX will develop ovaries [1]. Then mammalian sex determination is highly controlled by genetic programming. However, animals exhibiting Disorders of Sex Development (DSD), defined as ‘congenital conditions in which the development of chromosomal, gonadal, or anatomical sex is atypical’ [2]. Therefore, DSD constitutes a spectrum of disorders that affect the genitourinary tract and the endocrine-reproductive system [3]. 46, XY DSD includes the conditions of 46, XY partial or complete gonadal dysgenesis, and undervirilisation or undermasculinization of an XY male.

In humans, the DSD is associated with a wide spectrum of sex phenotypes. At least three major forms of androgen insensitivity syndrome (AIS) are recognized [4] Patients presenting complete androgen insensitivity syndrome (CAIS) exhibit female external genitalia with a blind-ending vagina, normal-sized testes located in the abdominal or inguinal area with hypoplastic Wolffian derivatives, and complete breast development with the absence of axillary and pubic hair. Individuals with partial AIS (PAIS) present a predominantly male phenotype with severe hypospadias or a predominantly female phenotype with clitoromegaly, ambiguous genitalia, and gynecomastia to variable degrees. A mild form (MAIS) includes male individuals that present solely with gynecomastia or infertility [5–7].

Recently, the definition of the disorders of sex development (DSD), a consensus terminology for the classification of ontogenetic errors and pathological phenotypes associated with sex abnormality within human medicine, has been extended to veterinary medicine for analogous pathologies of domestic animals [8]. Intersexuality (hermaphroditism) is a DSD resulting from pathological features present in the affected subject. These pathological aspects are chimerism, mosaicism, sex reversal syndrome, and male and female pseudohermaphroditism [8]. The criteria for the diagnosis should consider the subject morphology, the phenotypic appearance of the genital tract, the anatomopathological findings, cytological

karyotyping, and DNA analysis. An intersex condition can be determined by correlation. The true genetic sex of an animal with the phenotypic sex [9]. In this study, we report the histopathological, cytogenetic and DNA molecular findings in the case of a bovine Gyr with DSD, born from a delivery of twins, both with XY chromosomes.

2. Methodology

2.1 All animals used in this study were handled strictly in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The Animal Welfare Committee of CES University approved the protocols (Permit Number: 160).

2.2 Case history and evaluation

The animal analyzed was an apparent bovine female of the race Gyr, born from heterosexual multiple birth reported by a farmer. This female originated from a process of fixed-time artificial insemination. Initially, it was thought that was female freemartins, since the other individual, the male, exhibited normal phenotypic conditions while the apparent female, had feminized phenotype, smaller size, did not develop male gonads and had a highly developed clitoris in the form of the penis (Fig. 1). To confirm the diagnosis a PCR analysis, karyotype with R bands and histopathological analysis was performed to the subject. It was found that both individuals were males, which rules out a case of freemartins and lead us to a case of sexual development disorder.

2.3 Cytogenetic analysis

Peripheral blood sample (1 ml) was cultured in RPMI medium, enriched with FCS (10%), L-glutamine (1%) and phytohemagglutinin (1.5%) (as mitogen) for about 72

h at 37 ° C. In the latter, BrdU (R-banding) was added 6 h before harvesting to allow its incorporation into DNA. Cells from of cell cultures were harvested after Colcemid (0.3 µg/ml) treatment for 1 h and hypotonically treated (KCl 0.5%) and fixed three times in methanol: acetic acid (3: 1), the third time overnight. Then the drip of mitotic spreads was made on plates previously cleaned with ethanol to finally seal the plates with 0.3-0.5 mL of fresh fixative. The obtained metaphases were observed in a microscope at 100X with the help of the immersion oil looking for the presence of chimerism XX / XY and measuring the percentage of spreads that carried the chromosome "Y".

2.4 Diagnosis by PCR

Several samples (lymphocytes, lung, heart and kidney) were obtained from the apparent female calf and only lymphocytes sample were drawn from the male co-twin for the diagnosis of freemartin by polymerase chain reaction (PCR). Genomic DNAs of the calves were isolated using DNeasy Blood & Tissue Kits (Quiagen Cat No. /ID: 69504). The bovine DNA sequences, BOV97M and bovine 1.715 satellites, were used for sex determination. Amplification of BOV97M, located on the Y chromosome, produces a PCR product of 141 base pairs (bp). Amplification of the bovine 1.715 satellite, located on an autosome, produces a PCR product of 216 bp, indicating the success of the PCR procedure. The presence of both PCR products indicates a male embryo, and the presence of only the 216-bp product indicates a female embryo (Fig. 2). The BOV97M primers were forward, 59-GAT CAC TAT ACA TAC ACC ACT-39; and reverse, 59-GCT ATG ACA CAA ATT CTG-39. The sequence of the bovine-specific primers were forward, 59-TGG AAG CAA AGA ACC CCG CT-39; and reverse, 59-TCG TCA GAA ACC GCA CAC TG-39. One-third of a blastocyst lysate (2 ml of the total 6 ml of blastocyst lysate) was used in a final volume of 25 ml. The reaction mixture contained 13 PCR buffer (Invitrogen-Life Technology, Carlsbad, CA) without MgCl2, 2 mM MgCl2 (Invitrogen-Life Technology), 10 mM total dNTP, 1 U of Tag DNA polymerase (Invitrogen-Life technology) and 3 ng/ml of each BOV97M primer, 1.5 ng/ml of each bovine-specific

primer. Amplification was performed for a total of 40 cycles. Each consisted of template denaturation at 958C for 30 sec, primer annealing at 508C for 30 sec, and primer extension at 728C for 45 sec. After 40 cycles, the samples were incubated at 728C for 5 min and cooled to 48C. The amplified products were then electrophoresed on a 1.5% agarose gel (Invitrogen-Life technology), stained with ethidium bromide, and evaluated using UV light.

3. Results

3.1 Cytogenetic and molecular analysis

Cytogenetic and molecular analysis performed with R bands revealed that there is a XY chromosome constitution in all the mitosis evaluated (n=150 per animal), for both cases, the male and the supposed female (Fig 2). With the number of mitoses evaluated, cell chimerism was discarded in the two samples evaluated, ($P \leq 0.05$). Karyotyping results were further validated by PCR using primers specific for BOV97M, which is located on the Y chromosome (DNA amplified fragment size is 141 base pairs). The PCR products showed the presence of the region BOV97M in all evaluated tissues (Fig.3). These results suggest that the sexual disorder presented in the female is not a case of freemartins and that the female is actually a male who presented a case of disorders of sex development.

3.2 Necropsy findings and histological evaluation of the genital structures

Post-mortem traces of female reproductive tract were found, macroscopically vulvar presence, uterine horns and rudimentary ovaries were observed, all these structures presented a low morphological definition. In addition, there was a presence of testicles inside the mammary gland with dysplastic seminiferous tubules and without mature or immature sperm (Fig. 4). The presence of testes was also observed inside the mammary gland. The histological evaluation did not confirm the presence of

female genitalia. The areas where uterine horns and ovary were identified have epididymal histological structure (Fig 5.).

Fig 1. *A*. Bull Gyr used in the present case study. *B*. Presence of vulva with prepuce morphology

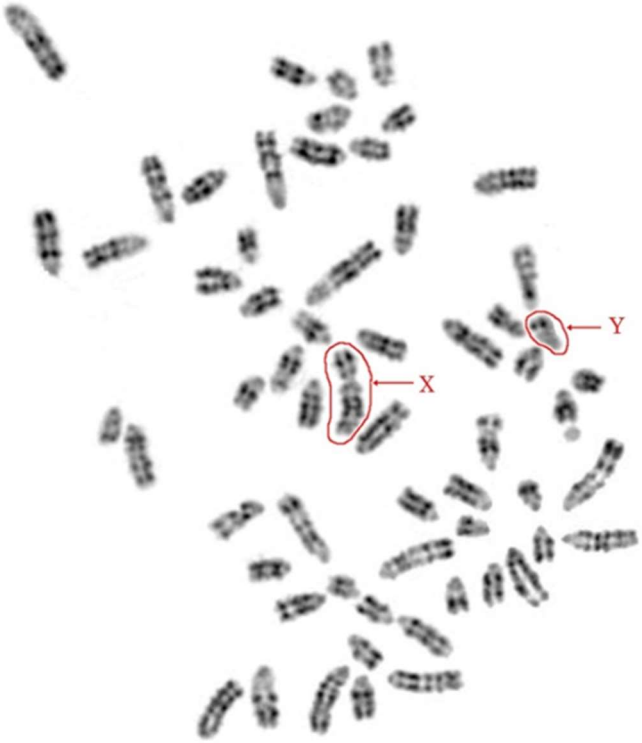

Fig 2. Chromosome analyses of the hermaphrodite subject. RBG-banded karyotype obtained from blood. The red arrows indicate the sex chromosomes (X-Y). No major rearrangements are visible.

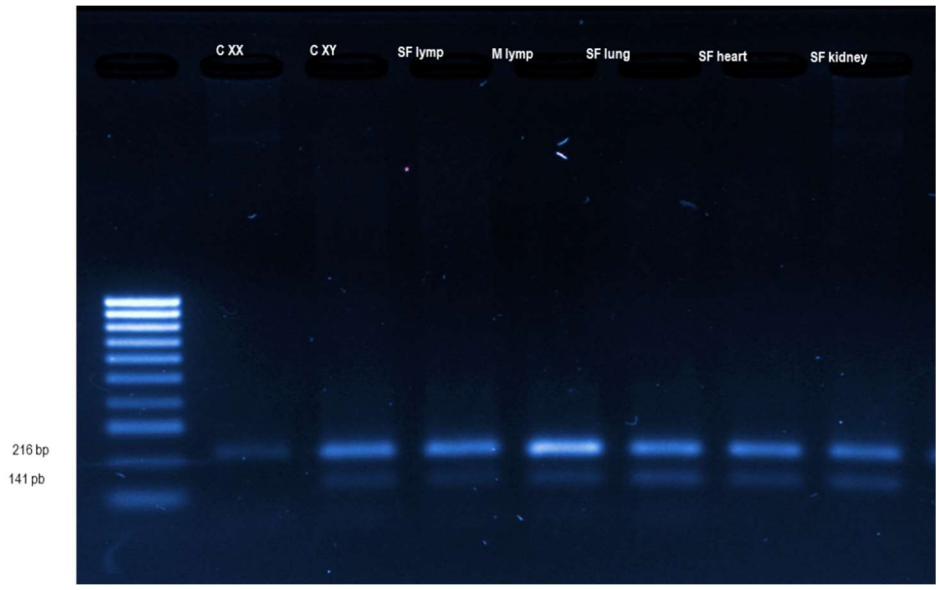

Fig 3. PCR analysis. Chromosome Y (BOV97M). CXX: Control female XX. CY: Control male XY. SF lymp: Supposed Female lymphocytes. M lymp: Male lymphocytes. SF lung: Supposed Female lung. SF heart: Supposed Female heart. SF kidney: Supposed Female kidney. Amplification of the region of chromosome Y is observed in all samples, except in the negative control (female XX).

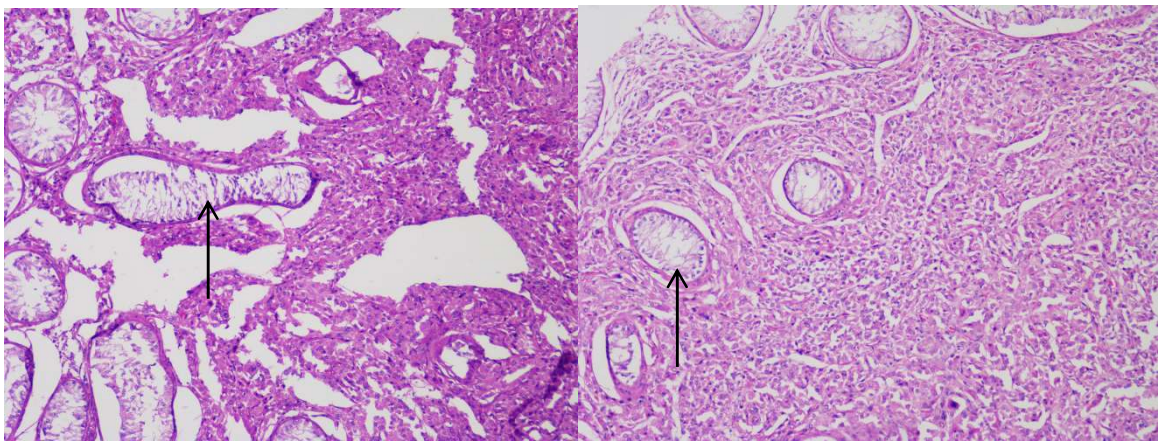

Fig 4. Microscopic findings. Testicle. The dysplastic seminiferous tubules are seen without mature or immature sperm, like the Sertoli cells, black arrow. Also, abundant highly undifferentiated interstitial cells are observed. 10x

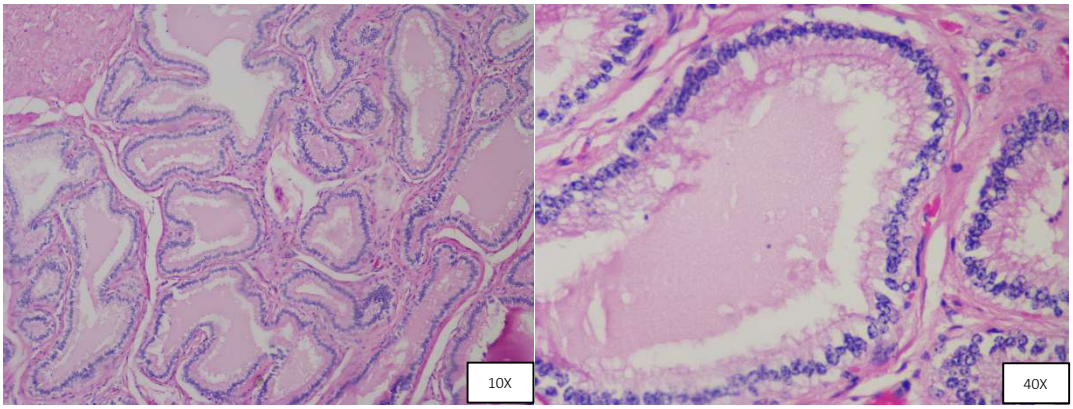

Fig 5. Areas where it was identified as uterine and ovarian horns with histological structure of seminal vesicles.

4. Discussion

The freemartin condition represents the most frequent form of intersexuality found in cattle, occasionally in other species [10–12] and in river buffalo [13]. Freemartinism occurs when vascular connections arise between the placentae of developing heterosexual twin fetuses and XX/XY chimerism develops, with masculinisation of the female tubular reproductive tract to different degrees. Furthermore, sex differentiation occurs about one week earlier in males than in females [11]. The alteration degree of the female reproductive organs is related to how early placental anastomosis occurs in fetal development, allowing the male's anti-Mullerian duct factor and testosterone to exert their effects [10,14].

Considering that 90% of cattle twin pregnancies in which fetuses are heterosexual the females are freemartins and sterile [11], and added to the feminized phenotype presented by the animal, represented as an under body size and clitoris with

appearance of foreskin (Fig 1), we assume the case as a typical of freemartins, where we perform a cytogenetic, molecular and histopathological evaluation.

The results of this study suggest that the case is not a freemartinism because all the mitoses evaluated in the two individuals corresponded to sex chromosomes XY. Therefore, we do not found cytogenetic evidence to confirm chromosomal chimerism XX/XY. All samples analyzed with PCR, showed amplification of the BOV97M region exclusive of the Y chromosome. In addition, the histopathological findings correspond to the male genital tract.

The present animal was diagnosed as a true hermaphrodite with an apparently normal male karyotype (60, XY) in peripheral lymphocytes, lung, heart and kidney. Besides, in the four tissues analyzed, amplification from a region of the Y chromosome was observed, indicating the presence of XY cells. The criteria for the diagnosis considered the subject morphology, the phenotypic appearance of the genital tract, the anatomopathological findings, cytological karyotyping, and DNA analysis. The true genetic sex of an animal can be determined, and when it is found not to correlate with the phenotypic sex, then an intersex condition is present [8,9]. Our results are in accordance with some reports that indicate that individuals with gonadal sex disorders can have an XX or XY sex chromosome constitution [8,15].

In the male, the presence of the SRY gene in the Y chromosome is required to initiate testicular differentiation pathways and to inhibit female-specific pathways [16]. When testicular differentiation is not achieved, whether because SRY expression fails to occur or is expressed after the permissive window for induction of the male pathway, gonadal morphogenesis is impaired and germ cells are not prevented to enter meiosis. Consequently, follicular structures are allowed to develop [1,17,18], and the embryonic gonad of an XY animal may develop either towards an ovotestis or an ovary. In this animal, a disordered sexual development was observed in the male reproductive tract; perhaps this suggests a mutation in the SRY gene that altered the testicular differentiation pathway.

In addition, this phenotype can be the result of androgen insensitivity, which is a disorder of sex development that exclusively affects individuals with XY. The abnormal development of both internal and external sex structures observed in these individual results from germline mutations in the androgen receptor (AR/NR3C4) gene[4,19]. Androgens bind to their receptors, regulating the transcription of genes involved in sex differentiation and the development and expression of the male phenotype. A defective androgen receptor fails to activate its target genes, which can lead to target-organ androgen-resistance and therefore a wide spectrum of sex phenotypes.

Likewise, in humans mutations of the NR5A1 gene encoding steroidogenic factor-1 have been reported in association with a wide spectrum of 46, XY DSD phenotypes including severe forms of hypospadias [2]. During early male development NR5A1 positively regulates the expression of two key genes involved in male sex determination and differentiation, SOX9 (Sry-box 9), and Anti-Mullerian Hormone (AMH) [20,21]. Recently, the range of phenotypes that are associated with NR5A1 mutations has broadened and now includes, XY complete and partial gonadal dysgenesis, penoscrotal hypospadias, microphallus with anorchidia [22,23].

In conclusion, the absence of XX / XY chromosomes characteristic of a freemartins, plus the results obtained in the molecular analysis and in addition to the histopathological results where there is no evidence of cells of the female reproductive tract, we can infer that the case is not a chimera as initially had been raised, but it is an XY male with sexual development disorder, which generates a phenotype with intersex characteristics, commonly known as pseudo-hermaphrodite.

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