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XY (*SRY*-positive) Ovarian Disorder of Sex Development in Cattle

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Dear Dr. Parma,
Some editorial changes as well as corrections have been made. I only highlighted those which need your special attention, but please carefully check the whole manuscript. Please note that the resolution of the figures will be higher in the final (printed and online) version of your manuscript.
Thank you very much.

Keywords
Histology · Microsatellite marker · *SRY* · XY constitution

Abstract
In mammals, the sex of the embryo depends on the *SRY* gene. In the presence of at least one intact and functional copy of this genetic factor (XY embryo) undifferentiated gonads will develop as testicles that subsequently determine the male phenotype. When this factor is not present, i.e., in subjects with 2 X chromosomes, an alternative pathway induces the development of ovaries, hence a female phenotype. In this case study, we describe a female cattle affected by a disorder of sex development (DSD). The subject, despite having a chromosomal XY constitution, did not develop testicles but ovaries, although they were underdeveloped. Moreover, genetic analysis highlighted the presence of the *SRY* gene with a normal coding region in both blood- and tissue-derived DNA. A chimeric condition was excluded in blood by sexing more than 350 cells and by allele profile investigation of 18 microsatellite markers. Array CGH analysis showed the presence of a not yet described 99-kb duplication (BTA18), but its relationship with the phenotype remains to be demonstrated. Gonadal histology demonstrated paired ovaries: the left one containing a large corpus luteum and the right one showing an underdeveloped aspect and very few early follicles. To our knowledge, we describe the first case of XY (*SRY*+) DSD in cattle with a normal *SRY* gene coding sequence.

In mammals, sex determination is one of the most complicated developmental processes. Indeed, the development of a male or female phenotype is governed by a complex network of genetic, epigenetic, and hormonal components [Windley and Wilhelm, 2015]. The first step of this delicate biological process, named sexual determination, is the formation of gonads in the developing embryo and requires the involvement of several genetic factors. The Y-linked *SRY* gene plays a relevant role; in fact its presence regulates the development of functional testicles through a genetic pathway of a series of gene activations and repressions. In embryos lacking this gene, namely XX embryos, another genetic pathway leads to the development of functional ovaries.

To date, most of the genetic factors involved in this process have been identified by analyzing subjects with a disorder of sexual development (DSD) [Bashamboo and McElreavey, 2016].

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Normally, mammals possessing a XY karyotype also have a copy of the *SRY* gene and develop testes. However, even in the presence of a functional copy of the *SRY* gene, there are other factors that may interfere with this process, giving rise to the condition called XY (*SRY*-positive) DSD, which is characterized by an incomplete or absent testes differentiation. Until now, this syndrome has been found, in addition to humans [McElreavey and Fellous, 1999], in other species such as horse, river buffalo, dog, sheep, and cat [Parma et al., 2016]. As for cattle, several different cases have been demonstrated prior to 1990, the year of the human *SRY* gene discovery [Sinclair et al., 1990]. Since then, 5 females with a XY constitution have been reported [Kondoh et al., 1992; [illegible] et al., 1994; Kawakura et al., 1996], but the cattle *SRY* gene sequence has been available only from early 1994 on [Payen and Cotinot, 1994]. Consequently, merely 3 related XY DSD cases could be analyzed according to the *SRY* gene [Kawakura et al., 1996]. In these 3 cases, despite the presence of a quite normal XY karyotype, PCR assays excluded the presence of the *SRY* gene, and later and more accurate investigations showed that the observed Y chromosome was represented by an Yp isochromosome without the *SRY* gene [Kawakura et al., 1997]. To our knowledge, no new case of XY (*SRY*+) DSD has been reported in the last 20 years. In this paper we report the first case of XY (*SRY*+) DSD in cattle with a normal *SRY* gene coding sequence.

Material and Methods

Case Report

The cow was taken to the Veterinary Hospital because of the lack of pregnancy despite repeated attempts. The animal was 24 months old and had a size according to the Holstein breed standard. No further anomaly has been reported.

Histology

Representative samples of the gonads, uterine tubes, uterus, cervix, and clitoris were dissected and immediately fixed by immersion in 10% neutral formalin for at least 7 days, then dehydrated in a graded series of ethanol, cleared with xylene, and routinely embedded in paraffin. For microscopic evaluation, 4–6 µm thick sections were routinely stained with hematoxylin and eosin (HE). Sections were viewed under an Olympus BX51 microscope equipped with a digital camera and DP software (Olympus, Italy) for computer-assisted image acquisition and management. A normal ovary of the same age and development has been considered as a normal control.

SRY Analysis

DNA was extracted from whole blood using a commercial kit (UltraClean Blood Spin, MoBio). DNA was also extracted from the paraffin-embedded right streak ovary as reported [McPherson, 1991]. In both cases, the DNA was diluted to 20 ng/µl, and then 1 µl was used as PCR starting material. In the blood sample, the presence of the *SRY* gene was detected by PCR using the following primers: BtSRY-F: 5′-aaacagtgcagtcgtatgcttctgc-3′ and BtSRY-R 5′-cttccttactctcgctaacaaaggc-3′. The 740-bp PCR amplification products were visualized on a 1.3% agarose gel with ethidium bromide.

In the tissue sample, where the extracted DNA showed degradation, the *SRY* gene was amplified with the following pair of primers: SRY-BT-SHF: 5′-actgccaggacgtattgagg-3′ and SRY-BT-SHR: 5′-gttgctgtaccacagctgga-3′. They amplified a region of 230 bp that overlaps with the beginning of the coding sequence (ATG) and does not include the HMG box. Amplifications were performed using the DNA polymerase enzyme (AmpliGold, Promega) with an annealing temperature of 56°C for 35 amplification cycles. DNA integrity was tested by amplifying a 220-bp genome portion of the *ABCA12* gene [Longeri et al., 2009], while *SRY* gene integrity was evaluated by direct sequencing of the *SRY* PCR products. The presence of the XY chromosomes in the tissue was verified using the amplification of *AMELX/Y* [Ballin and Madsen, 2007].

Microsatellite and SNP Analysis

DNA extracted from the blood was used as starting material for the amplification of 18 microsatellites (Table 1), commonly used for paternity tests and validated by ISAG (International Society of Animal Genetics). Microsatellites BM6017 and BMC6021 were also amplified in DNA from both blood and ovary, using the primers reported in Nguyen et al. [2007]. SNP rs29003723 and rs17871661 (https://www.ncbi.nlm.nih.gov/snp/) were amplified using the following primers: 723-F: 5′-CAGGACTTGTGGCCTCTTTC-3′, 723-R: 5′GTGGAGCTAGGAGGTTCAGG-3′, 661-F: 5′-GCCCTGGTGAGTCAGAGC-3′, 661-R: 5′CAGACTTGGAAGAGCGGCTA-3′. Both primer pairs amplify a very short genomic region (<120 bp) surrounding the SNP.

Table 1. Allele profile observed in blood-derived DNA

Marker	Chromosome	Allele a, bp	Allele b, bp
BM1824	1	182	182
TGLA57	1	90	96
BM2113	2	127	127
INRA023	3	206	214
MGTG4B	4	141	141
ETH10	5	219	225
AGLA293	5	230	230
ETH225	9	140	150
SPS113	10	151	153
INRA005	12	120	120
SPS115	15	256	258
TGLA53	16	158	158
TGLA227	18	91	97
ETH3	19	127	127
TGLA126	20	115	115
TGLA122	21	149	171
CYP21	23	189	189
BM1818	23	262	266

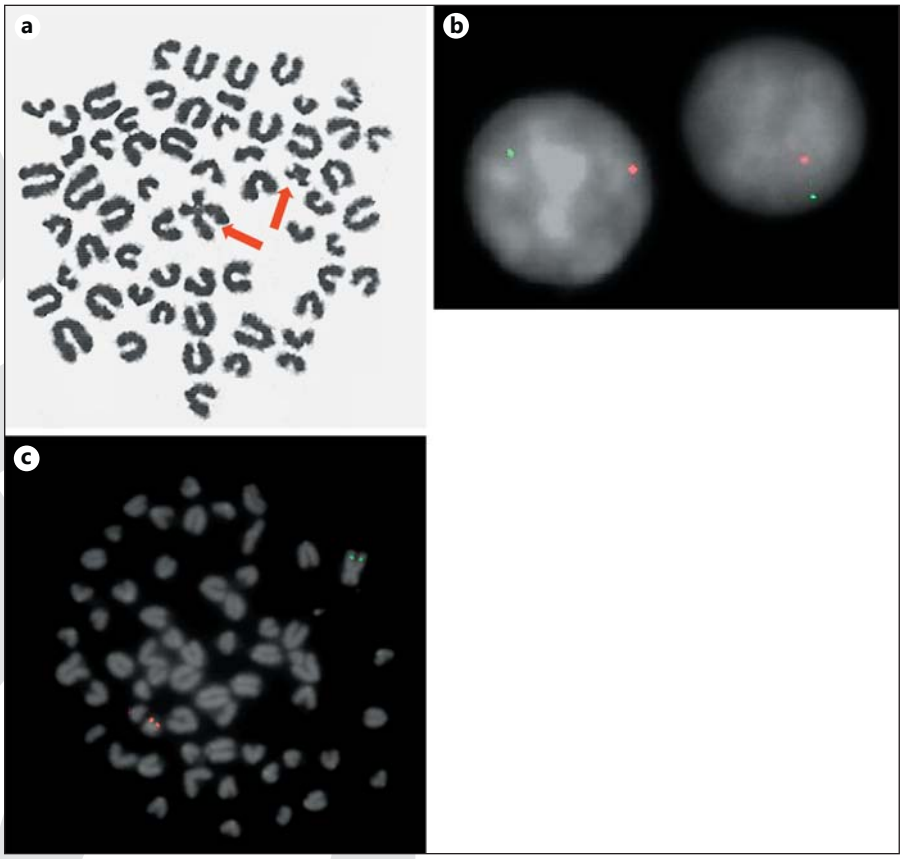

Fig. 1. Cytogenetic analyses of the XY (*SRY*+) DSD subject. **a** Metaphase obtained from blood. The 2 red arrows indicate the sex chromosomes. **b** FISH on blood nuclei. **c** FISH on blood metaphase. In both FISH analyses BAC 346G05 (Xq) is labeled in green and 787C06 (Y) is labeled in red.

Cytogenetic Analysis

Cytogenetic analysis was performed on metaphases obtained from peripheral blood lymphocyte cultures following a slightly modified standard method [De Grouchy et al., 1964]. FISH analyses were performed as reported in De Lorenzi et al. [2017]. BACs 787C06 (BTAYq, including the *SRY* gene) and 346G05 (BTAXq) [Eggen et al., 2001] were used as probes.

Array CGH

Array CGH was performed using DNA obtained from blood as reported previously [De Lorenzi et al., 2012].

Results and Discussion

The observation of 253 Giemsa-stained metaphases revealed the presence of a male karyotype (2n = 60,XY) with no sign of large structural abnormalities (Fig. 1a). The presence of the X and Y chromosomes in all 102 nuclei observed was also highlighted by FISH investigation (Fig. 1b). Finally, the localization of the *SRY* gene on the Y chromosome has been verified by FISH in all 5 metaphases tested (Fig. 1c). Considering that all 360 cells observed were XY, we can exclude the presence of a chimeric condition with more than 1% of XX with 0.95 confidence level [Hook, 1997]. Finally, given that a chimeric situation can also be detected by looking for more than 2 alleles in microsatellite markers, the analysis of the 18 microsatellites did not show the presence of extra alleles. Table 1 shows the allele condition of the 18 markers (a full report is available on request). Unfortunately, it was not possible to amplify these markers in the tissue-derived DNA, probably due to DNA degradation after extraction.

Considering the tests carried out to identify the *SRY* gene, it was possible to highlight the presence of this gene in both DNA derived from blood (Fig. 2a) and tissue (Fig. 2b). Moreover, the presence of the X and Y chromosome in the tissue was established (Fig. 2b). In addition, the comparison between the normal *SRY* cattle sequence (GenBank accession number EU581861) and the sequence of the PCR product revealed no changes. Array CGH analysis revealed a number of CNVs already described in previous studies [Fadista et al., 2010; Liu et al., 2010] and a variation not yet known: a duplication of the BTA18 genomic region 57,153,636–57,253,161 bp

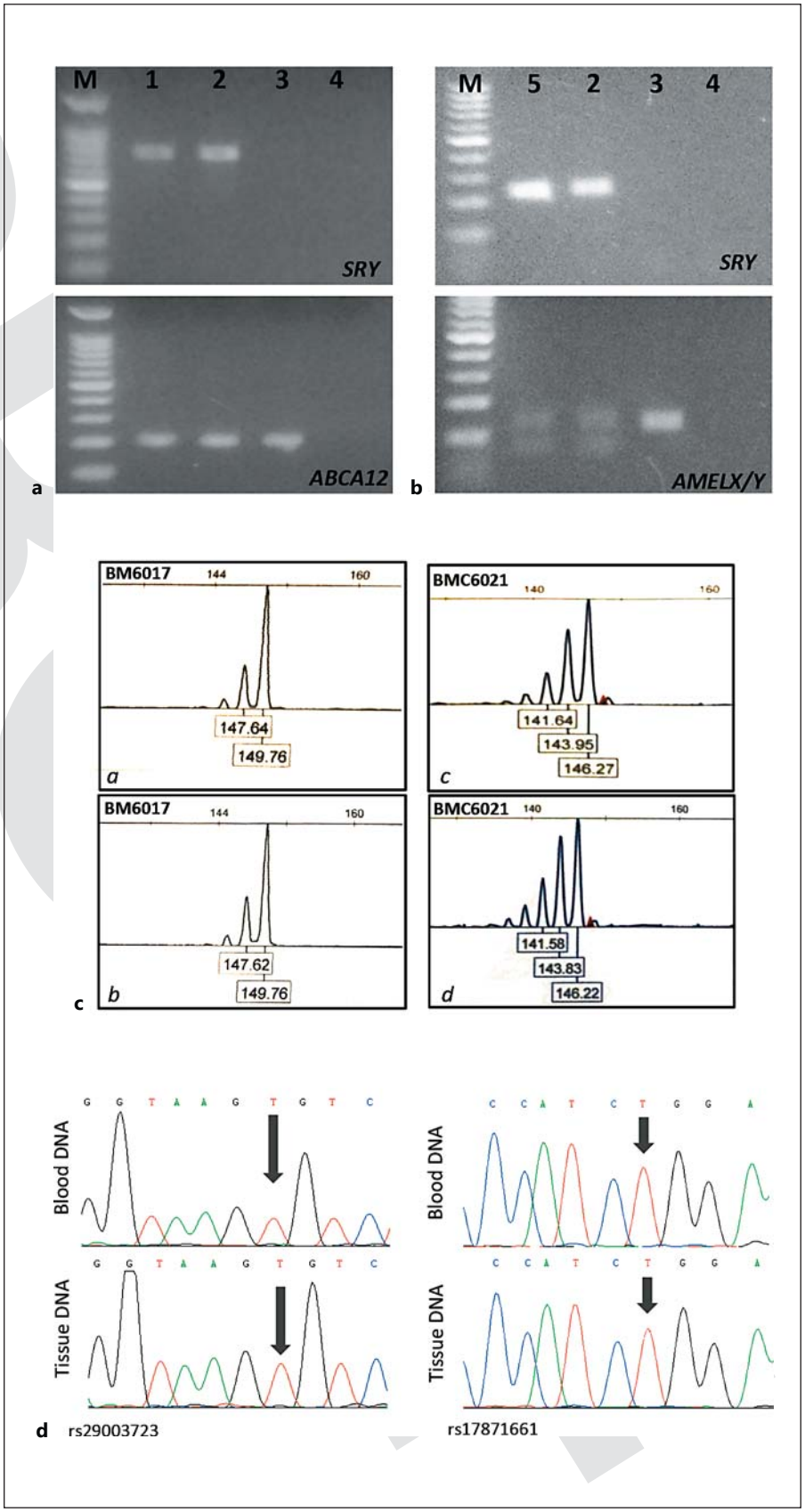

Fig. 2. *SRY* and marker analysis. **a** Results of *SRY* and control PCR using blood-derived DNA. **b** Results of *SRY* and *AMELX/Y* amplifications on DNA obtained from tissue. M, 100 bp DNA ladder; 1, DNA from XY (*SRY*+) DSD subject blood; 2, DNA from a normal male; 3, DNA from a normal female; 4, no DNA; 5, DNA from XY (*SRY*+) DSD subject tissue. **c** Results of the 2 X-linked microsatellites amplified in blood (*a*, *c*) and tissue DNA (*b*, *d*). **d** Sequence of the 2 SNPs amplified in blood and tissue DNA.

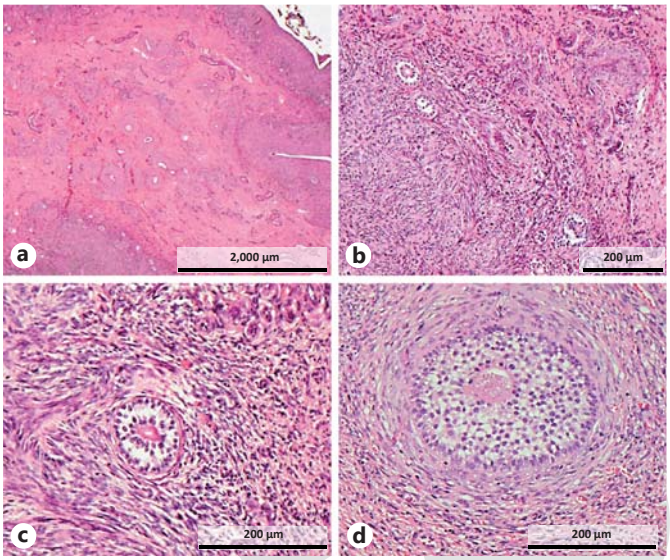

Fig. 3. Histology aspect of the right ovary, showing a quiescent appearance. **a** A vascularized connective stroma is mainly present, occupying the most part of the ovarian parenchyma. **b** Very small follicles can be seen in the more superficial ovarian rim. **c** A secondary follicle can be seen, devoid of an antrum and containing a very small oocyte with detectable zona pellucida. **d** For comparison, a similar stage follicle in a normal bovine ovary is shown at the same magnification as in **c**.

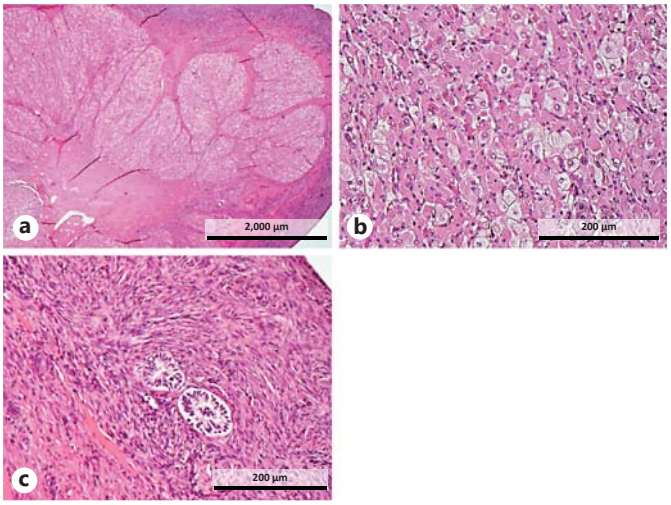

Fig. 4. Histology of the left ovary. **a** A very large corpus luteum almost occupies the entire gonad. **b** At higher magnification, different morphologies of the luteal cells can be noticed. **c** In the more superficial ovarian rim, very rare and small follicles at earliest stages can be detected.

(bosTau4 genome assembly). This 99-kb long genomic region includes at least 1 zinc finger gene, *ZF145* (zinc finger protein 415). In humans, this gene is widely expressed in several tissues, and a high expression level can be found in testis and brain. Further analyzes are necessary to establish a possible relationship between this alteration and the observed phenotype. Finally, it was possible to get information about some markers also in the DNA obtained from the tissue, even if the analysis was particularly complex due to DNA degradation. We demonstrated that the cell lines present in blood and tissue are identical, presenting the same allelic profile. Considering that the 2 analyzed microsatellites are located on the X chromosome, we could identify only one X chromosome as expected. The results are shown in Figure 2c and d.

Macroscopic examination of the gonads showed paired ovaries, the left one containing a large corpus luteum and the right one having an underdeveloped aspect and very few early follicles. The uterine tubes were normal, and the uterus had a normal appearance although it was underdeveloped, with the right uterine horn being smaller than the left and devoid of caruncles. The cervix was double with a normal structure, and the clitoris had a normal morphology.

Histological examination of the gonads revealed paired ovaries and confirmed the differences which had been noticed macroscopically. No evidence of testicular tissue was found by observing different regions of the gonads.

The right ovary had a quiescent aspect, being mainly composed of vascularized connective stroma (Fig. 3a) in which very small follicles could be rarely found, limited to the more superficial rim (Fig. 3b). The follicles were of the primary-secondary type, all of them devoid of an antrum and containing a very small oocyte (Fig. 3c). Figure 3d shows a follicle at a comparable stage in a normal bovine ovary (same magnification as Figure 3c).

The left ovary showed a very large corpus luteum, which almost occupied the entire gonad (Fig. 4a). It was of normal general morphology and vascularization. The luteal cells had different morphologies, reflecting different functional stages (Fig. 4b). In this ovary, early stage follicles were very rare and small (Fig. 4c).

Both uterine tubes were well developed, sharing the same morphology. The distinct, usual regions could be seen, i.e., infundibulum with fimbriae (Fig. 5a), ampulla (Fig. 5b), and isthmus (Fig. 5c). The columnar epithelium lining the ampulla region consisted of ciliated and nonciliated cells. The epithelium showed the aspect which is typical of the luteal phase in ruminants, i.e., it was characterized by cellular protrusions and nuclear extrusion by secretory cells (Fig. 5d, e). Figure 5f shows the aspect of

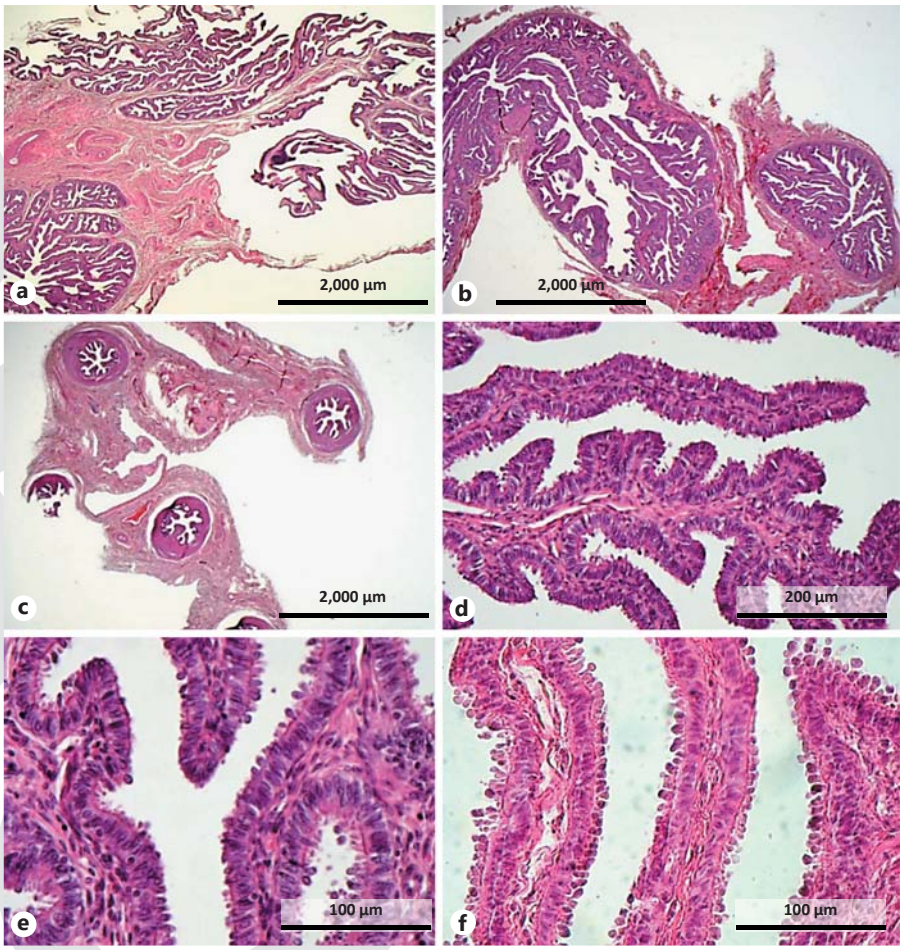

Fig. 5. Histology of the uterine tube. The different regions can be easily distinguished, i.e., infundibulum with fimbriae (**a**), ampulla (**b**), and isthmus (**c**). **d** Ciliated and nonciliated cells can be distinguished in the columnar epithelium lining the ampulla region. The secretory cells show protrusions and nuclear extrusion (**d**, **e**), which are a typical epithelial aspect during the luteal phase. **f** For comparison, the ampullary epithelium during the luteal phase in a normal ruminant oviduct is shown at the same magnification as in **e**.

the ampullary epithelium during the luteal phase in a normal ruminant oviduct.

The uterus had a normal although underdeveloped appearance. In particular, the right uterine horn was smaller than the left one and was devoid of caruncles. Compare Figure 6a (right horn) and Figure 6b (left horn) at the same magnification. In both horns, the branched uterine glands had the typical aspect of the secretory (luteal) phase. The cervix was double, each of them showing a normal thick-walled muscular structure. The cervix mucosa was thrown into prominent primary and secondary folds (Fig. 6c) lined by normal columnar epithelium.

The clitoris was normal. In a longitudinal section, a corpus cavernosum was present, richly supplied with nerve bundles. Normally conformed prepuce with lymphatic follicles could also be seen (Fig. 6d).

The absence of testicular development in the presence of a normal *SRY* gene, at least regarding the coding region, represents an interesting case. In humans, many genetic factors have been discovered to be responsible for such a condition. Several cases, but not all, have been imputed to mutation in *NR5A1* [Achermann et al., 1999], *CBX2* [Biason-Lauber et al., 2009], *MAP3K1* [Pearlman et al., 2010], *DHH* [Umehara et al., 2000], *AKR1C2* [Fluck et al., 2011], *ZFPM2* [Bashamboo et al., 2014], and *SOX8* [Portnoi et al., 2018]. XY sex reversal in humans could also be determined by duplication of the *NR0B1* gene [Bardoni et al., 1994] as well as deletions on chromosome 9p24.3 [Guioli et al., 1998] and deletion of a region upstream the *SOX9* gene [Kim et al., 2015]. With regard to the latter genetic factor, mutations are also responsible for this phenotype [Foster et al., 1994]. Often the mutations in these genetic factors give rise to a syndromic phenotype that also involves other organs outside the gonads and sexual development such as adrenal hypoplasia or failure, polyneuropathy, or campomelic dysplasia. Normally, in these cases the ovaries do not develop properly and the reproductive capacity is compromised. An exception is described by

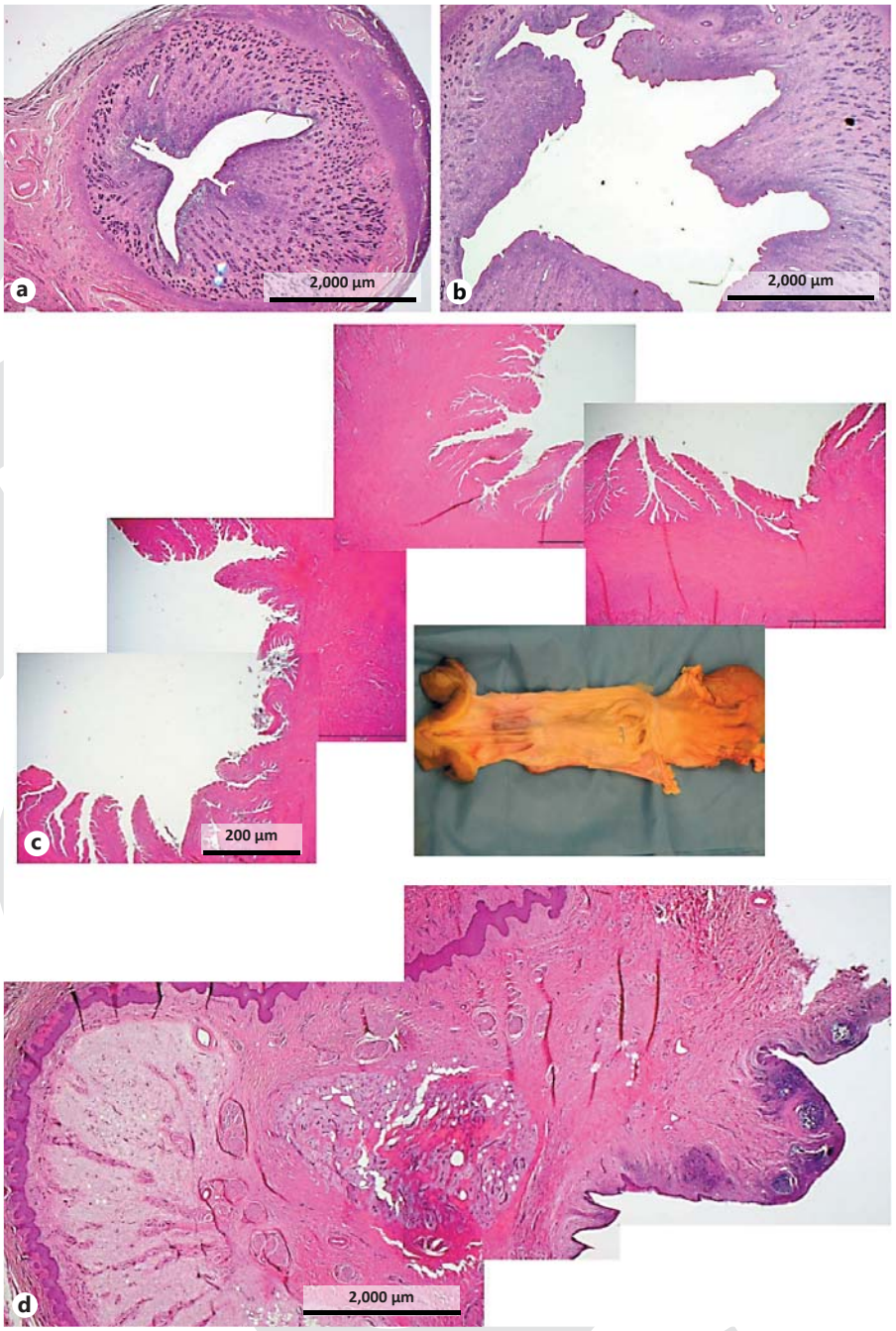

Fig. 6. Histology of the uterine horns, cervix, and clitoris. The right uterine horn (**a**) shows a smaller diameter than the left one (**b**) and is devoid of caruncles. In both horns, the uterine glands show the typical branched aspect of the secretory (luteal) phase. **c** The macroscopic picture shows a double cervix. Each cervix has a normal thick-walled muscular structure and prominent primary and secondary mucosa folds protruding into the lumen. **d** The clitoris was normal. In a longitudinal section, a corpus cavernosum was present, richly supplied with nerve bundles. A normally conformed prepuce with lymphatic follicles was also present.

Dumic et al. [2008] who reported a fertile XY woman with normal ovaries, but an in-depth analysis showed the presence of different cell lines in the ovarian tissue: 93% 46,XY, 6% 45,X, and less than 1% 46,XX.

However, despite the identification of all these causative genetic factors of XY DSD, at least a third of the cases remain unexplained. For this reason a non-artificially inducted syndrome in animal models represents a very important source of genetic information. In fact, once that a case of this type is well classified for both genetic and histologic features, it represents a good source of information for interspecific transverse studies.

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Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

The authors have no conflicts of interest to declare.

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