Applying real-time quantitative PCR to diagnosis of freemartin in Holstein cattle by quantifying *SRY* gene: a comparison experiment

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ABSTRACT

Background: Freemartinism generally occurs in female offspring of dizygotic twins in a mixed-sex pregnancy. Most bovine heterosexual twin females are freemartins. However, about 10% of bovine heterosexual twin females are fertile. Farmers mostly cull bovine fertile heterosexual twin females due to the lack of a practical diagnostic approach. Culling of such animals results in economic and genetic-material losses both for dairy and beef industry.

Methods: In this study, a comparative test, including qualitative detection of *SRY* gene by polymerase chain reaction (PCR), quantitative detection of relative content of *SRY* by real-time quantitative polymerase chain reaction (qPCR), and quantitative detection of H-Y antigen, was performed to establish the most accurate diagnosis for freemartin. Twelve Holstein heterosexual twin females were used in this study, while three normal Holstein bulls and three normal Holstein cows were used as a positive and negative control, respectively.

Results: Polymerase chain reaction results revealed that *SRY* gene were absent in three heterosexual twin females and only two of them were verified as fertile in later age. The qPCR results showed that relative content of *SRY* was more than 14.2% in freemartins and below 0.41% in fertile heterosexual twin females. The H-Y antigen test showed no significant numerical difference between freemartin and fertile heterosexual twin female.

Discussion: Our results show that relative content of *SRY* quantified by qPCR is a better detection method for diagnosis of freemartin in Holstein cattle as compare to qualitative detection of *SRY* gene by PCR or quantitative detection of H-Y antigen. To the authors' knowledge, this is the first time we applied qPCR to diagnosing freemartin by quantifying *SRY* gene and got relative *SRY* content of each freemartin and fertile heterosexual twin female. We concluded that low-level of *SRY* would not influence fertility of bovine heterosexual twin female.

Subjects Agricultural Science, Developmental Biology, Veterinary Medicine, Zoology
Keywords Heterosexual twin female, Freemartin, H-Y antigen, qPCR, *SRY*

INTRODUCTION

Freemartinism is an intersexual syndrome occurring in the female offspring of mixed-sex pregnancy, being one of the most important types of disorders of sexual development in domestic cattle (*Padula, 2005*; *Villagómez et al., 2009*). The most common consequences of freemartinism are masculinized phenotype and sterility (*Jeon et al., 2012*). Freemartin is a sterile female originated from heterosexual multiple pregnancies (*Esteves, Båge & Payan-Carreira, 2012*), usually born as co-twin to a male calf. Despite a rare event, freemartin cases have been reported born as triplets (*Zobel, 2011*), quadruplets (*Biswas et al., 2015*), amorphus globosus (*Weber, Rudolph & Freick, 2017*) and singleton which is due to loss of her male co-twin before parturition (*Kitahara et al., 2002*; *Szczerbal et al., 2014*).

The sterility of freemartin is explained through hormones and XX/XY chimera (*Capel & Coveney, 2004*; *Freeman, 2007*; *Gonelladiaza et al., 2012*). In the former scenario, male hormones such as testosterone and anti-Müllerian, originally due to Wolffian development and Müllerian regression (*Loriaux, 2016*), affect the female fetus through placental vascular anastomoses at 30–40 days of gestation and result in transformation of the general somatic habitus in female (*Jiménez, Barrionuevo & Burgos, 2013*; *Ayalavaldovinos et al., 2014*; *Refaat, Ali & Tharwat, 2015*). The chimera scenario explains that the XY cells of a male fetus transfer to his female twin through the shared vascular anastomosis branches, and result in formation of XX/XY chimeras which hinder the development of female gonads (*Peretti et al., 2008*).

It is observed that 82–97% of bovine females from heterosexual twins are freemartin and contain XX/XY chimera (*Zhang et al., 1994*; *Fujishiro et al., 1995*; *Esteves, Båge & Payan-Carreira, 2012*; *Kozubska-Sobocińska, Danielakczech & Rejduch, 2016*). The twinning rate is rising in last few years (*Szczerbal et al., 2014*) and it is expected that the number of fertile heterosexual twin female is also increasing. Normally heterosexual twin females are sold for meat or fetal bovine serum production which is economic and genetic material loss (*Hirayama et al., 2007*).

A rapid, sensitive, and inexpensive method for identification of freemartin at birth or early age becomes an urgent need to reduce unnecessary economic losses and to preserve important hereditary material, also to avoid the delivery of detrimental genetic materials by progeny (*Biswas et al., 2015*). Many diagnostic methods have been established for identification of freemartin, such as measurement of vaginal length (*Khan & Foley, 1994*), blood grouping test for degree of hemolysis (*Kästli & Hall, 1978*), karyotype analysis for XX/XY chimera (*Dunn, Johnson & Quaas, 1981*), polymerase chain reaction (PCR) or improved PCR techniques for detection of specific fragments located on the Y chromosome (*Hirayama et al., 2007*; *Ron et al., 2010*; *Ayalavaldovinos et al., 2014*), fluorescence in situ hybridization technique for detection of Y chromosome (*Sohn et al., 2007*; *Villagómez & Pinton, 2008*; *Rubes et al., 2009*), quantitative detection of hormones such as progesterone, estradiol, and anti-Müllerian hormone (*Rota et al., 2002*; *Cabianca et al., 2007*; *Remnant et al., 2014*; *Kitahara et al., 2018*), and detection of H-Y antigen qualitatively (*Wachtel et al., 1980*). Most of the methods are based on the assumption that

freemartin contains XX/XY chimera and H-Y antigen, whereas fertile heterosexual twin female does not contain XX/XY chimera or H-Y antigen. However, studies reported that three XX/XY chimeric heterosexual twin females were fertile (*Eldridge & Blazak, 1977*; *Smith, Camp & Basrur, 1977*; *Fujishiro et al., 1995*). Fertile chimeras could be an accidental phenomenon but it motivated us to design a more accurate and efficient diagnostic approach to recognize fertile heterosexual twin female and to assist farmers with their decision making with respect to heifer selection.

The sex-determining region Y (*SRY*) gene is located in the sex-determining region of Y chromosome, and its sequence is highly conserved and easily adapted to quantitative assays (*Nelson, 2002*; *Harley, Clarkson & Argentaro, 2003*). H-Y antigen, another potential marker for identification, is a male tissue-specific antigen secreted by the testis (*Wolf, 1998*), and was previously used to determine sex of bovine embryos (*Wachtel, 1979*). The objective of the current study was to establish an effective diagnostic approach for freemartin by either qualitative and quantitative detection of the *SRY* gene or quantitative detection of H-Y antigen. We hypothesized that cell exchange also occurred in fertile heterosexual twin female at low levels, thus slight cell chimera would not influence reproductive capacity of heterosexual twin female.

MATERIALS AND METHODS

Ethic statements

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 of the National Institutes of Health of China. The protocols were approved by the Animal Welfare Committee of China Agricultural University (Permit Number: DK1008).

Animals and samples

A total of 18 Holstein cattle of age 20 months ± 10 days (12 Holstein heterosexual twin females, three normal Holstein bulls and three normal Holstein cows) were selected in this study. All animals were on the same feeding regime and at same colony house. Animals were reared for two years. Blood samples were collected into sterile tubes with anticoagulant of EDTA by venipuncture for molecular biological analysis. For H-Y antigen determination, blood samples were collected into vacuum tubes without anticoagulant, and centrifuged at 3,500 rpm for 15 min before pipetting the supernatant serum into a 1.5 mL centrifuge tube. All blood samples and serum samples were transported back to the laboratory on ice within 6 h for subsequent DNA extraction and H-Y antigen determination, respectively.

Genomic DNA isolation and primer design

All blood samples were kept on a clean bench at room temperature for 10 min before being isolated for DNA according to the manufacturer's instructions (Tiangen Biotech, Beijing, China). The DNA concentration of all samples was determined by NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and then diluted to 50 ng/μL with buffer TB (Tiangen Biotech, Beijing, China). Primers (Table 1) of the target gene (*SRY*)

Table 1 Primer sequences for PCR and real-time quantitative PCR.

Gene	Sequence (5′ to 3′)	Amplicon size (bp)	Tm[1] (°C)	Accession No.
SRY	Forward: GCCACAGAAATCGCTTCC	229	60	NC_016145.1
	Reverse: CCGTGTAGCCAATGTTACCTT			
GAPDH	Forward: GTGAGAGACGGAACAGGAAGAA	110	60	AC_000162.1
	Reverse: ATGAGGGAAGACAGGACAAAGC			

Note:
[1] Melting temperature.

and housekeeping gene (*GAPDH*) for PCR and real-time quantitative polymerase chain reaction (qPCR) assays were designed by Primer 5.0 and were synthesized by Sangon Biotech (Shanghai, China).

PCR and relative quantitative PCR

Well optimized 20 μL reaction system was as follows: 10 μL of 2 × PCR FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany), 0.3 μL of each primer (10 μM), 1 μL of DNA template and 8.4 μL of DNase/RNase-Free water (Tiangen Biotech, Beijing, China). The PCR conditions included an initial incubation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, 72 °C for 15 s, and the final step of 72 °C for 5 min, amplified products were sent to Sangon Biotech (Shanghai, China) for Sanger sequencing. The qPCR program, executed using a Comparative Quantitation (Calibrator) Real-time PCR System, included a 10 min polymerase activation step at 95 °C followed by a 3-step PCR, which consisted of 40 cycles (95 °C × 15 s, 60 °C × 60 s, 72 °C × 15 s), and a melting curve was generated for the specificity assessment of each pair of primers. Both PCR and qPCR assays were performed in the Stratagene Mx3000P (Agilent Technologies, Wilmington, DE, USA), and qPCR was done in triplicate. Relative content of *SRY* was calculated using the comparative CT ($2^{-\Delta\Delta CT}$) method (*Schmittgen & Livak, 2008*) with normal bull as calibrator and *GAPDH* as the internal control to normalize the data, where CT refers to cycle threshold. ΔCT was calculated by subtracting the CT values of *GAPDH* from the CT values of the *SRY* of interest. $\Delta\Delta$CT was then calculated by subtracting mean ΔCT of the normal bull from ΔCT of tested heterosexual twin female, and relative content of *SRY* was calculated by the equation $2^{-\Delta\Delta CT}$.

H-Y antigen quantification

H-Y antigen concentration was determined by Bovine H-Y Ag ELISA Kit (MLBio, Shanghai, China). Competition assay in enzyme-linked immunosorbent assay (ELISA) was adopted in the determination. All samples were evaluated in a 96-well microtiter plate in duplicates, and the mean value of each sample was calculated. All steps were conducted by the same laboratory technician according to the manufacturer's instructions. Absorbance values were obtained by using an ELISA reader (Tecan, Männedorf, Zürich, Switzerland) at a wavelength of 450 nm.
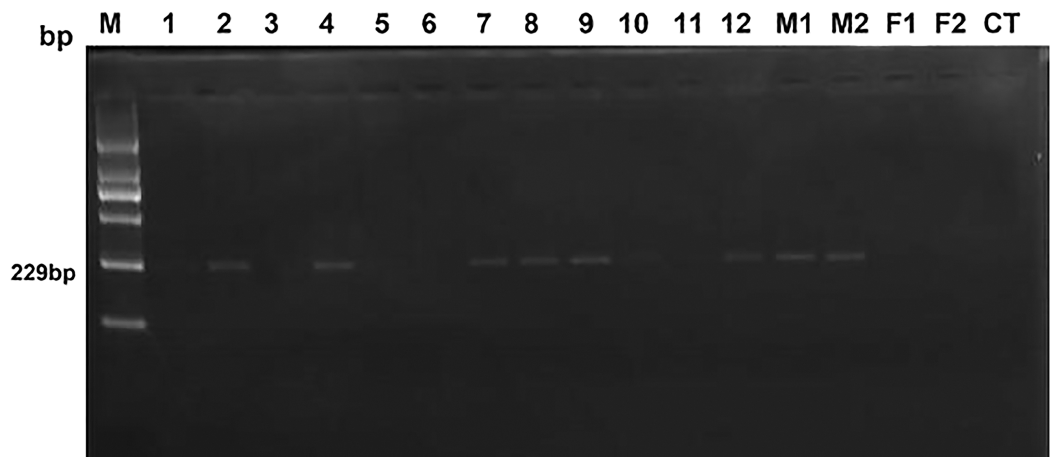
Figure 1 The electrophoresis results of test and control samples by PCR. Lane 1 (M): Molecular weight marker (2,000-bp DNA ladder); bands from bottom to top are listed as 100, 250, 500, 750, 1,000 and 2,000 bp; the size of target bands is 229 bp. Lanes 2 through 13 (No.1–No.12) display samples of 12 female heterosexual twins. Lanes 14 (M1) and 15 (M2) represent positive control. Lanes 16 (F1) and 17 (F2) show negative control. The rightmost lane (CT) is the blank control.

Full-size ▣ DOI: 10.7717/peerj.4616/fig-1

Statistical analysis

H-Y antigen concentration was calculated by Dose–Response Regression Models of Curve Expert Professional Version 2.6.3 Program (Hyams D.G., Starkville, MS, USA). Data were considered as valid only when $R^2 > 0.99$ in the standard curve.

RESULTS

After a two-year breeding of heterosexual twin females with three normal bulls, only two heterosexual twin females were successfully pregnant. Both pregnant heterosexual twin females gave birth single male calves. Gel electrophoresis results and sequencing results in PCR, fertility and relative content of *SRY* are shown in Fig. 1 and Table 2. Based on Fig. 1, samples No. 3, No. 5, and No. 6, negative samples (F1 & F2) and blank control (CT) were evaluated as negative clearly, while samples No. 1 and No. 11 could not be judged as negative or positive with naked eye, others were obviously positive. However, only No. 3 and No. 6 were verified as fertile heterosexual twin females, whereas No. 5 was verified as sterile after natural mating for several times even though it was shown to be negative in PCR (Fig. 1).

On the other hand, qPCR results showed that the relative content of *SRY* varied from 14.2% to 88.9% in freemartins, whereas 0.09% and 0.41% in fertile heterosexual twin females (Table 2).

Concentration of H-Y antigen was showed in Table 2. Average concentration of H-Y antigen in freemartin was 1.30 pg/mL, with a maximum of 1.89 pg/mL and minimum of 1.01 pg/mL, while 1.00 and 1.03 pg/mL in fertile heterosexual twin females, respectively.

DISCUSSION

All animals were on same feeding regime for two years. Environment and housing condition were also same throughout the experimental period. All heterosexual twin

Table 2 Detection results of amplifying *SRY* gene by PCR or qPCR and H-Y antigen determination.

Sample No.	*SRY* in electrophoresis[1]	Sequencing alignment[2]	Relative content of *SRY*[3] (%)	H-Y antigen (pg/mL)	Fertility[4]
1	Unknown	Consistent	20.7	1.89	N
2	Positive	Consistent	61.4	1.26	N
3	Negative	No product	0.41	1.03	Y
4	Positive	Consistent	41.4	1.04	N
5	Negative	Consistent	18.5	1.17	N
6	Negative	No product	0.09	1.00	Y
7	Positive	Consistent	59.0	1.28	N
8	Positive	Consistent	61.5	1.06	N
9	Positive	Consistent	53.6	1.04	N
10	Positive	Consistent	14.2	1.52	N
11	Unknown	Consistent	25.0	1.01	N
12	Positive	Consistent	88.9	1.69	N
M[5]	Positive	Consistent	100	37.1	Y
F[6]	Negative	No product	0	1.00	Y

Notes:
[1] Result of positive and negative means the band of sample could be judged precisely as presence and absence, respectively. While result of unknown means the band of sample could not be judged precisely as negative or positive with the naked eye.
[2] Sequencing alignment result of consistent means amplification sequence of sample is consistent with target sequence of *SRY*, and no product means no synthesis is detected in Sanger sequencing.
[3] Relative content of *SRY* was calculated in accordance to the standard of normal bull as 100%.
[4] Fertility was verified by calving or not after natural mating for several times over two years. Y represents fertile and N represents sterile.
[5] M means bull with normal fertility, serving as the positive control, and content of H-Y antigen was the mean of three normal Holstein bulls.
[6] F means cow with normal fertility, serving as the negative control, and content of H-Y antigen was the mean of three normal Holstein cows.

females had free access to mating after estrus. Therefore, it is excludable that subfertile female that requires more mating than female with normal reproduction would appear in our experiment. The obvious differences of qPCR results between freemartin and fertile heterosexual twin female indicate that quantifying *SRY* gene by qPCR technique might be a more accurate method for diagnosing freemartin. However, more cases are still needed to confirm the exact value of relative content of *SRY* between freemartin and fertile heterosexual twin female.

The frequency of XY cells in freemartin ranged from 2% to 99% in cytogenetic analysis (*Peretti et al., 2008*). However, improper diagnosis could occur if the frequency of XY cells in the heterosexual twin female is less than 5% in cytogenetic analysis (*Eldridge & Blazak, 1977*), and much below 1 in 500 by PCR-based assays (*Mcniel et al., 2006*). In our study, the relative *SRY* content of freemartin varied from 14.2% to 88.9%. This made the criterion ambiguous because congruent relationship between frequency of XY cells and relative content of *SRY* remained unknown. Previous reports have shown the relative proportion of XY to XX in karyotypes of freemartins was not related with the degree of masculinization (*Peretti et al., 2008*; *Kozubska-Sobocinska et al., 2011*; *Szczerbal et al., 2014*). It has also been reported that the proportion of XY cells had no relation to the inhibition degree of ovary or of Müllerian duct (*Vigier, Prepin & Jost, 1972*). However, no

report has shown the relationships between fertility and degree of masculinization in heterosexual twin female, thus the relationship between XY cells and fertility of heterosexual twin female remains unknown. Our data showed the presence of XX/XY chimera in fertile heterosexual twin female, indicating low-level of *SRY* would not influence fertility of bovine heterosexual twin female. Moreover, previous researchers have proven that XY to XX karyotype ratio was stable afterbirth for freemartin (*Greene, Dunn & Foote, 1977*; *Pessamorikawa, Niku & Iivanainen, 2004*). Therefore, our results could be applied equally to the newborn calf.

Low levels of *SRY* represents the low possibility of gonadal dysplasia. Primordial-gonad cells of fetus are in a bipotential status after conception (*Ottolenghi et al., 2007*; *Norling et al., 2013*; *Piprek et al., 2017*), which means the possibility to differentiate to either male cells or female cells. Testis-determining factor encoded by the *SRY* gene initiates testis differentiation of male, mainly via upregulating *SOX9* and *FGF9* (*Hiramatsu et al., 2009*; *Moniot et al., 2009*). The bipotential-gonad cells begin to differentiate into sertoli cells and leydig cells once *SOX9* reached proper levels (*Shoemaker et al., 2007*; *Benko et al., 2011*), resulting in formation of the testis (*Harikae et al., 2012*). In that situation, low-level of *SRY* is inadequate to initiate testis differentiation because of insufficient *SOX9*. Therefore, bipotential-gonad cells would not differentiate into sertoli cells and leydig cells which are vital for males' cell differentiation and proliferation (*Rebourcet et al., 2014*; *Ryan, 2014*). Anastomoses of two opposite-sex fetuses occur after the critical period of reproductive organ differentiation may lead to low-level of *SRY* (*Szczerbal et al., 2014*).

Interestingly, we observed certain H-Y antigen in all heterosexual twin females without any significant numerical difference between freemartin and fertile heterosexual twin female (Table 2). H-Y antigen was previously considered as male-specific cell-surface proteins (*Muller, 1996*). One possible explanation for the existing results is that H-Y antigen of the male fetus delivers to his co-twin female fetus via blood cells during the fetus period. The lack of numerical differences between freemartin and fertile heterosexual twin female indicated that quantitative detection of H-Y antigen was not suitable for selecting fertile heterosexual twin female. Our findings are in contradict with previous reported studies that a higher proportion of Y/X expressed more H-Y antigen (*Wachtel et al., 1975*; *Fraccaro et al., 1982*). Theoretically, concentration of H-Y antigen presents descending trend in normal bull, freemartin, and fertile heterosexual twin female. However, we only found that concentration of H-Y antigen in normal bull was higher numerically than freemartin and fertile heterosexual twin female, no visible difference between freemartin and fertile heterosexual twin female. Current results indicate that slight H-Y antigen would not hamper reproduction of a heterosexual twin female. The presence of H-Y antigen in fertile heterosexual twin female could be due to transfer of H-Y antigen to female fetus when hematopoietic tissue and cell interchange after vascular anastomoses during fetus period (*Niku et al., 2007*; *Abuelo, 2009*; *Kozubska-Sobocińska, Danielakczech & Rejduch, 2016*). It has been reported that H-Y antigen is activated during the formation of testes rather than triggering the formation (*Wolf, 1998*). Therefore, H-Y antigen not plays a decisive role in development of testis, and its presence not always leads to masculinization in female. *Wachtel et al. (1976)* found that H-Y antigen also

existed in women with XX/XY chimera as well as in XX true hermaphrodites, and reported that H-Y antigen expressing in the blood not hamper the presence of ovaries in women with XX/XY chimera or hermaphrodite (*Wachtel et al., 1976*; *Wachtel, 1977*). It can be hypothesized that concentration of H-Y antigen in fertile heterosexual twin female may be nearly the same or slightly lower than that of freemartin.

An alternative assumption that cannot be ruled out is that discrete sample size hinders the difference of H-Y antigen between freemartin and fertile heterosexual twin female. More heterosexual twin females are needed to eliminate individual variability.

To the best of the authors' knowledge, except for the qualitative experiment reporting in 2012 applying qPCR to quantifying *BRY4* gene to distinguish freemartin from fertile heterosexual twin female via characteristic fluorescence curve (*Artigas et al., 2012*), this is the first time we applied qPCR to diagnosing freemartin by quantifying *SRY* gene. Through this method, we got the relative *SRY* content of each freemartin and fertile heterosexual twin female. We declare that low-levels of *SRY* would not influence fertility of bovine heterosexual twin female.

CONCLUSION

In summary, we concluded that quantifying *SRY* gene by qPCR is a better detection method for the diagnosis of bovine freemartin compared with PCR or quantitative detection of H-Y antigen. Freemartin contains high relative content of *SRY*, whereas a fertile heterosexual twin female contains slight relative content of *SRY*. Our results indicate that low-levels of *SRY* would not influence fertility of a bovine heterosexual twin female. Further research is needed to confirm a potential cutoff value of relative content of *SRY* at different growth stages before puberty to make a strategic decision for a heterosexual twin female.

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ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Qinghua Qiu conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Taoqi Shao performed the experiments, authored or reviewed drafts of the paper, approved the final draft.
- Yang He analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Aziz-Ur-Rahman Muhammad analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Binghai Cao conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Huawei Su conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

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 of the National Institutes of Health of China (Permit Number: DK1008).

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in the Supplemental File.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4616#supplemental-information.

REFERENCES

Abuelo D. 2009. Clinical significance of chimerism. *American Journal of Medical Genetics Part C Seminars in Medical Genetics* **151C(2)**:148–151 DOI 10.1002/ajmg.c.30213.

Artigas R, Gagliardi R, Llambí S, Udela R. 2012. Real time PCR technique applied to the study of intersexed animals (bovines and canines). *Revista Electronica de Veterinaria* **13**:1–5.

Ayalavaldovinos MA, Galindogarcía J, Sánchezchiprés D, Duifhuisrivera T, Lemusflores C. 2014. A rapid multiplex PCR method for the diagnose of freemartin syndrome in domestic cattle (*Bos taurus*). *Advances in Animal & Veterinary Sciences* **2(2)**:120–123 DOI 10.14737/journal.aavs/2014/2.2.120.123.

Benko S, Gordon CT, Mallet D, Sreenivasan R, Thauvinrobinet C, Brendehaug A, Thomas S, Bruland O, David M, Nicolino M. 2011. Disruption of a long distance regulatory region

upstream of *SOX9* in isolated disorders of sex development. *Journal of Medical Genetics* **48(12)**:825–830 DOI 10.1136/jmedgenet-2011-100255.

Biswas J, Biswas S, Pan S, Mandal A. 2015. A cytogenetic study of heterosexual quadruplets of cattle (*Bos indicus*)—a case report. *Veterinary Archives* **85**:105–110.

Cabianca G, Rota A, Cozzi B, Ballarin C. 2007. Expression of AMH in female fetal intersex gonads in the bovine. *Anatomia, Histologia, Embryologia: Journal of Veterinary Medicine Series C* **36(1)**:24–26 DOI 10.1111/j.1439-0264.2006.00713.x.

Capel B, Coveney D. 2004. Frank Lillie's freemartin: illuminating the pathway to 21st century reproductive endocrinology. *Journal of Experimental Zoology Part A Comparative Experimental Biology* **301A(11)**:853–856 DOI 10.1002/jez.a.107.

Dunn HO, Johnson RH Jr, Quaas RL. 1981. Sample size for detection of Y-chromosomes in lymphocytes of possible freemartins. *Cornell Veterinarian* **71**:297–304.

Eldridge FE, Blazak WF. 1977. Chromosomal analysis of fertile female heterosexual twins in cattle. *Journal of Dairy Science* **60(3)**:458–463 DOI 10.3168/jds.S0022-0302(77)83888-5.

Esteves A, Båge R, Payan-Carreira R. 2012. Freemartinism in cattle. In: Mendes RE, ed. *Ruminants: Anatomy, Behavior and Diseases.* New York: Nova Science Publishers Inc, 99–120.

Fraccaro M, Mayerová A, Wolf U, Bühler E, Gebauer J, Gilgenkrantz S, Lindsten J, Curto FL, Ritzen EM. 1982. Correlation between the number of sex chromosomes and the H-Y antigen titer. *Human Genetics* **61(2)**:135–140 DOI 10.1007/Bf00274203.

Freeman G. 2007. Explaining the freemartin: Tandler and Keller vs. Lillie and the question of priority. *Journal of Experimental Zoology Part B Molecular & Developmental Evolution* **308B(2)**:105–112 DOI 10.1002/jez.b.21151.

Fujishiro A, Kawakura K, Miyake YI, Kaneda Y. 1995. A fast, convenient diagnosis of the bovine freemartin syndrome using polymerase chain reaction. *Theriogenology* **43(5)**:883–891 DOI 10.1016/0093-691x(95)00039-b.

Gonelladiaza AM, Duarte LZ, Dominguez S, Salazar PA. 2012. Abnormal position of lymph nodes in a freemartin sheep. *Veterinary Medicine Research & Reports* **2012(3)**:1–6 DOI 10.2147/VMRR.S28051.

Greene WA, Dunn HO, Foote RH. 1977. Sex-chromosome ratios in cattle and their relationship to reproductive development in freemartins. *Cytogenetics & Cell Genetics* **18(2)**:97–105 DOI 10.1159/000130753.

Harikae K, Tsunekawa N, Hiramatsu R, Toda S, Kurohmaru M, Kanai Y. 2012. Evidence for almost complete sex-reversal in bovine freemartin gonads: formation of seminiferous tubule-like structures and transdifferentiation into typical testicular cell types. *Journal of Reproduction & Development* **58(6)**:654–660 DOI 10.1262/jrd.2012-070.

Harley VR, Clarkson MJ, Argentaro A. 2003. The molecular action and regulation of the testis-determining factors, *SRY* (sex-determining region on the Y chromosome) and *SOX9* (*SRY*-related high-mobility group (HMG) box 9). *Endocrine Reviews* **24(4)**:466–487 DOI 10.1210/er.2002-0025.

Hiramatsu R, Harikae K, Tsunekawa N, Kurohmaru M, Kanai Y. 2009. 09-P100 FGF9 Signaling is crucial for a center-to-pole expansion of tubulogenesis in mouse testis differentiation. *Mechanisms of Development* **126**:S180 DOI 10.1016/j.mod.2009.06.430.

Hirayama H, Katagiri S, Kageyama S, Minamihashi A, Moriyasu S, Sawai K, Onoe S, Takahashi Y. 2007. Rapid sex chromosomal chimerism analysis in heterosexual twin female calves by loop-mediated isothermal amplification. *Animal Reproduction Science* **101(1–2)**:38–44 DOI 10.1016/j.anireprosci.2006.09.006.

Jeon BG, Rho GJ, Betts DH, Petrik JJ, Favetta LA, King WA. 2012. Low levels of X-inactive specific transcript in somatic cell nuclear transfer embryos derived from female bovine freemartin donor cells. *Sexual Development* **6(1–3)**:151–159 DOI 10.1159/000334050.

Jiménez R, Barrionuevo FJ, Burgos M. 2013. Natural exceptions to normal gonad development in mammals. *Sexual Development* **7(1–3)**:147–162 DOI 10.1159/000338768.

Kästli F, Hall JG. 1978. Cattle twins and freemartin diagnosis. *Veterinary Record* **102(4)**:80–83 DOI 10.1136/vr.102.4.80.

Khan MZ, Foley GL. 1994. Retrospective studies on the measurements, karyotyping and pathology of reproductive organs of bovine freemartins. *Journal of Comparative Pathology* **110(1)**:25–36 DOI 10.1016/S0021-9975(08)80267-8.

Kitahara G, Ali HE-S, Teh A, Hidaka Y, Haneda S, Mido S, Yamaguchi R, Osawa T. 2018. Characterization of anti-Müllerian hormone in a case of bovine male pseudohermaphroditism. *Reproduction in Domestic Animals* DOI 10.1111/rda.13149 [Epub ahead of print 9 February 2018].

Kitahara G, Kamimura S, Hamana K, Yoh-Ichi M. 2002. Cytogenetical, endocrinological, and histological analyses of a single-birth freemartin. *Journal of the Japan Veterinary Medical Association* **55(8)**:494–497 DOI 10.12935/jvma1951.55.494.

Kozubska-Sobocińska A, Danielakczech B, Rejduch B. 2016. Cytogenetic and molecular diagnostics of XX/XY chimerism in cattle, sheep, and goats—a review. *Annals of Animal Science* **16(4)**:989–1005 DOI 10.1515/aoas-2016-0028.

Kozubska-Sobocinska A, Rejduch B, Słota E, Sysa PS. 2011. New aspects of degenerative changes in reproductive system of freemartin heifers. *Annals of Animal Science* **11**:229–239.

Loriaux DL. 2016. Alfred jost (1916–1991). In: Lynn Loriaux D, ed. *A Biographical History of Endocrinology*. Hoboken: John Wiley & Sons Inc, 424–425.

Mcniel EA, Madrill NJ, Treeful AE, Buoen LC, Weber AF. 2006. Comparison of cytogenetics and polymerase chain reaction based detection of the amelogenin gene polymorphism for the diagnosis of freemartinism in cattle. *Journal of Veterinary Diagnostic Investigation* **18(5)**:469–472 DOI 10.1177/104063870601800508.

Moniot B, Declosmenil F, Barrionuevo F, Scherer G, Aritake K, Malki S, Marzi L, Cohen-Solal A, Georg I, Klattig J. 2009. The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. *Development* **136(11)**:1813–1821 DOI 10.1242/dev.032631.

Müller U. 1996. H-Y antigens. *Human Genetics* **97(6)**:701–704 DOI 10.1007/s004390050122.

Nelson JL. 2002. Pregnancy and microchimerism in autoimmune disease: protector or insurgent? *Arthritis & Rheumatism* **46(2)**:291–297 DOI 10.1002/Art.501.

Niku M, Pessamorikawa T, Taponen J, Iivanainen A. 2007. Direct observation of hematopoietic progenitor chimerism in fetal freemartin cattle. *BMC Veterinary Research* **3(1)**:29 DOI 10.1186/1746-6148-3-29.

Norling A, Hirschberg AL, Iwarsson E, Wedell A, Barbaro M. 2013. *CBX2* gene analysis in patients with 46, XY and 46, XX gonadal disorders of sex development. *Fertility & Sterility* **99(3)**:819–826 DOI 10.1016/j.fertnstert.2012.11.016.

Ottolenghi C, Uda M, Crisponi L, Omari S, Cao A, Forabosco A, Schlessinger D. 2007. Determination and stability of sex. *Bioessays* **29(1)**:15–25 DOI 10.1002/bies.20515.

Padula AM. 2005. The freemartin syndrome: an update. *Animal Reproduction Science* **87(1–2)**:93–109 DOI 10.1016/j.anireprosci.2004.09.008.

Peretti V, Ciotola F, Albarella S, Paciello O, Dario C, Barbieri V, Iannuzzi L. 2008. XX/XY chimerism in cattle: clinical and cytogenetic studies. *Sexual Development* **2(1)**:24–30 DOI 10.1159/000117716.

Pessamorikawa T, Niku M, Iivanainen A. 2004. Persistent differences in the level of chimerism in B versus T cells of Freemartin cattle. *Developmental & Comparative Immunology* **28(1)**:77–87 DOI 10.1016/s0145-305x(03)00104-6.

Piprek RP, Kolasa M, Podkowa D, Kloc M, Kubiak JZ. 2017. Cell adhesion molecules expression pattern indicates that somatic cells arbitrate gonadal sex of differentiating bipotential fetal mouse gonad. *Mechanisms of Development* **147**:17–27 DOI 10.1016/j.mod.2017.07.001.

Rebourcet D, O'Shaughnessy PJ, Monteiro A, Milne L, Cruickshanks L, Jeffrey N, Guillou F, Freeman TC, Mitchell RT, Smith LB. 2014. Sertoli cells maintain leydig cell number and peritubular myoid cell activity in the adult mouse testis. *PLOS ONE* **9(8)**:e105687 DOI 10.1371/journal.pone.0105687.

Refaat D, Ali A, Tharwat M. 2015. Ectopic kidney and lymph nodes and intra-abdominal testicular structure in a freemartin Holstein Friesian calf. *Theriogenology Insight* **5(1)**:47–52 DOI 10.5958/2277-3371.2015.00005.4.

Remnant JG, Lea RG, Allen CE, Huxley JN, Robinson RS, Brower AI. 2014. Novel gonadal characteristics in an aged bovine freemartin. *Animal Reproduction Science* **146(1–2)**:1–4 DOI 10.1016/j.anireprosci.2014.02.011.

Ron M, Porat B, Band MR, Weller JI. 2010. Chimaerism detection in bovine twins, triplets and quadruplets using sex chromosome-linked markers. *Animal Genetics* **42(2)**:208–211 DOI 10.1111/j.1365-2052.2010.02099.x.

Rota A, Ballarin C, Vigier B, Cozzi B, Rey R. 2002. Age dependent changes in plasma anti-Müllerian hormone concentrations in the bovine male, female, and freemartin from birth to puberty: relationship between testosterone production and influence on sex differentiation. *General & Comparative Endocrinology* **129(1)**:39–44 DOI 10.1016/S0016-6480(02)00514-2.

Rubes J, Pinton A, Bonnetgarnier A, Fillon V, Musilova P, Michalova K, Kubickova S, Ducos A, Yerle M. 2009. Fluorescence in situ hybridization applied to domestic animal cytogenetics. *Cytogenetic & Genome Research* **126(1–2)**:34–48 DOI 10.1159/000245905.

Ryan PL. 2014. Endocrine and exocrine function of the bovine testes. In: Richard M, ed. *Bovine Reproduction*. Hoboken: John Wiley & Sons Inc, 1024–1027.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols* **3(6)**:1101–1108 DOI 10.1038/nprot.2008.73.

Shoemaker C, Ramsey M, Queen J, Crews D. 2007. Expression of *Sox9*, Mis, and Dmrt1 in the gonad of a species with temperature-dependent sex determination. *Developmental Dynamics* **236(4)**:1055–1063 DOI 10.1002/dvdy.21096.

Smith GS, Camp SDV, Basrur PK. 1977. A fertile female co-twin to a male calf. *Canadian Veterinary Journal La Revue Vétérinaire Canadienne* **18**:287–289.

Sohn SH, Cho EJ, Son WJ, Lee CY. 2007. Diagnosis of bovine freemartinism by fluorescence in situ hybridization on interphase nuclei using a bovine Y chromosome-specific DNA probe. *Theriogenology* **68(7)**:1003–1011 DOI 10.1016/j.theriogenology.2007.06.022.

Szczerbal I, Kociucka B, Nowackawoszuk J, Lach Z, Jaskowski JM, Switonski M. 2014. A high incidence of leukocyte chimerism (60, XX/60, XY) in single born heifers culled due to underdevelopment of internal reproductive tracts. *Czech Journal of Animal Science* **59(10)**:445–449 DOI 10.17221/7707-cjas.

Vigier B, Prepin J, Jost A. 1972. Absence of correlation between XX/XY chimerism in the liver and the first signs of freemartinism in the calf fetus. *Cytogenetic and Genome Research* **11(2)**:81–101 DOI 10.1159/000130179.

Villagómez DA, Parma P, Radi O, Di GM, Pinton A, Iannuzzi L, King WA. 2009. Classical and molecular cytogenetics of disorders of sex development in domestic animals. *Cytogenetic & Genome Research* **126(1–2)**:110–131 DOI 10.1159/000245911.

Villagómez DA, Pinton A. 2008. Chromosomal abnormalities, meiotic behavior and fertility in domestic animals. *Cytogenetic & Genome Research* **120(1–2)**:69–80 DOI 10.1159/000118742.

Wachtel SS. 1977. H-Y antigen and the genetics of sex determination. *Science* **198(4319)**:797–799 DOI 10.1126/science.335511.

Wachtel SS. 1979. Primary sex determination. *Arthritis & Rheumatism* **22(11)**:1199–1210 DOI 10.1002/art.1780221107.

Wachtel SS, Hall JL, Müller U, Chaganti RSK. 1980. Serum-borne H-Y antigen in the fetal bovine freemartin. *Cell* **21(3)**:917–926 DOI 10.1016/0092-8674(80)90455-9.

Wachtel SS, Koo GC, Breg WR, Elias S, Boyse EA, Miller OJ. 1975. Expression of H-Y antigen in human males with two Y chromosomes. *New England Journal of Medicine* **293(21)**:1070–1072 DOI 10.1056/NEJM197511202932105.

Wachtel SS, Koo GC, Breg WR, Thaler HT, Dillard GM, Rosenthal IM, Dosik H, Gerald PS, Saenger P, New M. 1976. Serologic detection of a Y-linked gene in XX males and XX true hermaphrodites. *New England Journal of Medicine* **295(14)**:750–754 DOI 10.1056/NEJM197609302951403.

Weber J, Rudolph N, Freick M. 2017. Facets of clinical appearance and aetiology in an unusual bovine amorphus globosus. *Anatomia Histologia Embryologia* **46(5)**:502–506 DOI 10.1111/ahe.12286.

Wolf U. 1998. The serologically detected H-Y antigen revisited. *Cytogenetics and Cell Genetics* **80(1–4)**:232–235 DOI 10.1159/000014986.

Zhang T, Buoen LC, Seguin BE, Ruth GR, Weber AF. 1994. Diagnosis of freemartinism in cattle: the need for clinical and cytogenic evaluation. *Journal of the American Veterinary Medical Association* **204**:1672.

Zobel R. 2011. Amorphus globosus and freemartinism in triplets from a Holstein-Friesian cow—a case report. *Theriogenologygy Insight* **1**:105–110.