Case Report
J Vet Sci 2018, 19(6), 858-861 · https://doi.org/10.4142/jvs.2018.19.6.858

Freemartinism in replacement ewe-lambs of the Ripollesa sheep breed

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The freemartinism syndrome affects almost all female calves born as co-twins to male calves, whereas little is known about this phenomenon in female sheep. Within this context, 1,185 ewe-lambs from the Ripollesa sheep breed were genotyped for the presence of *oY1* polymorphism (a non-autosomal region of the Y chromosome). Neither ewe-lambs from single births (856) nor ewe-lambs from all-female multiple births (170) were revealed as freemartins, whereas five of 159 ewe-lambs from multiple births with male co-twins were freemartins (3.15 ± 1.38%). All freemartin ewe-lambs were confirmed by physical examination of external genitalia. The results confirm a low incidence of freemartinism from heterosexual twin pregnancies in Ripollesa sheep.

Keywords: Ripollesa breed, freemartin, sheep

In sheep, it has been reported that twins can affect each other [2,3]. From that perspective, freemartinism is one of the best-known twin-related phenomena, occurring in twins of different sexes, and in which the female twin exhibits imperfect masculinization and becomes sterile. Freemartin status is due to the establishment of placental anastomoses between the fetal membranes of co-twins before sexual differentiation of the fetuses (*i.e.*, early fetal life). Given that gonadal differentiation begins earlier in male than in female fetuses [9], sex-determining hormones from the male co-twin can move to the female fetus and suppress the normal development of female reproductive organs [9,17], as well as produce other morphological changes [7]. Thus, heterosexual twin pregnancies can avoid the freemartinism phenomena when placental anastomoses fail to fuse or when fusion occurs following the critical period of reproductive organ differentiation [9]. The incidence of freemartinism in female calves born as a co-twin to male calves is high (> 90%) [9,19], whereas little is known about this congenital disorder in sheep. Indeed, litters containing male and female lambs were common in sheep flocks, whereas freemartins have been generally considered as relatively rare (~1%), based on some classical studies focused at the population level [9,10,12]. Nevertheless, the overall incidence is highly influenced by the rate of multiple births in the population; note, if the rate of freemartinism is high, this may have a negative effect in sheep populations under selection for litter size. Thus, the incidence of freemartin ewes must be accurately estimated within the cohort of animals at risk (*i.e.*, ewes born from twin births with a male co-twins), in order to provide relevant information to the sheep industry.

The clinical symptoms of this syndrome are accompanied by XX/XY chromosomal chimerism in lymphocytes, which are typically examined using a polymerase chain reaction-based freemartin diagnosis method [14,19]. Within this context, the main objective of this short report focused on the detection of freemartin ewe-lambs in the Ripollesa sheep breed. Detailed data about birth type and the sex of the remaining siblings (if any) were available for all genotyped individuals. The study produced an accurate characterization of the incidence of freemartinism in Ripollesa sheep ewe-lambs with male co-twins, avoiding underestimations due to animals born from single births or female-only multiple births.

Between 2013 and 2016, and within the context of a paternity testing program for Ripollesa sheep [6], blood samples from 143 ram-lambs and 1,185 ewe-lambs were collected in 5 mL K3-EDTA Vacutainer tubes (BD, USA) by jugular venipuncture. The samples were marked, registered in a Ripollesa paternity testing data set, and kept frozen (−20°C) until processing in the laboratory. Relevant data for each individual were obtained from the relevant Ripollesa yield recording scheme and

Received 14 May 2018, Revised 28 Aug. 2018, Accepted 3 Sep. 2018
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pISSN 1229-845X
eISSN 1976-555X

included flock of origin, birth type, and sex of the lamb itself and its co-twin if from a multiple birth. A total of 13 purebred Ripollesa flocks were included in this study. All flocks were managed according to Mediterranean semi-intensive production systems and were grazed in the north-east region of Spain (*i.e.*, Catalonia) at elevations of 0 to 2,000 m above sea level. Differences in nutrition, management, and genetics among flocks were highlighted by the wide range of average litter sizes (1.05−1.55 lambs per litter) among the flocks.

Genomic DNA was extracted from all blood samples by using DanaGen Spin DNA kits (DanaGen-BioTed, Spain) and suspended to a concentration of 50 ng/μL (NanoDrop 2000; Thermo Fisher Scientific, USA). Genotyping was focused on the *oY1* single nucleotide polymorphism (SNP) [15], located in the 5′ region of the *SRY* gene in the non-autosomal region of the Y chromosome [16]. All samples were genotyped in the QuantStudio 12K Flex platform (Applied Biosystems, USA) at the Servei Veterinari de Genètica Molecular (Autonomous University of Barcelona, Bellaterra, Spain) by using custom TaqMan SNP probes (Thermo Fisher Scientific).

Freemartin ewe-lambs were detected in three of the 13 Ripollesa flocks examined. Only five animals exhibited the freemartin condition, and all were born from multiple births with one or more male siblings (three twin births and two triplet births with one and two males, respectively). Thus, indicating an average incidence of freemartinism in ewes with male co-twins of 3.15 ± 1.38% in Ripollesa sheep. Moreover, four of the five freemartin ewes had the adenine allele in the *oY1* SNP (the remaining ewe-lamb from Flock 9 had a cytosine allele). All of the freemartin ewes matched with the genotype of their sire. The freemartin status of each of the five ewes was confirmed by physical examination of external genitalia, each ewe having abnormal characteristics, such as in the shape and size of vulva and clitoris or in vaginal length. Note that the preponderance of the adenine allele was also observed in the Ripollesa ram-lambs (91.61 ± 2.32%; Table 1).

The incidence of freemartinism in the examined Ripollesa ewe-lambs from multiple births with male siblings was low (3.15%), and was within the range of percentages previously reported in the Merino and Clun Forest (1.2%) [5] and Rideau Arcott (4.4%) sheep breeds [1]. Moreover, the freemartinism incidence in the Ripollesa breed matched the estimate reported in a neighboring breed, the Rasa Aragonesa (3.1%) [14]; these results indicate the need for testing ewe-lambs born with co-twin males in order to prevent the selection of infertile individuals as breeding stock. Nevertheless, the incidence of freemartinism from population-based studies could be severely underestimated because of the inclusion of non-at-risk individuals (*e.g.*, ewe-lambs from singleton births or from homosexual twin pregnancies) [12,19]. Within this context, cohort-based studies are preferable in order to limit analyses to at-risk individuals. In our case, only 159 ewe-lambs out of 1,185 (13.42%) were born from a multiple birth with one or more male co-twins; thus, drastically reducing the size of the cohort of animals potentially exposed to freemartin-producing conditions. This prevents underestimating the risk of freemartinism when

Table 1. Number of Ripollesa ewe-lambs sorted by flock and birth type, and the number of ram-lambs by flock number and *oY1* single nucleotide polymorphism genotype

Flock	No. of ewe-lambs by birth type			Ram-lambs by *oY1* genotype	
	Single birth	Multiple birth			
		Female only*	Male sibs†	Adenine	Guanine
1	137 (0)	31 (0)	28 (0)	39	1
2	190 (0)	2 (0)	4 (1)	6	0
3	53 (0)	49 (0)	50 (3)	27	7
4	146 (0)	16 (0)	12 (0)	9	0
5	61 (0)	23 (0)	31 (0)	20	0
6	96 (0)	15 (0)	7 (0)	11	0
7	60 (0)	8 (0)	11 (0)	6	4
8	60 (0)	1 (0)	1 (0)	0	0
9	17 (0)	8 (0)	5 (1)	3	0
10	10 (0)	6 (0)	3 (0)	6	0
11	9 (0)	7 (0)	6 (0)	3	0
12	9 (0)	4 (0)	1 (0)	0	0
13	8 (0)	0 (0)	0 (0)	1	0
Overall	856 (0)	170 (0)	159 (5)	131	12

Number of freemartin ewe-lambs is given in parentheses. *All lambs in the litter were females. †One or more lambs in the litter were males.

females were effectively exposed to male co-twins during gestation, and establishes a framework for this phenomenon in commercial flocks of Ripollesa sheep. Stockbreeders must be cautious about this congenital disorder in prolific flocks in which an approximate 3% incidence of infertile ewe-lambs from male-female twin births may result in a significant number of freemartin ewe-lambs, potentially having important negative economic effects on flock profitability.

It is important to highlight that all rams were genotyped and had hemizygous genotype for the *oY1* SNP (Table 1). This indicated the usefulness of applying a genotyping approach to detect non-autosomal DNA in male samples, and, given that the expected percentage of chimeric lymphocytes in blood samples from freemartin ewes is high (~50%) [4,8,13], the incidence of false negatives must be considered minimal or null. On the other hand, no freemartins were detected among single-birth and all-female twin-birth ewe-lambs (Table 1); thus, almost removing the risk of false negatives. However, some authors have reported freemartins born from single births in cattle [11,18], and they linked them with the fetal death of a male co-twin during gestation. Although this has not been detected in the Ripollesa breed, this phenomenon cannot be disregarded. Further population monitoring is needed to confirm or discard this possibility.

Although the incidence of freemartinism may be biased downwards when estimated at a population level, the phenomenon is not rare in the sheep industry and accounts for an important percentage of ewe-lambs born from multiple births with one or more male siblings (~3%). This should be seen as a significant incidence in moderately-to-highly prolific breeds or flocks, where the chance of selecting multiple birth females with one or more male siblings as future ewes is high. Although some freemartin ewe-lambs can be easily detected and culled by direct inspection of external genitalia, appropriate genotyping for non-autosomal DNA characteristics of ewe-lambs at risk must be viewed as a useful tool within the sheep industry. For example, the inclusion of a Y chromosome marker in current parentage testing panels would not substantially increase laboratory costs, whereas it efficiently tests for unanticipated freemartins. Nevertheless, the economic convenience of laboratory testing for freemartin ewe-lambs must be evaluated in advance in low prolificacy sheep breeds with few candidate ewe-lambs from heterosexual twin pregnancies.

Acknowledgments

The authors are indebted to the Associació Nacional de Criadors d'Oví de Raça Ripollesa (Monells, Spain) for contributing field and genotyping data from its replacement breeding stock.

Conflict of Interest

The authors declare no conflicts of interest

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