

Application of Hormonal and Single Multiplex PCR Assays for Detection of Freemartinism in a Horned Goat

Mehrdad POURJAFAR^{1*}, Khalil BADIEI¹, Hassan SHARIFIYAZDI¹, Seyed Mojtaba NAGHIB¹,
Aliasghar CHALMEH¹, Mohammad Reza DIVAR¹

¹Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Corresponding Author: Mehrdad POURJAFAR Department of Clinical Sciences, School of Veterinary Medicine,
Shiraz University, Shiraz, P.O box: 71345, Iran
e-mail: dmp4m@yahoo.com

Geliş Tarihi / Received: 15.10.2011

ABSTRACT

The present study describes a rare case of freemartinism of the 60XX/60XY chimera type in an adult 7-year-old horned, mixed Lori breed phenotypically doe. Signs of masculinisation were evident in both the behavioral patterns and body conformation, but there was no structure similar to penis and hypospadias was observed in this goat. External genital organs were similar to that of a female one. In this study, a single multiplex PCR assay was used to determine the freemartinism status (XX-XY blood chimerism) based on semi-quantitatively co amplify a sex-based polymorphism in the amelogenin locus (AMX and AMY). In addition, serum concentrations of anti-Müllerian hormone (AMH), testosterone and progesterone hormones of this horned intersex goat were evaluated. Serum AMH levels in the present case (0.2 ng/ml) were similar to male (0.2 ng/ml) and lower than female (0.6 ng/ml) goat. The serum concentrations of testosterone (0.2 ng/ml) were remarkably high in comparison to normal control male and female ones (0.02 ng/ml). Progesterone level in the intersex goat (0.4 ng/ml) was close to control buck (0.7 ng/ml) and remarkably lower than control doe (10 ng/ml). The results of the present study showed that the use of hormonal pattern, especially AMH level, and single multiplex PCR technique provide an easy and alternative approaches for indication of XX/XY chimerism or mosaicism in intersex freemartin goats.

Key Words: Freemartin, XX/XY chimerism, intersex, horned goat, single multiplex PCR, anti-müllerian hormone

ÖZET

BOYNUZLU BİR KEÇİDE FREEMARTİNİZMİN HORMONAL VE TEKLİ MULTİPLEKS PCR ANALİZ UYGULAMALARI İLE TESPİTİ

Bu çalışmada, nadir bir olgu olan freemartinizmin dişi fenotipine sahip Lori ırkı melezi 7 yaşındaki boynuzlu ergin bir keçide 60XX/XY kimera tipi bildirilmektedir. Davranış biçimlerinde ve vücut yapısında maskülinizasyon belirtileri bulunur iken, penis veya hypospadias gözlenmemiştir. Dış genital organlar dişi keçi ile benzerlik göstermektedir. Bu çalışmada amelogenin lokusundaki (AMX ve AMY) cinsiyete bağlı polimorfizmin semi-kantitatif olarak çoğaltılması ile freemartinizm durumunun (XX-XY kan kimerizmi) belirlenmesinde tekli multipleks PCR yöntemi kullanılmıştır. Ayrıca anti-Müllerian hormon (AMH), testesteron ve progesteron hormonlarının serum konsantrasyonları da

değerlendirmeye alınmıştır. Mevcut vakadaki serum AMH düzeyleri (0,2 ng/ml) tekeye benzer (0,2 ng/ml) olarak, dişi keçiden (0,6 ng/ml) ise düşük olarak saptanmıştır. Serum testosteron konsantrasyonu (0,2 ng/ml) kontrol tekeye ve dişi keçiye (0,02 ng/ml) göre önemli düzeyde yüksek bulunmuştur. İnterseks keçide progesteron düzeyi (0,4 ng/ml) kontrol tekedeki düzeye (0,7 ng/ml) yakın, kontrol dişi keçideki düzeyden (10 ng/ml) ise belirgin olarak düşüktür. Mevcut çalışmanın sonuçları interseks freemartin keçilerde XX/XY kimerizmi veya mozaizmini belirlemede AMY düzeyi ve tekli multipleks PCR tekniğinin kolay ve alternatif bir yöntem olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Freemartin, XX/XY kimerizmi, interseks, boynuzlu keçi, tekli multipleks PCR, anti-müllerian hormon

Introduction

Freemartinism is a condition that occurs in twins of different sexes, where an imperfect masculinised sterile female twin is born with a male. The syndrome has been mostly reported for cattle and sheep (Cole et al., 1997; Cribiu and Chaffaux, 1990). In cattle, freemartinism is common because the fetal membranes from twin calves generally fuse, thereby permitting exchange of cells and hormones (testosterone and Müllerian inhibiting substance) between male and female fetuses (Padula, 2005). Twinning is also common in goats, but vascular anastomosis is either uncommon or occurs after the critical period for sexual differentiation (Basrur and Kochhar, 2007). Freemartinism may be considered as a form of intersexuality (Yadav, 1988). Caprine freemartins comprise approximately 6% of intersexes (Christensen et al., 2009). Intersexes are most frequent among polled goats, and the condition is thought to be caused by a recessive gene linked to that for polledness; however, intersexuality may rarely be seen in horned goats (Christensen et al., 2009). Horned intersex goats are rare and they usually present a chimerism 60XX/60XY (Bongso et al., 1982). Chimeras are individuals having cell lines from two different embryonic sources. This can occur experimentally or from the natural fusion of blastocysts in utero (Christensen et al., 2009).

Anti-Müllerian hormone (AMH) is a glycoprotein of 140 KDa belonging to the transforming growth factor beta family that is expressed only in the gonads (Cate et al., 1986). It inhibits the development of the Müllerian ducts (paramesonephric ducts) in the male

embryo (Behringer, 1994). Amounts of AMH that are measurable in the blood vary by age and sex. Presently, AMH is the best endocrine marker of the ovarian follicular reserve in human (van Rooij et al., 2002; Visser et al., 2006), mouse (Kevenaar et al., 2006) and cow (Rico et al., 2009). However, there is no information from the available literature regarding AMH levels in horned freemartin goats. The principal hormone produced by the gonads in caprine intersexes is usually testosterone, which accounts for masculine behavior (Christensen et al., 2009). As with the goats, the progesterone concentrations in the intersex sheep and the ram were never more than 1 ng/mL (Bosu and Basrur, 1984).

Caprine freemartins cannot be distinguished on the basis of external or internal genitalia from polled intersexes (Basrur and Kochhar, 2007). The diagnosis can be confirmed by demonstrating XX-XY blood chimerism (Bosu and Basrur, 1984; Ricordeau, 1981; Smith and Dunn, 1981). Although several diagnostic methods have recently been described for the diagnosis of freemartins in goats (Batista et al., 2000; Hafez et al., 2005; Szatkowska et al., 2004), according to the best of authors' knowledge, analysis of AMH and application of single multiplex PCR assay for detection of intersex goats have not been investigated. The aim of this study was to evaluate the serum concentrations of AMH, testosterone and progesterone along with single multiplex PCR assay for characterization of freemartinism goat with 60XX/60XY chimerism.

Materials and Methods

Animals

An adult 7-year-old horned, mixed Lori breed phenotypically doe was referred to Shiraz University Veterinary Clinic for reproductive examination. The main complaint of the owner was infertility of the goat.

Macroscopic Appearances

Signs of masculinization were evident in both the behavioral patterns and body conformation, especially in the head region but the appearance of its external genitalia was similar to that of a female. Physical examination revealed a narrow vulvar opening and a hypertrophic clitoris, approximately 1.8 cm long and 8 mm in diameter. The vagina was about 4 cm long and blind. Large testes with scrotum were palpated on the ventral part of abdominal wall. The anogenital distance was 1.5 cm and the udder was similar to that of a nulliparous goat. No structure similar to penis was detected and hypospadias was observed in the referred goat. Having been born co-twin to a male was found according to our signalments. A request for euthanasia and necropsy was declined by the owner.

Hormonal determinations

Serum concentrations of testosterone and progesterone were determined with a solid-phase ¹²⁵I radioimmunoassay (Coat-to-Count T kit, Diagnostic Products, Los Angeles, California, USA), according to the manufacturer's instructions. AMH was measured by commercially available Active MIS/AMH ELISA kits DSL (Diagnostic Systems Laboratories Inc., TX, USA). Moreover, blood samplings were performed from a non-pregnant doe and a fertile buck of the same breed, age and weight similar to those of the intersex goat, in order to control hormonal levels and molecular findings.

DNA extraction and PCR amplification

Genomic DNA was extracted from whole blood obtained from intersex freemartinism goat as well as normal male and female goats

using Rapid Genomic DNA Isolation kit (Qiagen DNAeasy) according to the manufacturers' instructions. The DNA was quantified spectrophotometrically and the integrity was assessed via agarose gel electrophoresis (0.8%). DNA was stored at -20°C for subsequent analysis.

The X and Y chromosome linked AMX and AMY gene (Ennis and Gallagher, 1994; Ennis et al., 1999; McNiel et al., 2006) was chosen as markers for diagnosis of freemartinism (XX/XY chimerism) in the goat. As previously described (Ennis and Gallagher, 1994; Ennis et al., 1999; McNiel et al., 2006) the forward primer SE 47 (5'-CAGCCAAACCTCCCTCTGC-3') and reverse primer SE 48 (5'-CCCGCTTGGTCTTGTCTGTTGC-3') were used for PCR amplification. This PCR technique amplifies a 262-bp fragment from the X chromosome and a 202-bp fragment from the Y chromosome in goat base on accession no. DQ469588 and DQ469589, respectively. All oligonucleotide primers used in this study were synthesized by Cinnagen Co. in Iran. DNA amplification was performed in a 25- μ l volume using thermal cycler (MG 5331, Eppendorf, Hamburg). The following PCR conditions were applied to each assay: 50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 1.5 mM MgCl₂, 200 μ M dNTPs, 10 pM of each primer, 1.25 U Taq DNA polymerase (Fermentas) and 2 μ l of template DNA. After the initial denaturation of template DNA at 94°C for seven min, the PCR profile was as follows: 35 cycles of 40 s of template denaturation at 94°C; 60 s of primer annealing at 64.7°C and 40 s of primer extension at 72°C; with a final extension at 72°C for five min. The presence of PCR products was determined by electrophoresis of 5 μ l of reaction product in a 3% agarose gel in TAE (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) electrophoresis buffer and were visualized by staining with ethidium bromide (0.5 μ g/ml) under UV light. Images were captured on a computer. Sterile water was used as the negative control, and samples were tested at least in duplicate.

Results

Serum concentrations of AMH, testosterone and progesterone of the control (male and female) and intersex goats are shown in Table 1. Level of AMH in the intersex goat was clearly similar to control buck. The serum concentration of testosterone was remarkably high in comparison to control ones. Progesterone level in the intersex goat was close to control buck and remarkably lower than control doe.

Two expected PCR product sizes of 262-bp fragment from the X chromosome and a 202-bp fragment from the Y chromosome were produced in intersex freemartinism goat as well as for normal male goat (Figure 1). Interestingly, a significant reduction in relative amounts of Y product (202 bp) was observed in freemartin goat as compared to normal male (Figure 1).

Table 1. Serum concentrations of testosterone, progesterone and AMH in male, female and intersex goats.

Tablo 1. Teke, dişi keçi ve interseks keçide testosteron, progesteron ve AMH serum konsantrasyonları.

Hormone (ng/ml)	Male	Female	Intersex
AMH	0.20	0.60	0.20
Testosterone	0.02	0.02	0.20
Progesterone	0.70	10.00	0.40

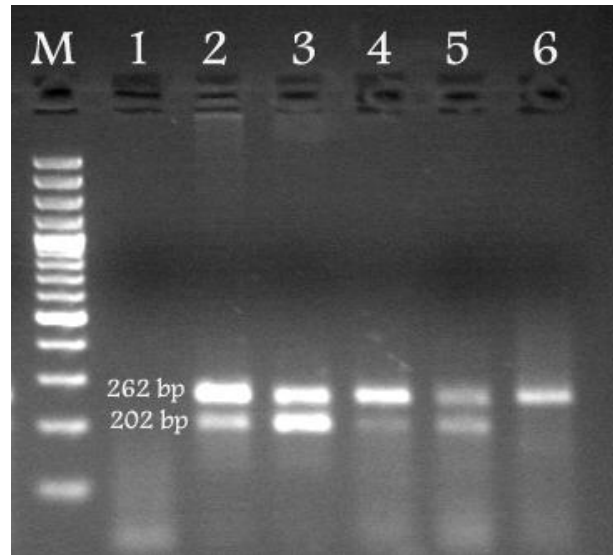


Figure 1. Gel analysis of the PCR amplification products from freemartinism (XX/XY chimerism), normal male and female goats.

Şekil 1. Freemartinizm (XX/XY kimerizmi), normal tekelerde ve dişi keçilerde PCR amplifikasyon ürünlerinin jel analizi.

A 100 bp ladder is used as a size marker (Lane M). An additional 202-bp band were found in freemartinism goat (Lane; 2 and 4) compared to normal female (Lane; 6) which produced only a 262-bp fragment from the X chromosome. However, a significant reduction in relative amounts of Y product (202-bp) was obviously observed in freemartinism (Lane; 2 (DNA=10 pg/PCR reaction); Lane 4 (DNA=1 pg/PCR reaction) compared to normal male (Lane; 3 (DNA=10 pg/PCR reaction); Lane; 5 (DNA=1 pg/PCR reaction)).

Discussion

In the present study, we applied an efficient molecular method (single multiplex PCR) for the diagnosis of freemartinism (XX/XY chimerism) in a goat in Iran. The PCR based testing has been explored as an alternative to cytogenetic examination for the detection of freemartinism. One such technique, described by Ennis and Gallagher (1994), is based on a polymorphism associated with the bovine amelogenin gene (AMX/Y). Cytogenetic analysis of cultured lymphocytes has been traditionally used to identify XY cells in suspected freemartins. Cytogenetic testing is thought to be 95%–99% accurate when 100 mitotic cells are studied (Dunn and Johnson, 1981). In addition, cytogenetic analysis for freemartinism allows detection of other reproductive problems, such as chromosomal centric fusions (Seguin et al., 2000). However, there are limitations to cytogenetic testing. Furthermore, cytogenetic testing requires extremely careful collection and handling of samples, is labor intensive, and requires cytogenetics expertise that is not readily available at most diagnostic centers (McNiel et al., 2006). PCR based testing has been explored as an alternative to cytogenetic examination for the detection of freemartinism (Fujishiro et al., 1995). Although a variety of PCR-based techniques can be used for sexing animals, an advantage of this technique is that a single pair of oligonucleotide primers is used to amplify both the AMX and AMY alleles; thus PCR failure cannot be mistaken as a negative test result for freemartinism (Seguin et al., 2000).

The AMX/Y allele residing on the Y chromosome contains a 60-bp deletion in the fifth exon when compared to the AMX/Y allele residing on the X chromosome (McNiel et al., 2006). Because a freemartin carries both XX and XY cells, this PCR technique can be used to diagnose this condition. In a previous study conducted in cattle (McNiel et al., 2006), showed that this diagnostic PCR assay is more sensitive than cytogenetic analysis for detection of XY cells in freemartinism in cattle. Semi-

quantitation was facilitated by use of a single pair of oligonucleotide primers which recognizes both templates (262-bp and 202-bp) during amplification; thus, PCR failure cannot be cause a false-negative result for freemartinism. In the current study, diagnostic value of this approach was also evaluated using DNA obtained from intersex freemartinism goat compared to normal male and female goats (Figure 1). Results showed that the proposed technique provides a suitable way for indication of XX/XY chimerism or mosaicism in intersex freemartin goats.

In female mammals, AMH is specifically expressed by granulosa cells and it has been found to be a highly reliable endocrine marker of the size of the ovarian pool of growing follicles in goats. Follicles of 1–5 mm in diameter had higher AMH expression in their granulosa cells and higher concentrations of AMH in follicular fluid compared with larger follicles. Moreover, the number of follicles of this size class was highly related to AMH concentrations in plasma and suggests that this follicular population is a major contributor to circulating AMH concentrations in goats (Monniaux et al., 2011). Measurement of AMH concentrations in blood can help to predict the capacity of goats to respond to superovulatory treatment and produce high or low number of embryos, so AMH is a landmark for reproductive performance of goats. Circulating AMH concentrations can be predictive of the ovulatory activities in goats. Also, in embryo transfer technique, AMH can be considered as an excellent candidate for the endocrine prediction of the capacity of a donor goat to produce high or low number of embryos (Monniaux et al., 2011).

According to our results, we found that plasma concentrations of AMH in normal male and the freemartin goat of the same age were equal (0.2 ng/ml). It is possible that low level of the AMH reflects changes in the proportions of primary follicles. So it could be concluded that our intersex goat had no active ovary.

Rota et al. (2002) showed that the plasma concentrations of AMH were very high in males and in freemartins calves at birth. AMH remains high in males for the first five months of life, but in the freemartins, it rapidly decreases to normal female levels, showing that AMH comes from the male twin.

Plasma testosterone concentration of freemartins was found to be indistinguishable from normal heifers (<10 pg/ml) and not useful for diagnosis (Saba et al., 1975). In young bulls, the trend of plasma testosterone concentrations is opposite to that of the AMH. The rise in testosterone production at puberty corresponds to a sharp decline in AMH concentrations (Rota et al., 2002). The serum concentration of testosterone in freemartin goat of our study was remarkably higher (0.2 ng/ml) than normal one (0.02 ng/ml). The present case and other infertile intersex goats can be used as teasers because the principal hormone produced by the gonads in caprine intersexes is usually testosterone (Christensen et al., 2009), although genetically they are female.

Progesterone concentrations in plasma of freemartins have been reported to fluctuate around baseline levels (<0.4 ng/ml) with occasional surges up to 1.6 ng/ml (Saba et al., 1975). The levels obviously depend upon the presence of functional luteal tissue, so according to our result, it could be concluded that low progesterone level (0.4 ng/ml) may be due to inactive ovarian (gonadal) tissue.

The results of the present study showed that the use of hormonal pattern especially AMH level and single multiplex PCR technique provide an easy and alternative approaches for indication of XX/XY chimerism or mosaicism in intersex freemartin goats.

REFERENCES

- Basrur, P.K., Kochhar, H.S., 2007.** Inherited Sex Abnormalities in Goats. In: Youngquist, R.S., Threlfall, W.R. (Eds.), *Current Therapy in Large Animal Theriogenology*, 2nd Ed. Saunders, USA, pp. 590-594.
- Batista, M., Gonzalez, F., Cabrera, F., Palomino, E., Castellano, E., Calero, P., Gracia, A., 2000.** True hermaphroditism in a horned goat with 6OXX/6OXY chimerism. *Canadian Veterinary Journal* 41, 562-564.
- Behringer, R.R., 1994.** The in vivo roles of müllerian-inhibiting substance. *Current Topics in Developmental Biology* 29, 171-187.
- Bongso, T.A., Thavalingam, M., Murkherjee, T.K., 1982.** Intersexuality associated with XX/XY mosaicism in horned goat. *Cytogenetics and Cell Genetics* 34, 315-319.
- Bosu, W.T.K., Basrur, P.K., 1984.** Morphological and hormonal features of an ovine and a caprine intersex. *Canadian Journal of Comparative Medicine* 48, 402-409.
- Cate, R.L., Mattaliano, R.J., Hession, C., Tizard, R., Farber, N.M., Cheung, A., Ninfa, E.G., Frey, A.Z., Gash, D.J., Chow, E.P., 1986.** Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45, 685-698.
- Christensen, B.W., Drost, M., Troedsson, M.T., 2009.** Diseases of the Reproductive System. In: Smith, B.P. (Ed.), *Large Animal Internal Medicine*, 4th Ed. Elsevier, pp. 1427-1470.
- Cole, J., Broadwell, M., Rogers, G., 1997.** Intersexuality in a Charolais heifer. *Veterinary Record* 141, 656-657.
- Cribiu, E.P., Chaffaux, S., 1990.** Intersexuality in domestic mammals. *Reproduction Nutrition Development Supplement* 1, 51-61.
- Dunn, H.O., Johnson, R.H., 1981.** Sample size for detection of Y chromosomes in lymphocytes of possible freemartins. *Cornell Veterinary Medicine* 71, 297-304.
- Ennis, S., Gallagher, T.F., 1994.** A PCR-based sex-determination assay in cattle based on the bovine amelogenin locus. *Animal Genetics* 25, 425-427.
- Ennis, S., Vaughan, L., Gallagher, T.F., 1999.** The diagnosis of freemartinism in cattle using sex-specific DNA sequences. *Research in Veterinary Science* 67, 111-112.
- Fujishiro, A., Kawakura, K., Miyake, Y.I., Kaneda, Y., 1995.** A fast, convenient diagnosis of the bovine freemartin syndrome using polymerase chain reaction. *Theriogenology* 43, 883-891.

- Hafez, S.A., Huckle, W.R., Caceci, T., 2005.** Anatomical, histological and genetic investigations of a sexually anomalous goat. *Veterinary Record* 157, 513-516.
- Kevenaer, M.E., Meerasahib, M.F., Kramer, P., van de Lang-Born, B.M., de Jong, F.H., Groome, N.P., Themmen, A.P., Visser, J.A., 2006.** Serum anti-mullerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology* 147, 3228-3234.
- McNiel, E.A., Madrill, N.J., Treeful, A.E., Buoen, L.C., Weber, A.F., 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, 469-472.
- Monniaux, D., Baril, G.R., Laine, A.L., Jarrier, P., Poulin, N., Cognié, J., Fabre, S., 2011.** Anti-Müllerian hormone as a predictive endocrine marker for embryo production in the goat. *Reproduction* 142, 845-854.
- Padula, A.M., 2005.** The freemartin syndrome: an update. *Animal Reproduction Science* 87, 93-109.
- Rico, C., Fabre, S., Médigue, C., di Clemente, N., Clément, F., Bontoux, M., Touzé, J.L., Dupont, M., Briant, E., Rémy, B., Beckers, J.F., Monniaux, D., 2009.** Anti-mullerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biology of Reproduction* 80, 50-59.
- Ricordeau, G., 1981.** Genetics: Breeding Plans. In: Gall, C. (Ed.), *Goat Production*, New York, Academic Press.
- 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. *General and Comparative Endocrinology* 129, 39-44.
- Saba, N., Cunningham, N.F., Millar, P.G., 1975.** Plasma progesterone, androstenedione and testosterone concentrations in freemartin heifers. *Journal of Reproduction and Fertility* 45, 37-45.
- Seguin, B.E., Zhang, T., Buoen, L.C., Weber, A.F., Ruth, G.R., 2000.** Cytogenetic survey of Holstein bulls at a commercial artificial insemination company to determine the prevalence of bulls with centric fusion and chimeric anomalies. *Journal of the American Veterinary Medical Association* 216, 65-67.
- Smith, M.C., Dunn, H.O., 1981.** Freemartin condition in a goat. *Journal of the American Veterinary Medical Association* 178, 735-737.
- Szatkowska, I., Zych, S., Udala, J., Dybus, A., Aszczyk, P.B., Sysa, P., Dabrowski, T., 2004.** Freemartinism: Three cases in goats. *Acta Veterinaria Brno* 73, 375-378.
- van Rooij, I.A., Broekmans, F.J., te Velde, E.R., Fauser, B.C., Bancsi, L.F., de Jong, F.H., Themmen, A.P., 2002.** Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Human Reproduction* 17, 3065-3071.
- Visser, J.A., de Jong, F.H., Laven, J.S., Themmen, A.P., 2006.** Anti-Mullerian hormone: a new marker for ovarian function. *Reproduction* 131, 1-9.
- Yadav, B.R., 1988.** Freemartinism in goat. *Indian Journal of Animal Science* 58, 123-126.