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Sex determination of ovine embryos by SRY and *amelogenin* (*AMEL*) genes using maternal circulating cell free DNA

Adel Saberivand[a,*], Sima Ahsan[b]

[a] *Division of Theriogenology, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran*
[b] *Graduate in Veterinary Medicine, Urmia University, Urmia, Iran*

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ABSTRACT

Simple and precise methods for sex determination in animals are a pre-requisite for a number of applications in animal production and forensics. Some of the existing methods depend only on the detection of Y-chromosome specific sequences. However, the detection of Y and X-chromosome specific sequences is advantageous. In the present study the accuracy of sex determination by SRY (sex-determining region Y) and AMEL (Amelogenin) gene detection was assessed using a polymerase chain reaction (PCR) of DNA extracted from free fetal cells in maternal blood, which is noninvasive for fetus and easier to collect. The PCR amplification of SRY primers produced a single band of 171 bp from ewes bearing a male fetus, whereas no band was amplified from the DNA extracted from ewes pregnant to a female fetus. Moreover, two bands of 182 and 242 bp in male and a single band of 242 in female fetuses were produced by AMEL gene primers in the PCR reaction. Using this technique 100% of samples were successfully sexed, excluding twins. In conclusion, we demonstrated that sex determination using DNA of free fetal cells in maternal plasma is efficient using both SRY and AMEL gene sequences. It also is evident that this method is not suitable for sex determination of twin pregnancies.

1. Introduction

One of the basic and important elements of animal production is scientific progress on reproductive technologies (Dervishi et al., 2011). On the other hand, fetal sex detection is a key practice in livestock management. It is extremely important in ovine industry to identify lamb gender for breeding, culling and eliminating expenses of the progeny test programs (Kadivar et al., 2013).

Several different methods have been used to determine mammalian prenatal sex. These methods are: trans-rectal ultrasonography (de Freitas Neto et al., 2010), cytogenetic analysis (King, 1984), detection of H-Y antigen (Watchel, 1984), measurement of X-linked enzymes before Barr body formation (Kageyama et al., 2004). The need to harvest cells in metaphase is a limitation of cytological detection. In situ hybridization and fluorescent in situ hybridization (FISH) is time consuming (Dervishi et al., 2011) and the success of trans-rectal ultrasonography is depended on the fetal position, age and the operator's experience (Ali, 2004).

Although the presence of fetal nucleic acids in the maternal circulation was discovered decades ago (Mandel and Métais, 1948), circulating cell free fetal DNA (ccffDNA), was just identified in maternal plasma in late 1990s (Lo et al., 1997) when they correctly identified male fetuses in 80% of the pregnant women carrying male fetus. This

* Corresponding author at: Division of Theriogenology, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tabriz, 29 Bahman Blvd., Tabriz, Iran.
E-mail addresses: a.saberivand@tabrizu.ac.ir, delsaberivand@yahoo.ca (A. Saberivand).

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revolutionary-like event has emphasized that the placenta is no longer thought to be an impermeable membrane (Swarup and Rajeswari, 2007) and provided new approach for prenatal molecular diagnosis such as fetal sex determination, screening for pregnancy-related complications and fetal diseases (Lo et al., 1998; Jimenez and Tarantal, 2003; Maron et al., 2007).

Embryonic sex determination using sex-specific DNA sequences by Polymerase chain reaction (PCR) has been used in cattle (Peura et al., 1991; da Cruz et al., 2012), pigs (Fajfar-Whetstone et al., 1993), humans (Handyside et al., 1989) and mice (Kunieda et al., 1992). Because of acceptable reliability, high sensitivity, inexpensiveness and rapidity, PCR is currently used as genotypic sex determination method (Dervishi et al., 2011).

The main drawback of this method is that the absence of amplification is interpreted as the fetus being female. To overcome this issue, a gene which is present in male and female should be amplified at the same time. Amplification of ZFX/Y region and Restriction Fragment Length Polymorphism analysis (Aasen and Medrano, 1990) and Duplex PCR co-amplifying a specific Y-chromosomal sequence and an autosomal sequence as a control have been used (Appao Rat and Totey, 1999; Mara et al., 2007).

Another issue is the type of ovine placenta. Unlike the humans (hemochorial), sheep has a synepitheliochorial placenta which does not permit a direct contact between the maternal blood and fetal trophoblast (Wooding, 1992). Therefore, the likelihood of the passage of fetal DNA to maternal circulation is very scarce, highlighting the importance of the sensitivity of DNA extraction methods to be used.

It is the presence or absence of the Y chromosome determines the sex of fetus during sexual differentiation. Therefore, the sex determining region, SRY has been the most frequent gene used for sex typing (Piprek, 2010), and seems to have greatest homology within the ruminant species (Pomp et al., 1995).

The amelogenin (AMEL) gene, which exists on both X (AMELX) and Y (AMELY) chromosomes, has been used to determine the sex in cattle (Ennis and Gallager, 1994), sheep and deer (Pfeiffer and Brenig, 2005; Dervishi et al., 2011; Kadivar et al., 2013).

The aim of the present study was to use cell free fetal DNA in pregnant ewe plasma to determine fetal sex in a PCR assay using SRY and amelogenin (AMELX/AMELY) genes.

2. Material and methods

2.1. Blood collection and plasma harvesting

Fifty one mature Ghezel ewes in gestational weeks of 8–20 from private sector and from animals brought to government-owned clinics were used in this study.

In addition, three normal non-pregnant ewes and three normal rams were used as control animals. As a source of ccffDNA, peripheral blood samples were obtained from the animals. A volume of 5 mL of blood samples was collected from jugular vein into the EDTA vacutainer tubes and centrifuged at 1600 × *g* at room temperature for 10 min to separate plasma from packed cells and buffy coat. Subsequently, they were centrifuged at 16,000 × *g* for 10 min to further separate cellular debris. The blood plasma samples were stored at −20 °C until analysis.

2.2. DNA extraction from maternal blood plasma

DNA was extracted from 100 μl plasma using a commercial DNA Purification kit (Sinaclon, Iran) as recommended by the manufacturer. The total DNA extracted from the cells was used as template in PCR for sex determination. The quality and quantity of DNA were determined using Biophotometer (Eppendorf, Germany).

The primers were synthesized (Sinagen, Iran) for SRY and AMEL according to Dervishi et al. (2011) and were as follows; SRY gene; upstream: 5′- GACAATCATAGCGCAAACGA-3′, downstream: 5′-CAGCTGCTTGCTGATCTCTG-3′; AMEL gene; upstream: 5′-CCGCCCAGCAGCCCTTCC-3′, downstream: 5′-CCCGCTTGGTCTTGTCTGTTGC-3′.

Amplification conditions were identical for two genes except the thermal profile as is stated. The amplification reactions were set in a final volume of 25 μl containing 5 pmol of each primer, 200 mM of each dNTPs, 2 mM $MgCl_2$, 50 mM KCL, 10 mM Tris–HCL, 0.5 U Taq DNA Polymerase and 100 ng of genomic DNA.

The thermal profile was as follows; initial denaturation at 94 °C for 5 min. Thirty-five cycles with the following step-cycle profile: denaturation at 94 °C for 45 s, followed by primer annealing at 63 °C (for AMEL) and 55 °C (for SRY) for 45 s, and primer extension at 72 °C for 45 s. The last extension step was 10 min longer. An aliquot of each reaction mixture was subjected to electrophoresis in 2% agarose gel and stained with safe stain (Sinaclone, Iran). The PCR-based sex of embryos were compared with the phenotypic sex and presented as percentage data. Efficiency of the method was evaluated in percentage (%) for both genes.

3. Results

Fetal male (SRY and AMELY) and female (AMELX) circulating DNA was successfully indicated in the maternal plasma in the gravid ewes. Out of 51 samples, 8 samples (15.68%) were twins that were not used in the analysis. For comparison, one sample of twins was used and analyzed (data are not included). The stage of pregnancy and PCR detected sex by PCR for both SRY and AMEL genes are summarized in Table 1. There was no significant difference between stages of pregnancy for both genes determining fetal sex.

The PCR amplification of SRY primers on DNA extracted from blood plasma of ewes bearing a male fetus produced a single band of 171 bp, whereas no band was amplified from the DNA extracted from ewes pregnant to a female fetus (Fig. 1).

Twenty one out of 23 (91.30%) female fetuses were correctly identified by SRY gene sex determination. The number of male fetuses identified by this gene was 19 out of 20 (95%). The overall test accuracy for correct sex determination for SRY gene was 93.15%.

Two bands of 182 and 242 bp in male and a single band of 242 in female fetuses were produced by AMEL gene primers in the PCR reaction (Fig. 2). Twenty two out of 23 (95.65%)