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A novel approach to prenatal fetal sex diagnosis by detecting an insertion in the Y-chromosome of ovine Amelogenin gene

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ABSTRACT

It is completely clarified that both intact fetal cells and cell-free fetal nucleic acids cross the placenta and circulate in the maternal blood stream during pregnancy. In the current study we employed a new approach in applying Amelogenin gene for fetal sex prediction in ovine species. The amount of fetal DNA in the maternal blood was quantified using quantitative real-time PCR technique. Peripheral blood was obtained from 45 pregnant sheep with age of pregnancy varying from 8 to 18 weeks. Optimal primers were designed on Amelogenin intron 4, a Y specific insertion region in Amelogenin gene. For 42 (93.3%) ewes, agreement was verified between fetal sex prediction using cffDNA (cffDNA) and birth outcome. The test sensitivity, specificity and accuracy of fetal sex prediction were 96.5%, 87.5% and 93.3% respectively. The result of relative quantification showed that the cell-free fetal DNA values were significantly higher in ewes with more than 3 months of pregnancy relative to those with less than it. In conclusion, an insertion part in the ovine Y-chromosome Amelogenin gene could be successfully used for fetal sex prediction in ovine species using cell-free fetal DNA.

1. Introduction

Nowadays it is completely clarified that the placenta does not form an impermeable barrier between mother and fetus and both intact fetal cells and cell-free fetal nucleic acids cross the placenta and circulate in the maternal blood stream. For the first time, Bianchi et al. isolated intact fetal nucleated red blood cells from peripheral blood of pregnant women using the monoclonal antibody against transferrin receptor (Bianchi et al., 1990). Since then, various methods of fetal cell enrichment from maternal blood with varying degrees of success have been developed (Sekizawa et al., 2007). However, scarcity and low efficiency of enrichment (Bianchi et al., 1997) and also persistence of some fetal cell types in the maternal circulation following pregnancy (Bianchi et al., 1996) have made the use of circulating fetal cells disappointing to date.

In 1948, Mandel and Metais discovered that the small amounts of extracellular DNA are present in the circulation of both healthy and diseased subjects (Mandel and Metais, 1942). Lo et al. (1997) were demonstrated that a part of cell-free DNA which is present in maternal circulation in healthy pregnant women had fetal origin (Lo et al., 1997). Since then, cell-free fetal DNA (cffDNA) is widely used for non-invasive prenatal diagnosis in human (Wright and Burton, 2009). The most common clinical application

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of cffDNA to date is non-invasive prenatal fetal sex determination. A number of Y chromosome specific sequences such as sex-determining region Y (SRY) (Costa et al., 2001), DYS (Deng et al., 2006) and DAZ (Stanghellini et al., 2006) have been investigated for this purpose.

In the ovine species, fetal sex prediction can be useful in the management decisions such as culling decisions, sex selection in breeding programs and decreasing the cost of progeny testing. Sex prediction in animal can improve the rationalizing management and commercialization of animals. Thus, the interest in offering this specialized service has increased in the word, because it allows one to more adequately plan the nutritional program of the pregnant ewes and increases the value of ewes carrying sexed fetuses to be traded (Santos et al., 2007).

For years, ultrasonography has been the method of choice for determining fetal sex in livestock industry. In ewe, fetal sex is determined by use of transrectal ultrasonography during days 60–69 of pregnancy (de Freitas Neto et al., 2010). However, this technique involves some disadvantages, because it requires extensive experience on the part of the operator, and reaching the fetus becomes difficult by progress of gestation. It causes fetal sex prediction increasingly difficult or sometimes impossible during the later stages of pregnancy (Ali, 2004). However, molecular sexing based on analysis of fetal DNA using the polymerase chain reaction (PCR) technique could be a reliable, sensitive, and practical alternative.

So far, many studies have investigated the use of cffDNA for sex determination in human (Devaney et al., 2011). However, studies on animal species are still in its infancy. Compared with humans with hemochorial placenta, ovine species have synepitheliochorial placental structure with no direct contact between the trophoblast and the maternal blood (Wooding, 1992). Hence, it is likely that the passage of fetal DNA to the ovine maternal blood would be scarce. In this study, we evaluated the feasibility of using Amelogenin gene for prenatal fetal sex determination in ovine species based on analysis of ccffDNA using the quantitative real-time PCR (RT-qPCR) technique.

The Amelogenin gene located on both X (AMEL X) and the Y (AMELY) chromosomes. The majority of studies have considered deletions in the AMELY, in a way that PCR amplification cause fragments with different sizes and sequences (Pfeiffer and Brenig, 2005; Weikard et al., 2006). In the current study, we employed a new approach in applying Amelogenin gene for fetal sex prediction. In this method, an insertion part in the ovine AMELY was used. Furthermore, the amount of fetal DNA in maternal blood was quantified using ovine AMELY.

2. Material and methods

2.1. Animal selection and biological samples

The present study included a group of 45 Lori–Bakhtiari pregnant sheep ranged in age of pregnancy from 8 to 18 weeks. Ewes were divided into two groups: less than 3 months and more than 3 months of pregnancy. As control, samples from three normal nonpregnant ewes and three normal rams were used. The animals belonged to an Agricultural Research Center farm (Shooli, Shahrekord, Iran). Approximately 10 mL of peripheral blood was obtained from the jugular vein. Samples were transported to the laboratory in EDTA containing tubes, on the ice and without freezing. To separate blood plasma, samples were centrifuged at 1000 × *g* for 10 min. Subsequently, they were centrifuged at 5000 × *g* for 10 min to further separate cellular debris. The blood plasma samples were stored at −20 °C until analysis. To determine the accuracy of gene results, the actual sex of fetuses was checked after birth.

2.2. DNA extraction

DNA extraction from maternal blood plasma has been described in detail elsewhere (Kadivar et al., 2013). Briefly, 1 mL blood plasma was mixed with Tris-ethylenediamine tetraacetic acid (TE) buffer. Then, proteinase K was added and kept at 56 °C for 3 h. Next, phenol and chloroform/isoamylalcohol were added. The tubes were centrifuged and the supernatants were transferred to fresh tubes. Ammonium acetate, ethanol and glycogen were added to supernatant and stored at −20 °C overnight. The tubes were centrifuged, the supernatant was discarded, and DNA was deposited with ethanol and then dried in air. The DNA was qualified using spectrophotometry. An absorbance ratio (at 260/280 nm) of 1.7–2 was indicative of pure DNA.

2.3. Primer design and PCR strategy

We used primers, forward (5′-TTTCTGCTGTCCA-CCCTGAG-3′) and reverse (5′-GACACTTGCCTTGG-ACACCT-3′), designed from the Amelogenin intron 4 (Gene Bank accession number: AY604731) to amplify a 111 bp region in AMELY. Intron 4 was reported as a 460–465 bp insertion located between the 4th and 5th exons in cetaceans and ruminants (Macé and Crouau-Roy, 2008). The Sequence similarity was checked by running BLAST over GenBank nr/nt nucleotide collection sequences with megablast algorithm (intended for high similarity sequences) (http://blast.ncbi.nlm.nih.gov) and with Ensemble genomic sequence (http://asia.ensembl.org/Multi/blastview) to find an AMELY fully dedicated region. Optimal primers were designed using Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). A second pair of primers, 5′-TGGCAAAGTGGACATCGTTG-3′ and 5′-TGGCGTGGACAGTGGTCATAAGTC-3′, were designed to amplify a 467 bp sequence from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as internal control.

Real-time qPCR analysis was performed using a Rotor-Gene Q 6000 System using the Maxima-TM-SYBR Green Master Mix (SinaClon, Karaj, Iran). The standard PCR was performed in a total volume of 20 μL, containing 5 pmol of each primer, 3 μL of DNA template, 4 μL of quantitative PCR master mix and DNase/Rnase free distilled water. Reaction conditions were an initial denaturation at 94 °C for 40 s, annealing temperature of 63 °C for 35 s, and an extension temperature of 72 °C for 32 s. The PCR amplification was performed in triplicate for each DNA sample with