The application of amplified TSPY and amelogenin genes from maternal plasma as a non-invasive bovine fetal DNA diagnosis

Arash Davoudi¹*, Ramin Seighalani¹, Seyed Ahmad Aleyasin², Alireza Tarang¹, Rouhollah Radjabi³, Farideh Tahmoressi¹

¹Department of Genomics and Animal, Branch of North Region of Iran, Agricultural Biotechnology Research Institute of North Region of Iran (ABRII), 4188988883 Rasht, Iran
²Department of Medical Biotechnology, National Institute for Genetic Engineering and Biotechnology (NIGEB), 14965161 Tehran, Iran
³Department of Plant Protection, Islamic Azad University, Dezful Branch, 6461645165 Dezful, Iran

*Corresponding Author: arash1983@gmail.com

INTRODUCTION

Invasive methods for prenatal diagnosis include chorionic villus sampling (CVS) and amniocentesis that entail the risk of fetal loss and mortality. During pregnancy, fetal cells including fetal DNA crossed the placenta and within maternal peripheral blood those valuable sources of the sex and genetics information fetuses. It is demonstrated fetal DNA in plasma and serum from healthy pregnant women (Lo et al. 1997). Recent studies have showed that fetal DNA in maternal plasma has mean of 3.4% and 6.2% of the total DNA of early and late gestation, respectively (Lo et al. 1998b) and cleared at an extremely rapid rate following birth (Lo et al. 1999). Fetal sex determination is now possible at 8th w of pregnancy, by testing of maternal blood sample. The great sensitivity of PCR technique, allows detecting small amounts of fetal DNA that present in maternal plasma. This test is based on the identification of specific regions of X and Y chromosome
circulating on maternal blood. Recent technical advances enable us to use both intact fetal cells (Bianchi 1998, Hahn et al. 1998, Steele et al. 1996, Hamada et al. 1998) and cell-free fetal DNA (Zhong et al. 2001, Bischoff et al. 2002, Lo et al. 1998) of maternal plasma and serum of non-invasive fetal gender and prenatal genetic diagnosis. However, the level of fetal DNA is low and DNA template obtained by these simple methods is not enough to reach the anticipated result. Most of these technical improvements such as fluorescence-based polymerase chain reaction (PCR) (Pertl et al. 2000, Genlin et al. 2010) and real-time PCR (Costa et al. 2001, Hromadnikova et al. 2003) methods are highly sensitive and technically demanding. However, expensive equipments limit their application in a routine setting. Some conventional PCR analyses of maternal plasma, serum and blood using the Y-specific sequences for example; DYS14, (Honda et al. 2002) DY32, (Honda et al. 2001) and DY31 (Zhao and Zou 2004), the Y-specific repeat sequences (Lo et al. 1998a) and sex determination region Y (SRY) (Al-Yatama et al. 2001, Zhong et al. 2000, Tungwiwat et al. 2003, Genlin et al. 2010) have been introduced for the diagnosis and fetal gender. But in a routine setting internal amplification control, examination of results is difficult to be interpreted (Tungwiwat et al. 2003). A synchronic amplification of the X-Y homologous region of the amelogenin was reported in human (Falcinelli et al. 1999) and bovine (Chen et al. 1999). also, bovine SRY gene sequence (Genlin et al. 2010), and zfx and zfy gene sequences in maternal blood using a pair primers has also been described for fetal sexing (Kirkpatrick et al. 1993). The aim of this study was to establish a rapid and reliable method for sexing of bovine fetuses. This has prompted us to develop another non-invasive method of bovine fetal sex determination applying PCR amplification of the X chromosome (467 bp amelogenin gene fragment), the Y chromosome (341 bp amelogenin gene fragment) and Y-encoded, testis-specific protein (TSPY) gene derived from a male-specific bovine DNA sequences simultaneously.

### MATERIAL AND METHODS

#### Blood sampling and plasma separation

Peripheral blood samples were obtained 38 pregnant heifers with gestational w of 12 to 38. Five normal heifers who had no pregnant history and five normal bulls served as positive control. 10 mL of maternal peripheral blood were collected and put into EDTA-containing tubes (20 mM). Tubes were centrifuged at 1000 r/min for 10 min with the brake and acceleration powers set to zero. Then tubes were centrifuged at 1200 r/min for 10 min with the brake and acceleration powers set to zero. Approximately 0.5 mL of supernatant (i.e., the plasma) was left in the tube to ensure that the buffy coat was not disturbed. Tubes were centrifuged at 2000 r/min for 5 min with the brake and acceleration powers set to zero. 350 μL of supernatant and samples were stored at -20°C for further processing.

#### DNA extraction from maternal plasma samples

350 μL maternal plasma and equal volume of TE were mixed in a 1.5 mL Eppendorf tube by addition of 5 μL proteinase K (20 mg/mL) solution. The mixture was digested at 56°C for 3 h, then 350 μL of equilibrium phenol and chloroform were added respectively. The tubes were centrifuged at 12000 r/min for 12 min, and then the supernatant was transferred to a fresh tube. Equal volume of chloroform and isoamyl alcohol (24:1) was added. After centrifuge (at 12000 r/min for 12 min), 1:10 of 3 mol/L sodium acetate and 2 volumes of 100% ethanol was added and the mixture was stored at -20°C for 14 h. Then tubes were centrifuged at 12000 r/min for 8 min at room temperature. The supernatant was discarded, DNA was purified and deposited with 70% ethanol, and was dried in the airing closet. Tubes were dried at 65°C for 3 min and finally, DNA was dissolved in 20 μL TE. Tubes were placed at 65°C for 40 min and
then stored at 4°C.

Quantification of the extracted DNA from maternal plasma
The concentration and purity of extracted DNA were detected by ultraviolet spectrophotometer (NANODROP 2000 Thermo). The results were read at 260 nm and 280 nm respectively.

PCR conditions for amplification amelogenin and TSPY genes
In this study, two primer pairs were used: one set was derived from a male specific bovine DNA “TSPY” gene (Lemos et al. 2005). This primer amplified sex-determination Y chromosome fragment and was thus representative of fetal DNA. The oligonucleotide sequence of the primers was: 5’- CCCGCACCTTCCAAGTTGTG-3’ and 5’-AACCTCCACCTCCTCCACGATG-3’. These primer pair were designed to produce a 260 bp DNA fragment. The other one set amplification of the bovine amelogenin (bAML) gene (Chen et al. 1999) on the X- and Y-chromosomes of bovine. The oligonucleotide sequence of this primer was: 5’-AAATTCTCTCACAGTCCAAG-3’ and 5’-CAACAGGTAATTTTCCTTTAG-3’. This primer was designed to allow the amplification of a 467 bp single fragment of the X-chromosomes in female cattle and two 467 and 341 bp fragments of the X and Y-chromosomes in male cattle. The PCR reaction mixture (25 μL), which contained 2.5 μL plasma DNA, 10 pmol each of primers TSPY and amelogenin, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 5 U/μL Taq DNA polymerase (Roche), was added to each sample in a 0.2 mL tube. The DNA sequence of TSPY and amelogenin were amplified by an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 63°C and 54°C for 60 sec, and extension at 72°C for 60 sec. The final extension was at 72°C for 5 min.

Quality of PCR products
8 μL of PCR product and 2 μL loading buffer were mixed. The amplification products were analyzed by electrophoresis on 1.5% agarose gel and stained with ethidium bromide.

Examination of PCR sensitivity
When the amount of extract template DNA from maternal plasma at PCR mixture was 2-3 μL (or 200-300 ng/mL), the most visible bond was seen (Fig. 1).

Results of PCR
The results were shown in Figs. 2 and 3, we found two clear bands, 341 bp and 467 bp in the template DNA extracted from heifers bearing male fetus (Lines 2, 5 and 6). There was one band at 467 bp after the template DNA extracted from heifers bearing female fetus amplified at the same condition (Lines 3, 4 and 7). After amplifying the DNA samples extracted from normal bull and heifer that had no pregnant history respectively, positive (Lines 8 and 9) results were obtained respectively. Also, the results were shown in Fig. 3, we found one clear band, 260 bp, in the template DNA extracted from heifers bearing male fetus (Lines 2, 4, 5 and 6). There was no band after the template DNA extracted from heifers bearing female fetus amplified at the same condition (Lines 3 and 7). After amplifying the DNA samples extracted from normal bull and heifer that had no pregnant history respectively, positive (Lines 8 and 9) results were obtained respectively. The results showed that fetal DNA could be found in heifers bearing male fetus.

RESULTS

DISCUSSION

Many studies agreed the placenta is the predominant source of fetal DNA in the maternal circulation. Fetal DNA in maternal plasma can be detected as early on day 32 of gestation. Since the feto-placental circulation is not established until 28–30 days post-conception, fetal DNA is thought may be derived from the trophoblast. Fetal DNA circulates in nucleosomes that are likely derived from ruptured apoptotic bodies (Kim et al. 2010).

In this investigation, first purpose was to detect fetal DNA to develop a non-invasive
prenatal diagnosis technique then fetal sex detection was considered a successful assay. This study allowed us to introduce an adequate methodology of DNA extraction, techniques of PCR amplification and analysis of the obtained fragments. It was confirmed that fetal DNA was detected from late first trimester (8th w of gestation) by conventional PCR. Follow-up throughout gestation confirmed that it was easy to detect fetal DNA as pregnancy advances. This investigation showed the diagnosis of female fetuses and demonstrated that it was possible to detect any sequence absent in the mother but present in the fetus. This technique of fetal sex detection has an important clinical application as a noninvasive tool in those cases of genetic disorders. The conventional PCR techniques, with a similar design, have been used by other groups to sex determination fetus successfully (Lo et al. 1989, Zhong et al. 2000, Al-Yatama et al. 2001, Honda et al. 2001, Honda et al. 2002, Tungwiwat et al. 2003, Zhao and Zou 2004 Genlin et al. 2010).

However, due to the overriding presence of maternal DNA, we were able to detect only fetal sequences of paternal origin in maternal plasma. These procedures could be used as an alternative method of CVS or amniocentesis for parents discouraged from having an invasive procedure. Understanding the technical parameters affecting the reliability of the detection of fetal DNA in maternal plasma is very important because of its application a routine prenatal diagnosis procedure. It is demonstrated that it is necessary to obtain the plasma as soon as possible and freeze it until time of DNA extraction, after phlebotomy of the mother. Maternal blood collected and processed for no longer than 24 h. A few samples were processed after this time (48 and 72 h), and by using these
samples we were able to detect maternal DNA but not fetal DNA. Previous studies indicated that fetal DNA in maternal plasma was stable even 24 h after collection (Angert et al. 2003, Gonzalez-Gonzalez et al. 2005). The time a sample spends in the tube before processing could not only affect the amount of total DNA by releasing DNA due to apoptosis of maternal cells, but also affect the stability of free-cell fetal DNA. The overriding maternal DNA interferes with fetal DNA amplification, and the degradation of fetal DNA is obviously inconvenient for PCR; therefore, it is important to consider the processing time as an important factor. These data need to be studied at length to establish protocols for the transmission of samples from distant places.

The DNA template was extracted from the plasma of 38 pregnant heifers (8 to 38 w). Fetus-derived Y sequence amelogenin and TSPY fragments were detected in 24 cases of maternal plasma samples from the 38 cases that we guesstimated. All samples taken after a gestational age of 8 w correctly predicted fetal gender. Previous studies (Zhong et al. 2001, Sekizawa et al. 2001, Rijnders et al. 2001) reported the diagnostic accuracy of fetal gender determination from maternal plasma samples obtained at early pregnancy. Among the 38 pregnant heifer, the sensitivity was 100%. The present data coincide with the literature reports showed that the accuracy of the technique was higher than other studies performed from the early pregnancy (Lo et al. 1998b, Costa et al. 2001, Honda et al. 2001, Sekizawa et al. 2001). In this study, we extracted DNA from the plasma by phenol-chloroform method and then developed PCR system for prenatal determination of bovine fetal sex. One of the benefits of DNA extraction with phenol-chloroform method is low cost of this method in comparison with extraction kit, indeed extracted DNA had appropriate quality and quantity. We used two rather than one Y-chromosome specific markers and one X-chromosome during validation of a laboratory-developed test. The result of $A_{260}/A_{280}$ was below 1.7 generally, indicating there was the possibility of existence of protein or RNA in the templates. This amount of protein or RNA contamination was expected due to the (considering the) nature of blood plasma. However, the purity of samples was sufficient. Though this conventional PCR method couldn’t be compared with the real-time quantitative PCR approach (Costa et al. 2001, Hromadnikova et al. 2003), but it could

Table 1. Results of fetal sex prediction by non-invasive approach using the conventional PCR analysis of maternal plasma DNA in 38 pregnant heifers at various gestational ages (w).

<table>
<thead>
<tr>
<th>Samples No</th>
<th>Gestational age (weeks)</th>
<th>Result of PCR</th>
<th>Birth outcome</th>
<th>Samples No</th>
<th>Gestational age (weeks)</th>
<th>Result of PCR</th>
<th>Birth outcome</th>
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<td>8.2</td>
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provide a more practical methodology with acceptable sensitivity and specificity. The simple and simultaneous amplification of amelogenin on the X and Y chromosome and TSPY sequence on the Y chromosome in a PCR reaction, respectively, could provide a satisfactory result for bovine fetal sex determination. As shown in Fig. 2 and Table 2, we were able to accurately identify the fetal sex using multiplex PCR analysis of 38 maternal plasma in the pregnant heifers. Among the 38 pregnant heifers, the final accuracy of 100% was obtained.

CONCLUSIONS

In general, the result showed that phenol-chloroform methods is a simple and sensi-
tional for isolation fetal DNA in maternal blood and PCR technique methods is non-invasive cost efficient, reliable and available for non-invasive bovine fetus sex determination.

ACKNOWLEDGEMENTS

The authors are grateful to the Agricultural Biotechnology Research Institute of North Region of Iran and Medical Biotechnology Department, National Institute for Genetic Engineering and Biotechnology for partial practical support to this study.

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Anne Plazmasından Elde Edilen Amplifiye TSPY ve Amelogen Genlerinin Sigir Fetüsü DNA Tanısı İçin Invaziv Olmayan Uygulanması

Özet