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The Potential and Accuracy of RNA-based Fetal Sex Determination during Early Pregnancy Using Cell-Free Fetal RNA from Korean Native Cows (*Bos taurus coreanae*)

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Abstract : Cell-free fetal RNA is useful to determine fetal sex and detect other inherent genetic disorders. However, non-invasive fetal sex determination methods using fetal RNA from maternal plasma is not yet well established in studies pertaining to bovine animals. Thus, the aim of this study was to systematically evaluate the presence of the male-specific ZRSR2Y gene transcript in maternal plasma using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assays, and to verify its accuracy, sensitivity, and specificity in determining fetal sex between 30 and 100 days of gestation. Overall accuracy, sensitivity, and specificity of the ZRSR2Y gene transcripts in determining fetal sex were 89.1%, 86.3%, and 100%, respectively. The 30 to 100 days of gestation were further classified into five stages of gestation, and each stage had relatively high accurate, sensitive, and specific results. Overall, these results indicate that the expression of the ZRSR2Y gene can be used for fetal sex determination in bovine animals using circulating cell-free RNA in maternal plasma during early pregnancy.

Key words **:** cell-free fetal RNA, early pregnancy, fetal sex determination, bovine.

Introduction

Early fetal sex determination is important in both the dairy and beef cattle breeding industries. Benefits of early fetal sex determination include increased commercial value of pregnant animals, taking culling decisions for marginal cows, the ability to predict replacement needs, and giving producers an advantage in decision-making regarding activity planning and financial benefits (4,5,16).

In the cattle breeding industry, transrectal ultrasonography based on the location of the genital tubercle has been the most frequently used method for fetal sex determination. However, transrectal ultrasonography requires a trained technician whose expertise plays an important role in fetal sex determination, also, the breed and age of animals must be taken into consideration (1). Determination of fetal sex becomes more difficult as pregnancy progresses past the first trimester, because the fetus moves more actively and the gravid horn is more likely to descend ventrally into the abdominal cavity (1,15). Furthermore, chorionic villus sampling and amniocentesis are invasive methods that potentially increase the risk of fetal loss (7,12). Thus, there is a demand for a non-invasive and reliable method for bovine fetal sex determination during early pregnancy.

Several studies have attempted to find non-invasive fetal sex determination methods. Lo *et al*. (10) first demonstrated that circulating cell-free fetal DNA (ccffDNA) can cross the placenta and circulates with the peripheral maternal plasma, with enough quantity to be used as a marker for fetal sex. Since then, ccffDNA has become a valuable source for fetal sex determination and genetic evaluation. However, there is some restriction of ccffDNA as a DNA-based sex determination method, because only paternally inherited fetal DNA sequences are detected in maternal plasma and it is difficult to detect fetal-specific gene expression patterns. For these reasons, the search for fetal-specific mRNA markers was of great interest to discriminate between fetal and maternal genetic materials. The presence of circulating cell-free fetal RNA (ccffRNA) in maternal plasma was first determined in pregnant women carrying male fetuses, using Y chromosome-specific zinc finger protein mRNAs (14). The presence and detectability of fetal-specific mRNA were subsequently verified, through the analysis of Y chromosome-independent mRNA, such as the mRNAs of human placental lactogen (hPL) and the beta-subunit of human placental chorionic gonadotropin (β-hCG) (13). In the bovine field, some studies had performed RNA-based fetal sex determination using tissues or embryos. For example, Hamilton *et al*. (8) evaluated characteristic expression patterns of eight Y chromosome-linked genes in bovine blastocysts and verified that the DDX3Y, USP9Y, and ZRSR2Y genes were good candidates for RNA-based fetal sex determination. Furthermore, Chang *et al*. (3) analyzed through direct testis RNA deep sequencing using eighteen Y chromosome-specific genes at three developmental stages: postnatal day 20, puberty (8 months), and maturity (2 years). In this study, comparative analyses revealed that the ZRSR2Y and RPL23AY genes were specific to cattle. However, non-invasive fetal sex determination

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using fetal RNA from maternal plasma is not yet well established. In a previous study, Lee *et al*. (9) confirmed that the ZRSR2Y gene is a good candidate for RNA-based fetal sex determination using ccffRNA from maternal plasma.

The aim of the present study was to systematically evaluate the presence of the fetal ZRSR2Y gene transcript in maternal plasma using RT-PCR assay, and to verify its accuracy, sensitivity, and specificity in determining fetal sex between 30 and 100 days of gestation.

Materials and Methods

Blood sampling and plasma separation

Blood samples were obtained from 92 pregnant Korean native cows between 30 and 100 days of gestation. Three heifers with no pregnant history and three bulls served as controls. About 10 mL of peripheral blood samples were collected from the jugular veins using evacuated tubes containing EDTA (BD Vacutainer Tubes, Becton Dickinson, UK Ltd, Oxfordshire, UK), and transported from the field to the laboratory in Styrofoam boxes containing ice, without freezing the samples. The blood samples were centrifuged at 1600 *g* for 10 min with the brake and acceleration powers set to zero. Following centrifugation, plasma was divided into 1 mL aliquots and stored at −80ºC before ccffRNA extraction.

ccffRNA extraction and reverse transcription

Total RNA extraction was performed using the PureLink® Viral RNA/DNA Mini Kit (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. RNA concentration was quantified using a NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). Reverse transcription was carried out for an equal amount of target RNA using the LeGene cDNA Synthesis Master Mix (2×) (LeGene Bioscience, San Diego, CA, USA). After reverse transcription, the cDNA stored at −80ºC before RT-PCR.

Detection of the ZRSR2Y gene

For the PCR reaction, 5 pmol of each primer, 5 µl of cDNA template, 4 µl of Hipi PCR PreMix (ElpisBiotech, Daejeon, Korea) was used and DNase/RNase-free H_2O was added to obtain a final reaction volume to 20 µl. The reaction conditions were an initial denaturation at 94ºC for 5 min, and 45 cycles of 94ºC for 30 s, 53ºC for 30 s, and 72ºC for 45 s. A final elongation step at 72ºC for 7 min was performed before maintaining the reaction at 4ºC. The details of the primers used in this study are outlined in Table 1. For visualization of the amplified product, 15 µl of the PCR product was electrophoresed through a 0.7% agarose gel (Lonza, Rockland, ME, USA) and stained with TopRed (Genomicbase, Seoul, Korea). The RNA bands were confirmed by i-MAXTM Gel Image Analysis System (Core Bio, Seoul, Korea). The PCR reactions were replicated three times for all samples.

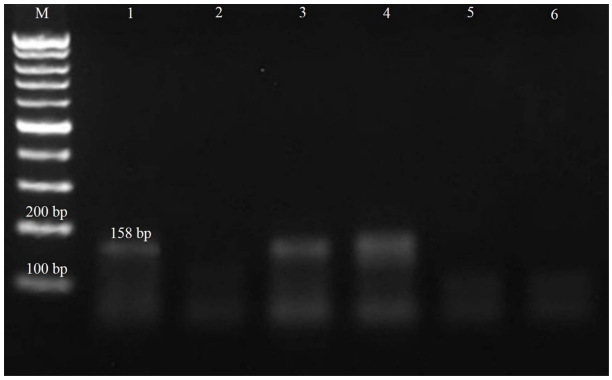

Fig 1. Results of electrophoresis for RT-PCR products from testing samples with the ZRSR2Y primer. M = DNA molecular weight marker (100 bp); line 1 = bull, positive control, 158 bp; line 2 = heifer with no pregnant history, negative control; line 3, 4 = cows bearing male fetuses, 158 bp; line 5, 6 = cows bearing female fetuses.

Statistical analysis

The efficiency of correct fetal sex determination and calf sex at birth were compared statistically by the chi-square test or the Fisher exact test. Contingency tables were suited to RT-PCR fetal sex determination and phenotypic sex at birth to calculate sensitivity, specificity, male predictive value (MPV), female predictive value (FPV), and accuracy. Sensitivity was defined as the number of males correctly identified by RT-PCR divided by the total number of males at birth. Specificity was the number of females correctly identified by RT-PCR divided by the total number of females at birth. MPV was calculated as the number of male fetuses correctly identified divided by the total number of (correct and erroneous) male determinations. FPV was calculated as the number of female fetuses correctly identified divided by the total number of (correct and erroneous) female determinations. Accuracy was defined as the proportion of males and females that were correctly identified (Quintela et al. 2012). Statistical significance was considered at $p < 0.05$. All statistical analyses were performed using the GraphPad InStat software (version 3.05).

Results

The 158bp bands of RT-PCR products from all the control bull samples were confirmed by electrophoresis, and no bands were seen for the control heifers that had no pregnant his-

Table 1. Primer sequences used for RT-PCR

Primer name	Primer sequences (5' → 3')	Size (bp)	Source (GenBank accession number)	Gene name and symbol
ZRSR2Y-F	GTCAGTTGCAACCTGGAACC	158	GQ426330	Zinc finger (CCCH type), RNA binding motif and serine/arginine rich 2, Y-linked (ZRSR2Y)
ZRSR2Y-R	GCCATATTCCATTGGGTCAC			

Table 2. Sensitivity, specificity, male predictive value (MPV), female predictive value (FPV), and accuracy of fetal sex determination by RT-PCR using the ZRSR2Y gene

	Gestational age (days)											
	30-39		40-49		50-59		60-69		70-100		Total	
	male*	female*	male*	female*	male*	female*	male*	female*	male*	female*	male*	female*
n	16	2	16	5	17	5	11	3	13	4	73	19
Sensitivity	87.5		93.8		82.4		81.8		84.6		86.3	
Specificity	100		100		100		100		100		100	
MPV	100		100		100		100		100		100	
FPV	50		83.3		62.5		60		66.7		65.5	
Accuracy	88.9		95.2		86.4		85.7		88.2		89.1	

*Fetal sex at birth

tory. Likewise, the bands of the RT-PCR products from plasma samples of cows bearing male fetuses were visualized by electrophoresis and no bands were visualized from the plasma samples of cows bearing female fetuses (Fig 1). RT-PCR amplification of the 92 tested plasma samples from the pregnant cows was classified into five groups: 30-39 days of gestation, 40-49 days of gestation, 50-59 days of gestation, 60-69 days of gestation, and 70-100 days of gestation. The accuracy of RNA-based fetal sex determination in each group was 88.9%, 95.2%, 86.4%, 85.7%, and 88.2%, respectively. The sensitivity of fetal sex determination in each group was 87.5%, 93.8%, 82.4%, 81.8%, and 84.6%, respectively. The specificity of fetal sex determination in all groups was 100%. The overall accuracy, sensitivity, and specificity were 89.1%, 86.3%, and 100%, respectively (Table 2). There was a strong correlation between the possibility of correct fetal sex determination and calf sex at birth ($p < 0.0001$).

Discussion

It has been generally believed that the placenta forms an impermeable barrier between the mother and the fetus, and that the maternal and fetal circulations are separated by placental membranes during pregnancy. However, several studies showed that circulating fetal nucleic acids could cross the placental membrane because of the following three possibilities: 1) apoptosis of fetal hematopoietic cells regulated by the maternal immune system; 2) abundant cellular activity in the placenta; and 3) a concentration gradient leading to the direct transfer of fetal nucleic acid molecules across placental membranes (2,6,10,17). After the circulating fetal nucleic acids were first detected by Lo *et al*. (10), several studies have investigated the standard model of fetal sex determination using the circulating fetal nucleic acids in maternal plasma and serum. The efficacy and accuracy are dependent on the selection of sex-specific genes. In this regard, many studies have aimed to search for the best candidate genes for sex determination. A previous study has revealed that the ZRSR2Y gene had the highest performance among three candidate genes for RNA-based sex determination using ccffRNA in maternal plasma (9). For that reason, we used the same sequence of the ZRSR2Y gene to determine fetal sex, and the results of the present study showed that the expression of ZRSR2Y gene was detectable in early gestation periods.

Several studies have reported changes in the concentration of circulating fetal nucleic acids at different gestational stages. Lo *et al*. (11) demonstrated that the mean levels of fetal nucleic acids in early and late gestation were 3.4% and 6.2%, respectively, of the total amount of nucleic acids existent in maternal plasma. Devaney *et al*. (7) verified that the sensitivity of detecting circulating fetal nucleic acids prior to 7 weeks of gestation was below 74%, as compared to above 94% sensitivity past 7 weeks of gestation. Poon *et al*. (14) mentioned that the detection rates of fetal male-specific mRNA in maternal plasma increased 3-fold in the late stages of pregnancy as compared with the early stages of pregnancy. Thus, the accuracy and sensitivity of fetal sex determining tests tend to increase with gestational age. For example, da Cruz *et al*. (5) reported that the rate of success of prenatal sex determination using PCR for bovines between 5 and 6 weeks of gestation was approximately 50%. Additionally, Xi *et al*. (18) reported that they could successfully predict fetal sex from pregnant cow blood with the overall accuracy rate of 60% during 30-59 days of gestation. However, both studies indicated that the accuracy rate increased to 99.9% if the tests were performed from the 7th week of gestation onward. In the present study, we classified 30 to 100 days of gestation into five groups; each group had relatively high accuracy, sensitivity, and specificity for RNA-based fetal sex determination. In addition, there was no evidence of increasing detection rates with gestational age. Our results in the overall accuracy, sensitivity, and specificity of fetal sex determination are similar with the results of Lee *et al*. (9). Our study had a high accuracy of fetal sex determination prior to 7 weeks as compared with prior studies, and the ZRSR2Y gene might be a good candidate for the RNA-based sex determination in the first trimester of gestation. For further studies, we plan to determine the correct target sequences for the selection of optimal primers to increase the sensitivity and the accuracy of the ZRSR2Y gene in bovine fetal sex testing.

In conclusion, the present study demonstrated that the male-specific ZRSR2Y gene was useful for early bovine fetal sex determination using ccffRNA. This result contributes to the current knowledge of fetal sex gene expression patterns in early gestational stages.

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