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Confirmation of Male Specific Fetal Free RNA in Maternal Plasma and Comparison of Accuracy on the Sex Determination using Real-time PCR Method in Korean Native Cattle

Sang-Ho Lee[1], Chul-Ho Park[1], Jun-Tae Park[1], Sang-Guk Park[2], Jin-A Lee[3], Guk-Hyun Suh[1], Ki-Seok Oh[1] and Chang-Ho Son[1,†]

[1]Laboratory of Veterinary Obstetrics, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea
[2]Rural Development Administration, National Institute of Animal Science, Chungnam 331-801, Korea.
[3]Laboratory of Veterinary Infectious Disease, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea

ABSTRACT

Cell-free fetal RNA has been highlighted as useful tools for the fetal sex determination or other genetic inherent disorder. However, there is no knowledge about the sex determination using cell free fetal RNA in bovine field. Thus, the present study aimed to evaluate the presence of transcripts of *DDX3Y*, *USP9Y* and *ZRSR2Y* genes in maternal plasma of pregnant cows to determine the sex of the fetus using real-time quantitative polymerase chain reaction assay, and verify its accuracy, sensitivity and specificity compared with the molecular testing and the calf sex at birth. Transcripts of *USP9Y* and *DDX3Y* genes were expressed in the all plasma of males and females both the control group and the experimental group. However, *ZRSR2Y* gene was matched up with the molecular testing and the true sex in control group and has an overall accuracy of 82.6%, a sensitivity of 75%, and a specificity of 100% in experimental group. Therefore, these results indicated that real time PCR technique, as a noninvasive and cost-efficient method, is possible to determination fetal sex in the bovine species using circulating cell free RNA in maternal plasma and especially *ZRSR2Y* gene could be a good candidate for the RNA based sex determination work.

(Key words : cell free fetal RNA, real-time PCR, sex determination, *ZRSR2Y*, bovine)

INTRODUCTION

The development of fetal sex determination methods in livestock still remains animal science, veterinary and zootechnical challenge (da Cruz *et al*., 2012). Various methods have been used for determining the sex of fetus to direct the management of animals, giving producers a merit in decision-making regarding activity planning and financial profits (Shea, 1999).

In the livestock industry, transrectal ultrasonography based on location of the genital tubercle has been the most frequently used method of early fetal sex determination. The best time for fetal gender determination by ultrasonography in cows is between days 56 and 98 of gestation, although the breed and age should be taken in consideration (Ali, 2004). However, there are some limitations using ultrasonographic method in fetal sex determination as pregnancy progresses after the first trimester (Quintela *et al*., 2012). As the fetus grows, it becomes more difficult to obtain the desired ultrasound image because the fetus moves more actively.

In mammals, primary sex differentiation is rigidly regulated by chromosome and is not influenced by the environmental agent (Ali, 2004). DNA carries the genetic blueprint which contains all characteristics of an organism including sex determination related components. Therefore, DNA-based techniques, especially polymerase chain reaction (PCR) has been used to sex determination over the last few decades. Lo *et al*. (1997) first reported that human free fetal DNA can cross the placenta and is present in peripheral maternal plasma with enough quality to be used as a template in PCR. Also, it has been demonstrated that the mean level of fetal DNA in early and late gestation consist of 3.4% and 6.2%, respectively, of the total DNA existent in maternal plasma (Lo *et al*., 1998). Since then, circulating cell free fetal DNA (ccffDNA) has emerged as a valuable source for prenatal fetal sex determination and

† Correspondence : E-mail : chson@jnu.ac.kr

genetic evaluation. For that reason, noninvasive fetal sex determination by PCR using ccffDNA recently has been highlighted in veterinary fields. da Cruz *et al*. (2012) reported that the fetal sex predicted by PCR using ccffDNA isolated from maternal plasma was in accordance with the sex of the calf at birth in 88.6% of case in bovine. However, there was some limitation of using DNA in maternal blood, because the DNA based sex determination method is only possible to detect paternally inherited fetal DNA sequences and it is difficult to detect gene expression patterns in fetus, as well. For these reasons, the development of gender independent nucleic acid markers, such as RNA, was greatly interested. Poon *et al*. (2000) demonstrated the presence of circulating cell-free fetal RNA (ccffRNA) in maternal plasma that might be used as a marker for noninvasive fetal sex determination in human. In bovine field, noninvasive fetal sex determination method by using of ccffRNA has not been well established yet. However, Hamilton *et al*. (2012) analyzed consistency of expression of eight Y chromosome linked genes, such as *DDX3Y*, *EIF1AY*, *HSFY*, *SRY*, *TSPY*, *USP9Y*, *ZFY* and *ZRSR2Y* in bovine blastocyst and confirmed that *DDX3Y*, *USP9Y* and *ZRSR2Y* were the strong candidate for the RNA based sex determination work. This previous study prompted us to examine the RNA based sex determination method in bovine ccffRNA, especially using *DDX3Y*, *USP9Y* and *ZRSR2Y* genes. Therefore, this present study aimed to evaluate the presence of transcripts of *DDX3Y*, *USP9Y* and *ZRSR2Y* genes in maternal plasma using real-time PCR assay, and verify its accuracy, sensitivity and specificity in terms of sex determination in bovine practical field.

MATERIALS AND METHODS

1. Blood Sampling and Plasma Separation

Blood samples were obtained from 23 pregnant Korean native cattle with gestational weeks of 7～40. Three normal heifers which had no pregnant history and three normal bulls served as control group. 10 mL of maternal peripheral blood samples were collected from the jugular vein 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 blood samples were centrifuged at 1000 g for 10min with the brake and acceleration powers set to zero. Following centrifugation, plasma was divided into 1 ml aliquots and stored at −80℃ before the ccffRNA extraction.

2. ccffRNA Extraction and Reverse Transcription

Total RNA extraction was performed using PureLink® Viral RNA/DNA Mini Kit (Invitrogen, Grand Island, NY, USA) according to manufacturer's instrument. The RNA concentration was quantified using NanoDrop ND-1000 (Thermo Scientific, Waltham, MA, USA). Reverse transcription of an equal amount of target RNA was performed using QuantiTect® reverse transcription kit (Qiagen, Valencia, CA, USA). Following reverse transcription, the cDNA stored at −80℃ before real-time PCR.

3. Quantitative Real-time PCR Analysis

The transcripts of *ZRSR2Y*, *USP9Y* and *DDX3Y* genes were analyzed for expression of ccffRNA in the maternal plasma and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the Ct method. The details of all the primers used in this present study are outlined (Table 1).

Quantitative real-time polymerase chain reaction (PCR) was performed with iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using MyiQ™2 thermocycler and SYBR green detection system (Bio-Rad Laboratories). The real-time PCR conditions were 95℃for 10 min, then 50 cycles at 95℃, 30 sec; 57℃, 30 sec and 72℃, 30 sec.

4. Statistical Analysis

The efficiency of correct fetal sex determination and the calf sex at birth were compared statistically by chi-square test or Fisher exact test. Contingency tables were suited to real-time PCR fetal gender assignment 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 real-time PCR examination, divided by the total of males at birth. Specificity was the number of females correctly identified by real-time PCR examination, divided by the total of females at birth. Male predictive value was calculated as the number of male fetuses correctly diagnosed divided by the total (true and erroneous) male diagnoses. Female predictive value was calculated as the number of female fetuses correctly diagnosed divided by the total (true and erroneous) female diagnoses. Accuracy was the proportion of males and females that were correctly identified (Quintela *et al*., 2012). Statistical significance was

Table 1. Primer sequences used for real-time PCR experiment

Primer name	Primer sequences (5' → 3')	Size (bp)	Source (GenBank accession number)	Gene name and symbol
GAPDH-F	TTCCTGGTACGACAATGAATTTG	153	NM_001034034.1	Glyceraldehyde-3-phosphate, dehydrogenase (GAPDH)
GAPDH-R	GGAGATGGGGCAGGACTC			
DDX3Y-F	GGACGTGTAGGAAACCTTGG	225	NM_001172595.1	DEAD (Asp-Glu-Ala-Asp) box, polypeptide 3, Y-linked (DDX3Y)
DDX3Y-R	GCCAGAACTGCTACTTTGTCG			
USP9Y-F	GCCAGATGACCAAGAAGCCCCA	285	NM_001145509.1	Ubiquitin-specific peptidase 9, Y linked (USP9Y)
USP9Y-R	GGACTGTAAGGCCTAATAGCCTGGT			
ZRSR2Y-F	GTCAGTTGCAACCTGGAACC	158	GQ426330	Zinc finger (CCCH type), RNA binding motif and serine/arginine rich 2, Y-linked (ZRSR2Y)
ZRSR2Y-R	GCCATATTCCATTGGGTCAC			

considered at $P<0.05$ significance level. All the statistical analyses were performed using GraphPad Instat (version 3.05).

RESULTS

In this study, transcripts of gene *ZRSR2Y*, *USP9Y* and *DDX3Y* were expressed both the control group and the experimental group. There was amplification in all samples for transcripts of *GAPDH* qPCR demonstrating 100% efficiency of ccffRNA extraction. In *ZRSR2Y* gene, there was 100% accordance between the sex of the control group identified by the molecular testing and the true sex. However, transcripts of gene *USP9Y* and *DDX3Y* were expressed regardless of gender (Fig. 1 and Table 2). Furthermore, there was a strong relationship between the possibility of correct sex determination and the calf sex at birth using transcripts of *ZRSR2Y* gene in the experimental group ($p = 0.0013$). But, prediction of fetal gender using transcripts of *USP9Y* and *DDX3Y* genes were not considered significantly ($p = 1.000$).

Transcripts of *ZRSR2Y* gene had an accuracy of 82.6% (19/23), and a sensitivity of 75%, it correctly identified 12 of 16 male fetuses, whereas the specificity was 100%, all 7 female fetuses were correctly identified. And male and female predictive values were 100% and 63.6%, respectively (Table 3).

DISCUSSION

Many researches agreed that the placenta is a predominant source of the circulating fetal DNA in the maternal plasma (Bianchi, 2004). During pregnancy, placental membrane separates the maternal and fetal circulations. However, ccffDNA can cross the placental membrane by the destruction of fetal cells result from the interaction between regulated apoptosis of fetal cells and the maternal immune system (Bianchi, 2004; de Leon *et al.*, 2012).

In ruminants, the structure of placenta is different compared with human. Bovine species has a synepitheliocorial placenta which has no direct contact between the trophoblast and the maternal blood (Wooding, 1992). Furthermore, Poon *et al.* (2000) reported that the detection of fetal RNA in maternal plasma is quiet difficult because of the low amount of fetal RNA. For that reason, our study is performed by real-time qPCR because it has a large dynamic range of over five orders of magnitude and a sensitive detection rate of very low copy numbers of nucleic acids (Heid *et al.*, 1996; Finning and chitty, 2008).

In the present study, the Y-linked gene of *ZRSR2Y*, *USP9Y* and *DDX3Y* were examined in maternal plasma to determine the existence and consistency of their expression and to verify which genes may represent good candidates for sex determination especially in the RNA-based gene expression studies. Transcripts of the *ZRSR2Y*, *USP9Y* and *DDX3Y* genes were expressed in the plasma of the control group and the experimental group. However, only transcript of *ZRSR2Y* gene was matched up with the molecular testing and the true sex in control group, whereas transcripts of *USP9Y* and *DDX3Y* genes were expressed in the all plasma of males and females both the control group and the experimental group. Several studies

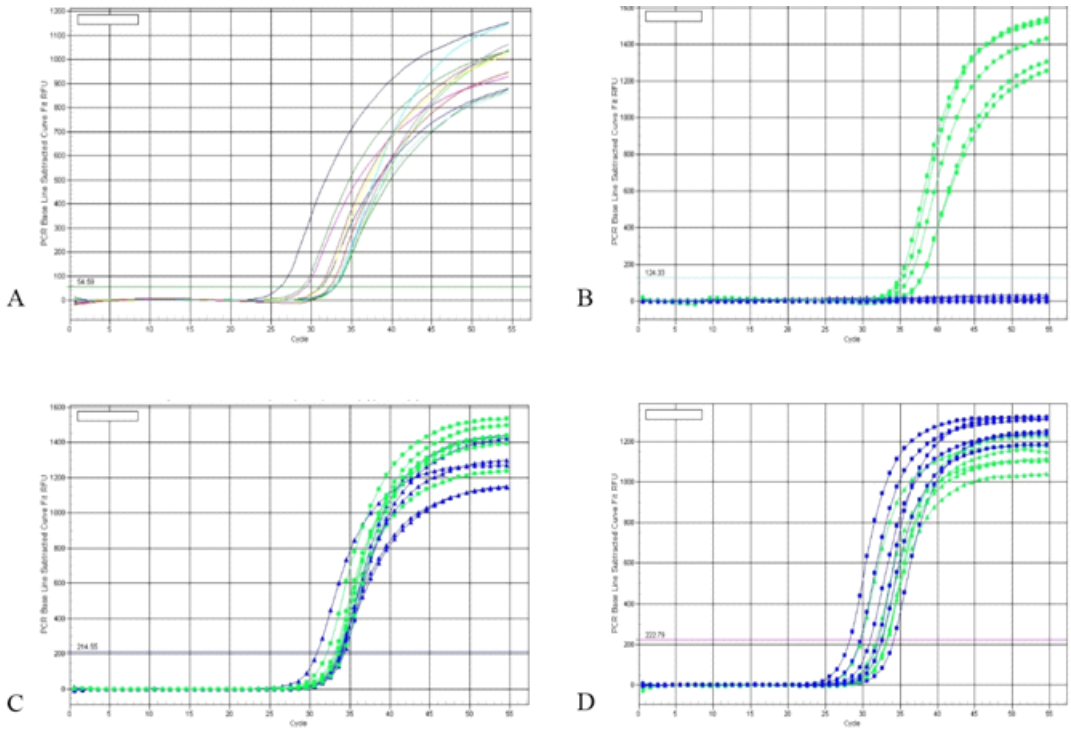

Fig. 1. Genes amplification plot of male genomic RNA as a positive control (squares) and female genomic RNA as a negative control (triangles) : (A) amplification plot GAPDH; (B) amplification plot ZRSR2Y; (C) amplification plot USP9Y; (D) amplification plot DDX3Y.

have reported the extraction of nucleic acids from maternal plasma for fetal sex determination using real-time PCR in various animals. Khorram Khorshid *et al*. (2013) used the *SRY*, *DYS14* and *DAZ* genes for fetal gender determination in the first trimester of pregnancy in human. Their results showed that sensitivity and specificity were 97.3% and 97.3%, respectively. Jimenez *et al*. (2003) reported that fetal sex prediction in rhesus monkeys has 100% sensitivity and no false-positive results. And de Leon *et al*. (2012) detected the *SRY* gene from extracted nucleic acids in blood plasma of pregnant mares. They confirmed that sensitivity and specificity were 90.9% and 95%, respectively. Similarly, Kadivar *et al*. (2013) used the circulating cell free fetal nucleic acids in ewe blood plasma to determine fetal sex using real-time PCR assay. Their results reported that the sensitivity and specificity were 100% and no false negative or false positive results. In the present study, transcripts of *ZRSR2Y* gene showed that an overall accuracy and sensitivity and specificity were 82.6%, 75% and 100% in experimental group, respectively. Hence, this result suggested that there was some possibility to influence the rate of gene

Table 2. Accordance between the sex of molecular testing and the true sex by ZRSR2Y, USP9Y and DDX3Y genes using real-time PCR in control group

Samples	The true sex	Result of ZRSR2Y	Result of USP9Y	Result of DDX3Y
1	Male	Male	Male	Male
2	Male	Male	Male	Male
3	Male	Male	Male	Male
4	Female	Female	Male	Male
5	Female	Female	Male	Male
6	Female	Female	Male	Male

Table 3. Sensitivity, specificity, male predictive value (MPV), female predictive value (FPV) and accuracy of fetal sexing by real-time PCR method using ZRSR2Y gene

n	The molecular testing results	
	Male	Female
	16	9
Sensitivity	75	
Specificity	100	
MPV	100	
FPV	63.6	
Accuracy	82.6	

expression. Hamilton *et al*. (2012) reported that primer selection is obviously an important consideration. Their results showed that there were practical differences in expression, even though different pairs of primers were targeting the same gene, but different regions. Also, Revil *et al*. (2010) suggested that there is an excessive expression of genes that seems to be required for proper development.

To our knowledge, this is the first investigation demonstrating the existence of ccffRNA in plasma of pregnant cows for fetal sex determination. Although our study has less accuracy and sensitivity compared with prior studies, this discovery provides a novel potentiality for prenatal fetal sex determination of ccffRNA with non-invasive process and an opportunity for detecting genetic diseases by expression of transcripts of target genes. Therefore, we are planning to choice the correct target sequence for selection of optimal primer to increase the sensitivity and the accuracy of the gene for further study. Also, we intend to confirm the correct detection point of ccffRNA in maternal plasma of pregnant cows, and measure the quantitative changes of ccffRNA during pregnant period in maternal plasma.

In conclusion, our results confirmed that the real time PCR technique, as a noninvasive and cost-efficient method, is possible to determination fetal sex in the bovine species using circulating cell free RNA in maternal plasma and *ZRSR2Y* gene might be a good candidate for the RNA based sex determination work.

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