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Original Research

Developing a Nested Real-Time PCR Assay for Determining Equine Fetal Sex Prenatally

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ABSTRACT

The aim of this investigation was to develop a real-time polymerase chain reaction (PCR) assay for detecting circulating cell-free fetal DNA in Arabian pregnant mare plasma and predicting fetal sex through identifying SRY gene. Peripheral blood samples of 28 pregnant mares were collected into tubes containing EDTA, at a single time point, between 8 and 20 weeks of gestation. Circulating cell-free fetal DNA was extracted from 3 mL of maternal plasma. Using outer and inner primers, real-time PCR was performed for the SRY gene and for the glyceraldehyde-3-phosphate dehydrogenase gene as the internal control. Amplicons were labeled as positive, negative, or inconclusive according to threshold cycle and melting curve analysis. Of the total 28 Arabian pregnant mares, 16 were carrying male and 12 were carrying female fetuses. On first round of real-time PCR, results were inconclusive for SRY gene; however, on the second round, conclusive results were obtained. In total, the accuracy of test was 88% (22/25) (confidence interval [CI]: 65.5, 95.6); it had a sensitivity of 85.7% (CI: 65.6, 92.5), whereas its specificity was 90.9% (CI: 65.3, 99.5). We had a positive predictive value of 92.3% (CI: 70.7, 99.6) and a negative predictive value of 83.3% (CI: 59.9, 91.2). To conclude, SRY gene can permit detection of equine fetal sex with a good accuracy by analyzing cell-free fetal DNA in maternal plasma just after 8 weeks of gestation.

1. Introduction

Accurate diagnosis of fetal sex in domestic and zoo species has substantial research and commercial applications. For equine breeders, fetal sex prediction is valuable in decision making and commercial strategies [1]. For years, equine fetal sex determining has been conducted through transrectal ultrasonography to identify and locate the genital tubercle between 54 and 84 days of gestation [2]. This embryonic structure develops on the fetal midline, between the hind legs [3]. At around 55 days of pregnancy, genital tubercle of male fetus moves toward the umbilicus to become the penis, whereas in female fetus, it moves toward the anus to become the clitoris [3].

Nevertheless, early fetal sex determination by ultrasonography has limitations: First, it requires a very experienced inspector, and second, in horses, the positioning of the transducer on the fetus and the visualization of its structures are hampered by the large amount of allantoic fluid and high fetal mobility due to long umbilical cord. Although the use of color Doppler ultrasonography and transabdominal techniques has made possible the identification of sex in older equine fetuses [4], again, the

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evaluator should have sufficient experience and require enough time to rule out potential artifacts and make an accurate diagnosis.

In contrast, molecular sexing based on analysis of circulating cell-free fetal DNA (ccffDNA) present in maternal plasma using the polymerase chain reaction (PCR) technique can be a reliable, sensitive, and practical alternative [5]. Sex typing by PCR has been used for preimplantation embryos in horses using the SRY, AMELX-AMELY, and ZFX/ZFY genes [6,7]; also, fetal molecular sexing in the final 3 months of pregnancy has been described in Thoroughbred mares [8]. Moreover, molecular fetal sex determination in human based on identifying sequences derived from the Y chromosome has a reported accuracy rate of approximately 80% and 100% by 7 and 20 weeks of gestation, respectively [1], and depending on the protocol and methods used, accuracy increases with increasing gestational age [9,10].

However, isolation of equine cell-free fetal DNA (cffDNA) in an overwhelming background of maternal cell-free DNA is a considerable technical challenge in itself: Overall concentration of circulating cell-free DNA is relatively low (cell-free fetal DNA represents only 3% to 6% of the total free DNA in maternal plasma throughout pregnancy) [11]; cell-free fetal DNA molecules are outnumbered by maternal plasma cell-free DNA molecules [12]. Furthermore, compared with human placenta (hemochorial placenta), horses have a relatively noninvasive epitheliochorial placenta [13]. Hence, it is likely that the passage of fetal DNA into the equine maternal blood would be scarce and should be amplified.

Despite the histological differences in fetomaternal layers, researchers have used PCR assays during pregnancy to predict fetal sex in farm species. Among these, prenatal fetal sex determining in horse has its special economic importance and is highly regarded by producers of this species [14,15]. To our knowledge, only one study has evaluated the possibility of using cffDNA to determine horse fetal sex in the last 3 months of gestation [8]. In the present study, the objective was to use a PCR assay to detect ccffDNA in plasma of Arabian pregnant mares and predict fetal sex through identifying SRY gene.

2. Materials and Methods

2.1. Animals and Sample Collection

During August to November 2014, 28 pregnant Arabian mares (*Equuscaballus*) with singleton fetuses were selected. Approximately 10 mL of peripheral blood was collected from each pregnant mare, at a single time point, between 8 and 20 weeks of gestation (Table 1). Gestational age was obtained by natural mating dates. Blood samples were collected in tubes containing EDTA and kept at 4°C before being sent to the laboratory. In all cases, fetal sex was unknown at the time of blood sampling and was subsequently confirmed by direct clinical examination after birth. All specimens were coded to facilitate blind testing. The study was approved by our University's Ethical Committee, and the owners of all participating animals gave informed written consents.

Table 1
Amplification of fetal GAPDH and SRY sequences from maternal plasma.

Row	Gestation (mo)	GAPDH	Outer Primer	Inner Primer
Mares with male fetuses				
1	2	+	−	+
2	2.5	+	−	+
3	2.5	+	−	+
4	3.5	−	−	−
5	3.5	+	−	+
6	3.5	+	−	+
7	3.5	+	−	+
8	4	+	−	+
9	4	+	−	+
10	4	+	−	+
11	4	+	−	−
12	5	+	+	+
13	5	+	−	+
14	5	+	−	+
15	5	−	−	−
16	5	+	−	−
Mares with female fetuses				
17	3	−	−	−
18	3	+	−	−
19	3	+	−	−
20	3.5	+	−	+
21	3.5	+	−	−
22	3.5	+	−	−
23	4	+	−	−
24	4	+	−	−
25	5	+	−	−
26	5	+	−	−
27	5	+	−	−
28	5	+	−	−

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.
The sign plus (+), means the case showed amplification in PCR.

2.2. Sample Processing and cffDNA Extraction

Blood samples were processed within a few hours after collection. To separate plasma from the cellular fraction, samples were centrifuged at low speed (10 minutes at 3,500*g*), and the supernatant was transferred to fresh tubes, during which great care was taken to ensure that the buffy coat or the blood clot remained intact. The plasma samples then underwent a second centrifugation step at high speed (10 minutes at 10,000g and 4°C) to remove all residual intact cells. The recentrifuged plasma was collected into fresh tubes and stored at −80°C until DNA extraction. Cell-free fetal DNA was extracted from 3 mL of maternal plasma using the DNP kit (Sinaclon, Karaj, Iran) according to the manufacturer's protocol with slight modifications. Reagents were increased proportionally to accommodate the 3-mL sample size. Because the manufacturer indicates to dilute the DNA in 200 μL, it was diluted in 100 μL elution buffer (provided in the kit) to increase the concentration. DNA samples were kept at −80°C until PCR analysis.

2.3. Real-Time PCR

The real-time PCR assays were performed in the real-time PCR equipment (Rotor-Gene Q System, Qiagene, Foster City, CA). The Y chromosome–specific region, a single-copy gene (SRY), was systematically analyzed in all cases. The glyceraldehyde-3-phosphate dehydrogenase