Diagnostic Performance of RT-PCR for Fetal Sex Determination Using Cell-Free Fetal DNA in Maternal Plasma during the Last Third of the First Trimester in Mares

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Abstract

Accurately determining fetal sex early in mares is essential for horse breeding. In this study, we investigated the detection of cell-free fetal DNA (cffDNA) in horse serum using RT-PCR during pregnancy. Blood samples were obtained from 30 pregnant mares in the last third of the first trimester of gestation at stables located in the provinces of Semnan, Golestan, and Tehran. In addition, blood samples were taken from one male foal and one female foal with no history of mating to serve as positive and negative controls, respectively. The phenotypic sex of the foals was recorded after parturition and compared with RT-PCR results performed on maternal plasma to evaluate the accuracy of fetal sex determination using the sex-determining region Y (SRY), equine Y⁻ linked microsatellite marker YH12 (Eca.YH12), and microsatellite locus TKY270 on the equine X chromosome (TKY270) gene markers. A total of 30 pregnant mares were sampled, with 27 carrying to term and three aborting. Fetal sex was confirmed postnatally (11 males, 16 females). The SRY marker had a sensitivity of 72.7%, specificity of 100%, and accuracy of 88.9%. Eca.YH12 marker yielded the same results as SRY and did not enhance sensitivity when used in combination. The X-linked marker TKY270 was detected in all samples, indicating that the samples were of high quality and could serve as an internal control. These markers supported our results, strengthening the diagnostic process. RT-PCR for the SRY gene detected cffDNA in the last third of the first trimester and worked well, especially for identifying female fetuses. These findings were further supported by the results obtained from the Eca.YH12 and TKY270 genetic markers.

Keywords: cffDNA, Fetal sex determination, Mare, RT-PCR, SRY gene

1. Introduction

Sex determination is a fundamental biological process in all sexually reproducing eukaryotes (1). In many species, sex is genetically established through the expression of one or more genes located on the sex chromosomes. Among vertebrates, the most common systems are male heterogamety (XX females and XY males) and female heterogamety (ZW females and ZZ males). Sex chromosomes have evolved independently multiple times, typically emerging when a member of an autosomal pair acquires a gene or allele responsible for sex determination. The frequency of new sex chromosome emergence varies greatly across species. Notably, frequent turnovers, defined as changes in the location or identity of sex-determining genes, have been observed in several taxa, including many species of fish and amphibians (2, 3). Over the years, scientists have developed several methods to determine genetic sex, including karyotyping, testing for H-Y antigens, and analyzing enzymes associated with the X chromosome (4). In addition to molecular methods, some studies have examined hormonal indicators such as maternal testosterone concentration to predict the sex of the fetus in horses (5). However, PCR-based methods have taken the lead. Why? Because they are they are faster, more accurate, and more reliable than the older techniques. Additionally, PCR is relatively easy to use and budget-friendly, making it a popular choice—especially in commercial settings (4). Fetal DNA fragments, originating from the trophoblastic cells covering the chorionic villi, are released into the maternal circulation following trophoblast apoptosis. Additionally, mRNA and DNA fragments derived from placental cell apoptosis contribute to a minor portion of fetal genetic material detectable in maternal blood (6). It has been well established that circulating cffDNA can be detected in maternal blood throughout all stages of pregnancy (7). cffDNA becomes detectable as early as the fifth week of gestation and remains present until delivery (8). Due to its short half-life (approximately 16.3 minutes), cffDNA is rapidly cleared from the maternal bloodstream within a few hours postpartum, minimizing interference from previous pregnancies. This transient nature makes cffDNA an ideal target for non-invasive prenatal genotyping studies (9). De Leon, Campos (10) provided the first evidence of circulating cffDNA in the plasma of pregnant mares, opening a new avenue for non-invasive prenatal diagnostics, including fetal sex determination. Molecular techniques based on cffDNA in maternal blood present a highly accurate and non-invasive alternative to traditional invasive procedures. Beyond sex determination, these methods have the potential to facilitate early diagnosis of genetic disorders and pregnancy-related complications (11).

Early, non-invasive determination of fetal sex in horses has the potential to become a valuable tool in equine breeding and genetic diagnostics. By analyzing cffDNA present in maternal plasma, fetal sex can be identified during the first trimester of gestation. Determining a foal's sex early is economically crucial in the horse world, as it significantly impacts a pregnant mare's value. This study investigates the diagnostic performance of RT-PCR to detect cffDNA in the maternal plasma of pregnant mares during the last third of the first trimester of gestation.

2. Material and methods

2.1. Study Population

The study population consisted of horse breeding farms located in the provinces of Semnan, Golestan, and Tehran. Sampling was conducted over an 8-month period in two phases, from December 2019 to March 2021. A total of 30 pregnant mares were examined to determine the fetal sex during the last third of the first trimester of pregnancy. The results regarding the sex of the fetus (male or female) were used to evaluate the accuracy of the test after the mares gave birth and were subsequently recorded.

2.2. Sampling

Blood samples were collected from 30 pregnant mares during the last third of the first trimester of gestation to determine fetal sex. Additionally, blood was drawn from one male and one female foal

with no prior breeding history to serve as positive and negative control samples, respectively. The actual fetal sex (male or female) was recorded after parturition to assess the accuracy of the test. Blood was collected from the jugular vein and transferred into tubes containing EDTA-anticoagulant. The samples were quickly transported to the laboratory on ice. To isolate the plasma, the EDTA-treated blood samples were centrifuged at 4,000 RPM for 10 minutes.

2.3. PCR analysis

The separated plasma was stored at -80°C until it was time for DNA extraction. DNA was extracted from the plasma samples, as well as from the genomic DNA of the control mare and female blood samples, using a commercial DNA extraction kit (GeNet Bio, Korea). The amount and purity of the extracted DNA were assessed using a NanoDrop spectrophotometer. To identify the SRY gene marker, PCR was carried out on DNA obtained from the plasma of pregnant mares. The primers used for amplification of the SRY gene were forward primer 5′-CGCCAGCATAGATCACAGAA-3′ and reverse primer 5′-CGCAAGGTAGCTGAAAGACC-3′, as previously described by De Leon, Campos (10). To verify the reliability of the SRY gene detection, additional genetic markers were included in the analysis: ECAY (a Y-chromosome-specific marker), ECAX (an X-chromosome marker), and an autosomal marker to serve as a positive control for PCR. Specifically, the Eca.YH12 marker (291 bp), a Y-linked sequence specific to horses, was used as a secondary indicator of male DNA; TKY270 (274 bp) was used to detect X-chromosomal sequences; and the GAPDH gene (150 bp), a commonly used housekeeping gene, served as an autosomal internal control. The primer sequences for these markers were as follows:

The primers used for TKY270 were 5′-CTGCTTTAGAGAAACAAACT-3′ (forward) and 5′-CCATGGTGAGAAAAATGAGA-3′ (reverse), as described by Wallner, Piumi (12). For GAPDH, the forward primer was 5′-GCCGTAACTTCTGTGCTGTG-3′, and the reverse primer was 5′-AATGAAGGGGTCATTGATGG-3′, as reported by Kakoi, Tozaki (13).

For GAPDH, the primers used were 5′-GCCGTAACTTCTGTGCTGTG-3′ (forward) and 5′-AATGAAGGGGTCATTGATGG-3′ (reverse), as described by De Leon, Campos (10).

2.4. Statistical analysis

The outcomes of RT-PCR fetal sex determination were systematically compared with the phenotypic sex identified at birth during the latter part of the first trimester of gestation. The results were subsequently presented as percentages to facilitate clear interpretation. To evaluate the performance of the SRY gene detection method in the last third of the first trimester of gestation, the following metrics were calculated using SAS software (version 9.1, SAS Institute, 1997): sensitivity, specificity, positive predictive value, negative predictive value, overall accuracy, and method efficiency**.**

3. Results

A total of 30 blood samples were collected from mares during the latter portion of the first trimester of gestation, with pregnancy confirmation achieved through ultrasonography. Among these mares, 27 successfully delivered foals, while three unfortunately experienced abortions. The findings related to mare parturition, along with the outcomes of gene amplification for SRY, TKY270, and Eca.YH12 and GAPDH, derived from the analyzed blood samples, are detailed in Table 1.

Table 1. Results of mare parturition and the outcomes of gene amplification for SRY, TKY270, Eca.YH12, and GAPDH from blood samples collected from pregnant mares

Female					Male				
No	SRY	TKY270	Eca.YH12	GAPDH	No	SRY	TKY270	Eca.YH12	GAPDH
1	-	+	-	+	1	-	+	-	+
2	-	+	-	+	2	+	+	+	+

3	-	+	-	+	3	+	+	+	+
4	-	+	-	+	4	+	+	+	+
5	-	+	-	+	5	-	+	-	+
6	-	+	-	+	6	+	+	+	+
7	-	+	-	+	7	+	+	+	+
8	-	+	-	+	8	-	+	-	+
9	-	+	-	+	9	+	+	+	+
10	-	+	-	+	10	+	+	+	+
11	-	+	-	+	11	+	+	+	+
12	-	+	-	+					
13	-	+	-	+					
14	-	+	-	+					
15	-	+	-	+					
16	-	+	-	+					

An analysis of fetal sex following parturition indicated the presence of 11 male foals and 16 female foals. A comparison between the postnatal sex and the gene amplification results for SRY and TKY270 was not conducted for three mares that gave birth to male foals. In male foals (n=11), SRY, a Y-chromosome marker, was amplified in 8 of 11 cases (72.7%), with no amplification in the remaining three. Eca.YH12, another Y-linked marker, showed identical results. The X-linked marker TKY270 and the autosomal control GAPDH were amplified in all male samples. In female foals (n=16), neither SRY nor Eca.YH12 was amplified, but both TKY270 and GAPDH were present in all samples. These findings confirm that TKY270 and GAPDH are reliable internal controls for DNA quality and amplification (Table 1). Diagnostic performance was then calculated. Using SRY alone, sensitivity for detecting male fetuses was 72.7%, specificity 100%, positive predictive value (PPV) 100%, negative predictive value (NPV) 84.2%, and overall accuracy 88.9% (Table 2).

Table 2. Fetal sex determination in 27 pregnant mares using RT-PCR targeting the SRY and Eca.YH12 genes from free fetal DNA in maternal blood plasma during the last third of the first trimester of gestation and confirmed after parturition

Marker	True Positive (Male correctly identified)	False Negative (Male misclassified)	True Negative (Female correctly identified)	False Positive (Female misclassified)	Total
SRY	8	3	16	0	27
Eca.YH12	8	3	16	0	27

When combining SRY and Eca.YH12 in an “either/or” approach (male if any Y marker is positive), the results were identical in this dataset, as Eca.YH12 did not rescue the three SRY-negative male cases (Table 3).

Table 3- Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of fetal sex determination based on the detection of free fetal DNA by RT-PCR during the last third of the first trimester of gestation in pregnant mares

Marker	Accuracy	Sensitivity	Specificity	PPV	NPV
SRY	88.9%	72.72%	100%	100%	84.21%
Eca.YH12	88.9%	72.72%	100%	100%	84.21%

Figures 1 and 2 illustrate the amplification curves for samples containing the SRY and TKY270 genes in male fetuses. In contrast, Figure 3 presents the amplification curve for samples with the Eca.YH12 gene in female fetuses.

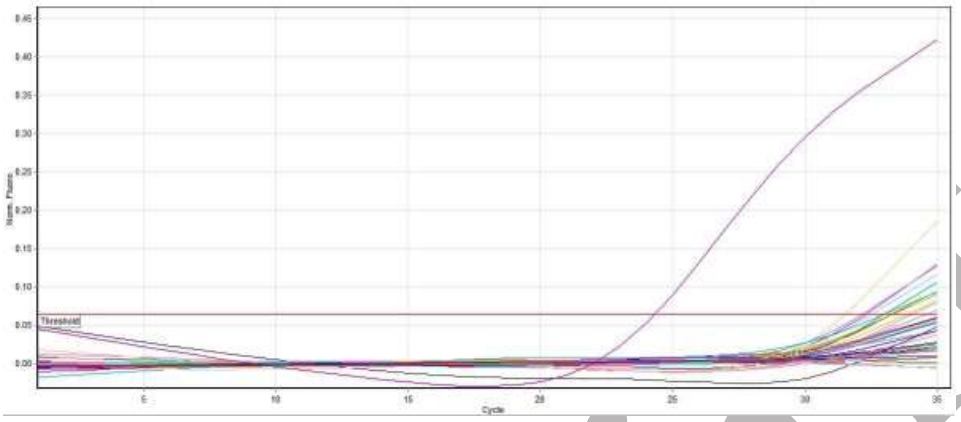

Figure 1. Real-Time PCR Analysis in some fetal DNA samples extracted from the maternal plasma. Amplification Plot of SRY.

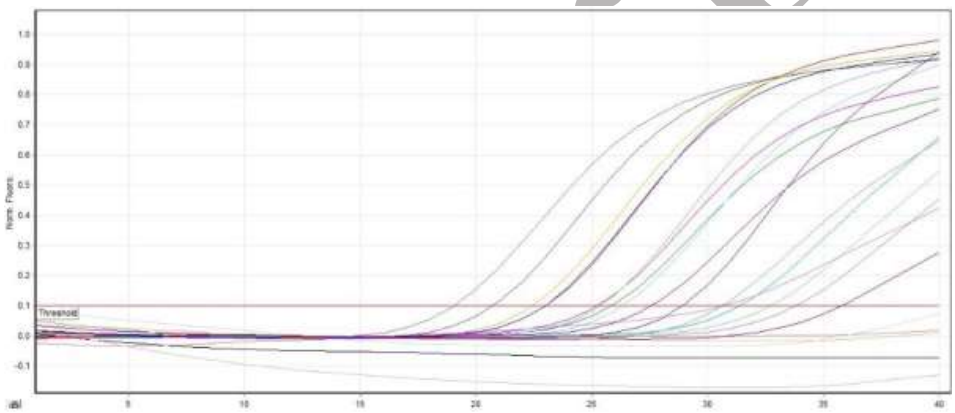

Figure 2. Real-Time PCR Analysis in some fetal DNA samples extracted from the maternal plasma. Amplification Plot of TKY270.

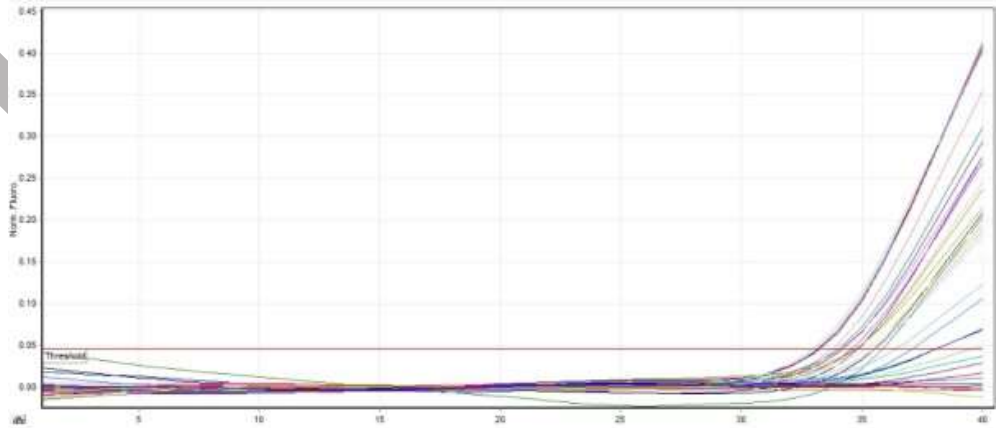

Figure 3. Real-Time PCR Analysis in some fetal DNA samples extracted from the maternal plasma. Amplification Plot of Eca.YH12.

4. Discussion

The presence of cell-free nucleic acids in blood was first reported by Mandel and Metais in 1948. Although initially regarded as a significant scientific discovery, it received limited attention until 1994, when the detection of mutated RAS gene fragments in the plasma of cancer patients brought renewed interest to the field (14). Lo, Corbetta (9) successfully isolated fetal-derived nucleic acids from maternal plasma using Y-chromosome-specific sequences. They demonstrated that these nucleic acids could be detected in maternal blood as early as the initial weeks of gestation and are rapidly cleared postpartum, minimizing interference from previous pregnancies. This fetal DNA enters maternal circulation primarily through apoptosis and immunologic or physical degradation of placental and fetal cells.

Molecular approaches utilizing fetal DNA in maternal plasma provide a non-invasive and reliable alternative to traditional invasive procedures. Such techniques not only enable prenatal sex determination but also have broader applications in detecting genetic abnormalities and pregnancy-related complications (11). In equine reproduction, non-invasive fetal sexing based on the analysis of cffDNA extracted from maternal plasma is a promising tool that can contribute to early genetic diagnosis. De Leon, Camops (10) introduced a notable approach for fetal sexing in horses using cffDNA. In their study, SRY gene amplification via PCR in 20 pregnant mares during the third trimester yielded 72.7% sensitivity and 85% overall accuracy. When second-round PCR and qPCR were employed, these values improved to 90.9% sensitivity and 95% accuracy, respectively.

Kadivar, Tafti (16) further expanded this research by employing real-time PCR to detect SRY in 28 pregnant Arabian mares between 8 and 20 weeks of gestation. The test achieved an overall accuracy of 88%, with 85.7% sensitivity and 90.9% specificity. The PPV and NPV were reported at 92.3% and 83.3%, respectively.

Similar advancements have been reported in bovine fetal sex prediction using maternal plasma DNA. JF (17) successfully identified fetal sex by amplifying the SRY gene in pregnant cows between days 30 and 59 of gestation, with an overall accuracy of 60%. Later, Wang, Cui (18) employed nested PCR on 110 plasma samples from pregnant cows and reported fetal sexing accuracies of 100% for male fetuses (43/43) and 91% for female fetuses (61/67). Likewise, Da Cruz, Silva (19) achieved 88.6% accuracy in sex prediction by amplifying Y-specific sequences in plasma from 35 pregnant cows. The estimated accuracy rose to 99.9% when testing was conducted at or after day 55 post-fertilization.

Further validation was provided by Lemos, Takeuchi (20), who used TSPY gene primers to accurately match PCR results with phenotypic sex in all 47 male and 37 female bovine fetuses studied.

In sheep, Kadivar, Hassanpour (21) reported the successful application of real-time PCR targeting the SRY gene for sex determination in 46 pregnant ewes between the second and fifth months of gestation. Their approach showed 100% sensitivity and specificity, with no false positives or negatives. Quantitative real-time PCR further demonstrated a significant increase in fetal DNA levels as pregnancy progressed. Similarly, biometric variations in the genitalia of other livestock species, such as the Arbia goat, have been examined to enhance understanding and diagnosis of reproductive status and pregnancy (22). Kadivar, Hassanpour (23) introduced a novel approach using the amelogenin gene for fetal sexing in sheep. In a group of 45 pregnant ewes (8–18 weeks of gestation), the test demonstrated 96.5% sensitivity, 87.5% specificity, and 93.3% overall accuracy. The relative quantification showed a significantly higher level of cffDNA in ewes beyond 12 weeks of gestation compared to earlier stages.

Our findings revealed that no false-positive results occurred in fetal sex determination using cffDNA across all trimesters, aligning with previous research in mares (24, 25) and ewes (21, 23), which also utilized the detection of TKY270 and Eca.YH12 gene fragments alongside the SRY marker. However, some other studies have reported occurrences of false positives (10, 13, 16, 17). On the other hand, the false-negative results we found were similar to those reported in previous studies (10, 13, 16, 24). Interestingly, these false negatives became less common as the pregnancy advanced in mares, which

helped improve the accuracy of the test. This trend was also supported by our observation that the Eca.YH12 gene fragment was missing in every sample that gave a false-negative result.

Detection of cffDNA using an RT-PCR assay targeting the SRY gene during the last third of the first trimester of pregnancy in the studied mares demonstrated acceptable diagnostic performance (including accuracy, specificity, and positive predictive value), especially for female fetus detection. Additionally, the identification of the Eca.YH12 and TKY270 gene markers have further confirmed these findings.

The main limitation of our study was the occurrence of these false negatives in male fetuses. The most likely explanations include a low concentration of cell-free fetal DNA (cffDNA) during the last third of the first trimester of gestation, as well as the single-copy nature of SRY and Eca.YH12, and potential technical constraints in DNA extraction or PCR efficiency. Previous reports in horses have emphasized similar challenges: for example, De Leon and Campos (10) reported a sensitivity of 72.7% using SRY PCR and an increased sensitivity of up to 90.9% using nested-PCR or qPCR approaches. Kadivar, Tafti (16) employed nested real-time PCR for mares between 8 and 20 weeks of gestation, achieving a sensitivity of 85.7% and an accuracy of 88%. Increasing gestational age, adopting quantitative or nested PCR, or utilizing multi-copy Y markers, such as TSPY, may enhance sensitivity in future studies.

The auxiliary markers had distinct roles. Eca.YH12 mirrored the performance of SRY in this dataset and, therefore, did not enhance sensitivity when combined; however, it provided methodological robustness as an independent Y target. TKY270, being X-linked, was consistently amplified in all samples and functioned as a reliable internal control, ensuring that negative Y results reflected the actual absence of signal rather than amplification failure.

Taken together, our results support the feasibility of early fetal sex determination in horses using non-invasive cffDNA testing during the last third of the first trimester. However, the occurrence of false negative results highlights the importance of gestational age and technical optimization. A practical diagnostic system was that in a case of positive detection of either of the marker (SRY or Eca.YH12), male sex should be noted; on the other hand, in a situation where neither of the markers (Y markers) could be detected on the sample, the status of the sample should be female sex, as long as internal controls (TKY270, and GAPDH) are satisfactorily identified. The assay with unknown ambiguity must be retried at a slightly later stage.

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Authors' Contribution

Study concept and design: M.A.H., and R.N.S
Acquisition of data: M.A.H., R.N.S., and S.T
Analysis and interpretation of data: M.A.H., and H.S
Drafting of the manuscript: M.A.H.
Critical revision of the manuscript for important intellectual content: M.A.H., and H.S
Statistical analysis: M.A.H.
Administrative, technical, and material support: M.A.H., R.N.S., and S.T

Ethics

This study has fully observed ethical considerations.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Funding/Support

We have no financial interests related to the material in the manuscript.

AI Use Declaration

The generative AI tool used was ChatGPT (GPT-4, OpenAI), which was applied during manuscript writing and editing in June 2025, primarily for language refinement and minor grammatical corrections. According to Grammarly Premium, just 5% of the content was flagged as potentially AI-generated. The tool played no role in the creation of scientific content, data analysis, interpretation, or study design.

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