Veterinary Integrative Sciences 2022; 20(2): 267-277. DOI; 10.12982/VIS.2022.021

Vet Integr Sci

Veterinary Integrative Sciences

ISSN; 2629-9968 (online)

Website; www.vet.cmu.ac.th/cmvj

Research article

Early determination of fetal sex in singleton pregnant ewes by real-time polymerase chain reaction and ultrasonography, a comparative study

Karar Yaser Hussain1 and Abbas Fadhil Daham[1,*]

[1]Department of Surgery and Obstetrics, Faculty of Veterinary Medicine, University of Al-Qadisiyah, Diwaniya city 58001, Iraq

Abstract

This study aimed to compare the efficiency of real-time polymerase chain reaction (PCR) and ultrasonography in fetal sexing of early singleton pregnant ewes. Forty-five ewes were examined using ultrasonography to confirm singleton pregnancies and to diagnose the sex of the conceptus. Blood samples were collected from all tested ewes for SRY and AMLX detection in circulating cell-free fetal DNA of these maternal blood specimens using real-time PCR. The definite sex of the fetuses was confirmed by the true sex of offspring after birth. The total percentages of correctly diagnosed cases in both diagnostic techniques, male and female fetuses, and the percentages of true fetal sex diagnosis regarding the gestation periods of tested animals were counted and compared. The results demonstrate the superiority of real-time PCR in accurate diagnosis compared to ultrasound in all the tested parameters. The total percentage of fetal sex diagnostic technique accuracy was 95.55% (43/45) and 48.89% (22/45) for real-time PCR and ultrasonography, respectively. The percentages of the accuracy of detected male and female fetuses were 38.46% (10/26) and 63.16% (12/19), and 92.31% (24/26) and 100% (19/19) for ultrasonography and real-time PCR, respectively. The accuracy of fetal sexing was 66.66% (6/9), 42.85% (3/7), 36.36% (4/11), 50% (9/18), and 88.89% (8/9), as well as 85.71% (6/7), 100% (11/11), and 100% (18/18) in the gestation periods of 50–55, 56–60, 61–65, and 66–70 days for ultrasonography and real-time PCR, respectively. In conclusion, we assessed the potential of early fetal sex diagnosis in singleton pregnant ewes by real-time PCR and ultrasonography, identifying the significant superiority of real-time PCR.

Keywords: Fetal sex, Pregnant ewes, Real-time PCR, Ultrasonography

Corresponding author: Abbas Fadhil Daham, Department of Surgery and Obstetrics, Faculty of Veterinary Medicine, University of Al-Qadisiyah, Diwaniya city—58001, Iraq Email: abbas.daham@qu.edu.iq Tel: 0096 47729126962

Funding; Department of Surgery and Obstetrics, Faculty of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniya, Iraq.

Article history; *received manuscript: 8 August 2021, revised manuscript: 21 September 2021, accepted manuscript: 10 February 2022, published online: 14 February 2022*

Academic editor; *Korakot Nganvongpanit*

INTRODUCTION

Early pregnancy identification is critical for the ovine business to reduce the expenses of nutrition supplements for non-pregnant ewes, aids reproduction maintenance, and adds to the selection process by identifying subfertile animals (Paula et al., 2003). Detecting pregnant ewes with teaser rams and discovering animals in estrus that are not pregnant after breeding or have had an early embryonic loss are both time-consuming and labor-intensive processes (Arashiro et al., 2018). Ultrasonography is the most widely used procedure for this purpose; nevertheless, accurate pregnancy identification in sheep may require approximately 25 additional days after mating (Dal et al., 2019). Embryo sexing is a valuable technique for producers because it helps them to manage their breeding stock more efficiently and effectively (Tavares et al., 2016). This may be achieved using ultrasonography with reasonable accuracy, particularly in advanced pregnancies. However, this efficiency declines, and the diagnostic errors increase in early pregnancies, requiring substantially greater experience and utilizing a longer time in large-sized livestock, particularly in small ruminant herds. Recently, using several molecular methods for early fetal sex detection has garnered attention in several animal species, including goats, cattle (Quirino et al., 2010), sheep (Asadpour et al., 2015), and Arabian camels (Abdulla et al., 2020), and also in humans (D'Aversa et al., 2018). These molecular techniques used for fetal sex detection are based on detecting specific genes related to fetal sex in circulating cell-free fetal DNA (ccffDNA) in the maternal blood, such as SRY and amelogenin (AML). Conventional polymerase chain reaction (PCR) and real-time PCR are the most commonly used molecular techniques. These techniques have several advantages over ultrasonography and other classical methods, namely, high accuracy, ease of use, inexpensiveness, lower time consumption, and simple sample collection. In human and bovine reproduction, these new and definite techniques have been well-developed and widely applied either for fetal or early embryonic sexing or early in the invasive diagnosis of a wide range of hereditary and teratological conditions. Transrectal ultrasound (depending on localizing the genital tubercle) is a commonly used technique for fetal sex diagnosis in the livestock industry. For example, transrectal ultrasound (Neto et al., 2010) has been used to detect the fetal sex in sheep on days 60–69 of gestation; conversely, fetal sexing using transrectal ultrasound necessitates consideration of the age and breed of the tested animals, as well as the use of expensive technology (Asadpour et al., 2015). Moreover, in many studies, fetal DNA in the maternal blood comprises 3.4% and 6.2% of total DNA in early and advanced gestation, respectively (Tein et al., 1998). Fetal cells enter the maternal bloodstream during the first trimester and remain there throughout pregnancy (Ramos, 2006). The use of free fetal DNA in maternal circulation to detect the fetal sex in ewes is a non-invasive and valuable method (Allen et al., 2017). The ccffDNA has served as a vital source for determining fetal sex and genetic screening throughout pregnancy (Asadpour et al., 2015). Both ccffDNA and intact fetal cells can be collected for non-invasive early genetic diagnostics (Bischoff et al., 2002). The SRY, AMLY, and AMLX genes have been used to define sheep molecular sexing (Kadivar et al., 2013). SRY represents a single copy gene on the Y chromosome responsible for determining male sex during

embryogenesis in most placental mammals (King et al., 2007). Owing to its placement on the highly unstable Y chromosome, SRY is a weak and partially crippled gene, and its conformation and regulatory sequence may be destroyed (Kashimada and Koopman, 2010). AML is frequently employed in both animal and human sex diagnostic studies, and depending on the difference in the size of the AML gene between both sex chromosomes, AML is employed for forensic casework, prenatal diagnosis, and fetal sexing prenatally (Grzybowsk et al., 2006). Several studies have proven that different sets of AML gene primers provide credible biomarkers for sex detection in various domestic animals, including cows, swine, does, and ewes (Quirino et al., 2010). Real-time PCR tests have a high sensitivity for detecting low levels of fetal DNA quantities (Bischoff et al., 2002). PCR for fetal sexing is cheap (the average cost of electrophoresis and PCR is less than 2$/sample) (Tavares, et al., 2016). A molecular procedure using fetal DNA obtained from the maternal blood can be an alternative method for ultrasonography in sexing embryos (Tavares, et al., 2016). This study aimed to compare the efficiency of real-time PCR and ultrasonography in the fetal sexing of early singleton pregnant ewes to determine the best and the most accurate method for early fetal sexing in sheep.

MATERIALS AND METHODS

Early diagnosis of pregnancy and fetal sex by ultrasonography

Forty-five farmer-raised Iraqi singleton pregnant ewes aged 1.5–3 years had been confirmed by days 50–70 of conception. The sex of the fetuses was determined by the location of the genital tubercles (Erdogan, 2012) when female fetuses had tubercles closer to the tail, and males had tubercles closer to the umbilical cords (Reichenbach et al. 2004). The sexing of all identified fetuses was performed in conjunction with scanning to ascertain the number of fetuses. During the inspection, the location of the genital tubercle was precisely determined. Pregnancy was diagnosed using a real-time B-mode ultrasonic scanner with a 5-MHz external probe (L80, China). Before scanning early the following day, water and food were denied for 12 h. Scanning was performed in the fleece less inguinal region of the animal. One person held the animal slightly against the fence in a standing stance. Just at the scan for correct instrument placement, one sheep's hindquarters was packed away. During each session, an ultrasonic partnering gel was placed on the probe to ensure proper friction and remove air from between the probes and the animal's skin (Figure 1: a & b).

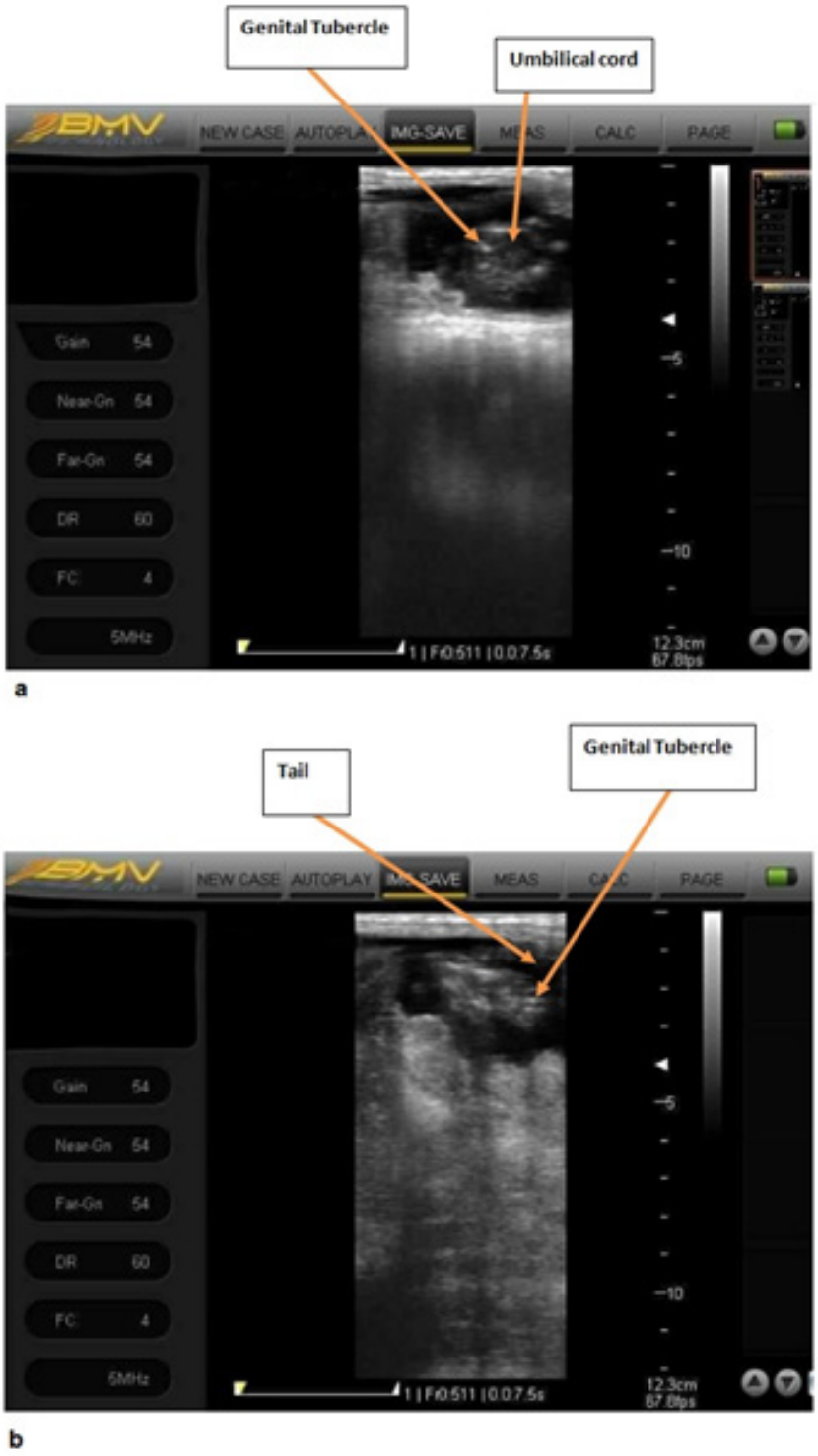

Figure 1 a- The location of the genital tubercle (GT) (male fetus). b- The location of the genital tubercle (GT) (female fetus).

Blood specimens collection

We collected blood specimens (3 mL/ewe) from the jugular veins of the same 45 ewes examined using ultrasonography. Samples were collected after ultrasound diagnosis ended placed in a tube containing EDTA and left outside the cooling for 10–0 min, then placed in a cooled incubator, and then transported to the laboratory for freezing until the collection of all 45 specimens was completed and used for DNA extraction and subsequent real-time PCR

Primers

The PCR primers for determining the male type (SRY gene) in pregnant sheep blood samples were designed in this study using NCBI-Pick primers (AY604733.1), whereas the female-type AMLX gene was designed according to a previous study (Miura et al., 2011), and these primers (Table 1) were provided by (ScientificResercher.Co.Ltd/ Iraq).

Table 1 Primers used in this study.

Primers	Sequence (5'-3')		Amplicon
Male type SRY gene	F	AATGGTCGAGCGAAAATGGC	139 bp
	R	TGGACTAACCGAATCACTGAGC	
Female type AMLX gene	F	CCGCCCAGCAGCCCTTCC	243 bp
	R	CCCGCTTGGTCTTGTCTGTTGC	

Genomic DNA Extraction

Genomic DNA was extracted from the blood specimens using the G-spinTM Total DNA Extraction Kit (Blood protocol) (Genfine, Beijing, China) according to the manufacturer's instructions. The purity of the isolated genomic DNA was checked and measured using a Nanodrop spectrophotometer (Thermo, USA), which read the absorbance at 260/280 nm by select the relevant program after opening the Nanodrop software (nucleic acid, DNA).

Sex determination using real-time PCR

Real-time PCR technique was performed for highly sensitive female type (AMLX gene) and male type (SRY gene) in pregnant sheep blood specimens. The following stages were used to carry out this procedure, as described previously (Asadpour et al., 2015).

PCR master mix was prepared using (RealMODTM Green SF 2X qPCR mix), which was completed according to the company's requirements, as follows (DNA template 5 ng in a volume of 5 µL, AMLX Forward primer (10 pmol) in a volume of 1 µL, AMLX Reveres primer (10 pmol) in a volume of 1 µL, 2X qPCR master mix in a volume of 10 µL and a PCR water volume of 3 µL, the total volume was 20 µL.

The PCR master mix components of the male-type SRY gene (DNA template 5 ng in a volume of 5 µL, SRY Forward primer (10 pmol) in a volume of 1 µL, SRY Reveres primer (10 pmol) in a volume of 1 µL, 2X qPCR master mix in a volume of 10 µL, and PCR water in a volume of 3 µL; therefore, the total volume was 20 µL.

All components of the master mix were placed in an Exispin vortex centrifuge and spun at 3000 rpm for 3 min. The samples were then placed in a real-time PCR thermo cycler (MiniOpticon-BioRad, USA).

The qPCR thermocycler conditions for AMLX and SRY genes were carried out using Real-Time PCR thermocycler equipment were as follows: initial activation at 95°C for 10 min in 1 cycle, denaturation at 95°C for 15 s in 40 cycles, and annealing and extension at 60°C for 30 s in 40 cycles.

Real-time data analysis was performed by analyzing the threshold cycle number (CT value) that presented positive amplification in the real-time PCR cycle number.

The analysis of the melt or dissociation curves was used to identify the number and approximate size of our PCR products. A highly specific assay was confirmed by providing a single melt peak at a high temperature.

Melt curve analyses revealed a sharp peak of a specific product at temperatures greater than 80°C, with very few nonspecific products at lower temperatures.

Statistical analysis

Statistical analysis was conducted using SPSS version 27, and the data were presented as numbers and percentages. The $\chi 2$-test was used to compare pregnancy diagnosis procedures and was considered significant if the P-value was less than 0.05 (Schiefer, 1980).

RESULTS

The overall accuracy of the ovine fetal sex diagnostic technique was 95.56% (43/45) and 48.89% (22/45) for real-time PCR and ultrasonography, respectively, while the percentages of the accuracy of detected ovine male and female fetuses by both diagnostic techniques were 38.46% (10/26) and 63.16% (12/19), and 92.31% (24/26) and 100% (19/19) for ultrasonography and real-time PCR, respectively (Table 2). Our results of using B-mode ultrasonography to determine the early fetal sex in 45 singleton pregnant ewes were 48.89% (22 out of 45) vs. 51.11% (23 out of 45) false diagnoses divided into 16 males and seven females. The definite sex of the offspring confirmed the results of our study after birth.

Table 2 Comparison between ultrasonography and real-time polymerase chain reaction (PCR) technique in early determination of fetal sex in ewes

Technique	Sample			NO. (%)					
	Total NO.	True	False	Male			Female		
				Total No.	True	False	Total No.	True	False
Ultrasonography	45	22 (48.89)	23 (51.11)	26	10 (38.46)	16 (61.54)	19	12 (63.16)	7 (36.84)
Real-time PCR	45	43 (95.56)	2 (4.44)	26	24 (92.3)	2 (7.69)	19	19 (100)	0 (0)
X^2		24.42			8.581			16.65	
P-value		< 0.0001			0.003			0.0001	

The percentages of the accuracy of ovine fetal sex diagnostic techniques in different gestation periods ranged between 36.36% (4/11) and 66.66% (6/9) for ultrasonography, and between 85.71 (6/7) and 100% (18/18) for real-time PCR in gestation periods tested, which ranged between 50 and 70 days (Table 3).

Table 3 Comparison of the true diagnosis of fetal sex (number & percentage) according to the gestation period tested using ultrasonography and real-time polymerase chain reaction.

Technique	50-55(day) No=9		56-60(day) NO=7		61-65(day) NO=11		66-70(day) No=18		χ^2	P-value
	no	%	no	%	no	%	no	%		
Ultrasonography	6	66.66	3	42.85	4	36.36	9	50	1.94	0.585*
Real-time PCR	8	88.9	6	85.71	11	100	18	100	3.88	0.274*
χ^2	1.28		2.80		10.26		12			
P-value	0.257*		0.094*		0.001**		0.001**			

DISCUSSION

Our ultrasonography results depend on the accurate localization of the genital tubercle (Erdogan, 2012) because it is a hyperechoic structure (Santos et al., 2018). The gestation period of the tested ewes ranged from 50 to 70 days, based on the findings of (Neto et al., 2010), who suggested that fetal sexing is possible in sheep from the 50th day of gestation onward in embryos derived from natural selection mating. These results were less than those of Amer (2010), who recorded 82% accuracy for ewes in pregnancy between 61 and 70 days. Our results were also lower than those of a previous study (Santos et al., 2018), which recorded a reality of 82.61% and 95.83% in ewes at the gestation period of 55 and 65 days, respectively, and they suggested that the false diagnosis, particularly in the male fetuses, maybe due to the delayed migration of genital tubercle occurring later in male fetuses that had been misdiagnosed as females. Their suggestions may elucidate the more significant percentage of false diagnoses in our study regarding the high number of male fetuses compared to that of female fetuses (Table 2), particularly if we know that the male fetuses generally completed their genital tubercles migrate later than those of females, probably due to the increased distance that the genital tubercle must travel in males (Aguiar Filho et al.,2010). However, the misdiagnosis of female fetuses as males could be due to structures with similar echogenicity that are mistaken for the genital tubercle, resembling the edges of crumpled hind limbs, or possibly the umbilical cord garbed around the abdomen, as well as the misdiagnosis could frequently happen if diagnoses have been made too early (Santos et al., 2018), as well as the difficulty of manipulating the uterus of sheep and goat during an ultrasonographic investigation, which affected the accuracy of this technique (Santos et al., 2007) and might have been due to differences in the ages and breeds of tested ewes (Asadpour et al., 2015) or due to the fetal status during examination (Ali, 2004). In addition to the differences in the experience of the technician using ultrasonography.

To detect fetal sex in sheep, assessing free fetal DNA in the mother's blood is a non-invasive and valuable method. Circulating-free fetal DNA serves as a restorative material for fetal sex diagnosis and genetic screening throughout pregnancy (Asadpour et al., 2015). In the plasma of pregnant women, fetal DNA is considered to constitute 3% to 13% of the total circulating free DNA (Drury et al., 2016). The mechanism of fetal DNA leakage into maternal circulation is not understood; however, cell lysis resulting from physical and immunological damage and developmentally regulated apoptosis of fetal tissues could allow fetal DNA to cross the placental membrane (Lo et al., 1997).

Although the synepitheliochorial structure of the ovine placenta is expected to prevent transplacental cell leakage, prior research in species with the same placental type, such as cows, revealed that fetal DNA may be transferred across the placenta (Asadpour et al., 2015). Bischoff et al.(2002) suggested that the concentration of fetal DNA in maternal plasma may not depend on the circulating intact fetal cells, but rather is a product of growth and cellular turnover during embryonic or fetal development. Pfeiffer and Brening (2005) demonstrated that amelogenin amplification by PCR is a reliable approach for determining sex in sheep in a prior investigation. The presence of fetal male SRY gene and male and female AML circulating DNA in the maternal blood of ewes with single fetuses was successfully demonstrated in this study using real-time PCR. Using real-time duplex PCR for the detection of the two genes, that is SRY and female AMLX, we found an accuracy of 95.5% (43 out of 45) distributed as 92.3% (24/26) of male fetuses and 100% (19/19) of female fetuses (Table2).

Our findings were identical to those of Tavares, et al. (2016), who reported 95.9 % accuracy in cows and sheep. Additionally, this result is consistent with that of Dervishi et al., (2011), Kadivar et al., (2013), Saberivand et al., (2015), who reported 100% accuracy when using conventional PCR to detect SRY, or both SRY and AML genes, in sheep, and higher than that of Asadpour et al., (2015), who reported that the superiority of the overall test accuracy for correct sex determination using plasma samples was equal to 78±1 (95% distributed as 12/15 [80%] cases from known male pregnancies and 13/17 [76%] cases from known female pregnancies).

The low affinity of these researchers might have been due to differences in the techniques used, in that they detected only the SRY gene, which increases the false-negative results, while we used multiple genes (SRY and female AMEL), thus decreasing the false-negative results. Regarding the sex of the fetus diagnosed, our results were in agreement with those of Wang et al. (2010), who recorded an accuracy of 100% in male and 91% in female bovine fetuses, while we obtained an accuracy of 92.3% in male vs. 100% in female fetuses. These differences may be due to differences in the tested animal species. Regarding the period of conception, the percentage of diagnosing accuracy increased with the advancing of pregnancy (100% from days 61 to 72 vs. 88.89% and 85.71% on days 50–55 and 56–60 of gestation, respectively). This could be attributed to the increase in fetal DNA quantities in the mother's blood as the pregnancy progresses (Zhou et al., 2015).

PCR has been used to detect male and/or female fetal genes in the ccffDNA in the maternal blood in various domestic animals, including goats, cattle (Quirino et al., 2010), sheep (Asadpour et al., 2015), and Arabian camels (Abdulla et al., 2020). The exact molecular technique for fetal sex detection in ccffDNA has been applied in humans (D'Aversa et al., 2018).

When we compared the ultrasonography with the real-time PCR method for the early detection of fetal sex in ewes, real-time PCR was more efficient and highly accurate than ultrasonography in terms o f total percentage in diagnosing male and female fetuses (Table 2), and in diagnosing the fetal sex in different periods of conception (Table 3). Moreover, another advantage was that real-time PCR did not require extensive time and needed no experience for diagnosing ovine fetal sex compared to the time and experience required for the ultrasound method.

CONCLUSION

In comparison to that of ultrasonography, fetal sex determination in early pregnant singleton ewes using real-time PCR to detect SRY and AMLX exhibited a high accuracy. Because the accuracy of SRY and AMLX using real-time PCR improves as gestation progresses, utilizing real-time PCR to diagnose the early fetal sex in sheep is easier and more definite than ultrasonography.

ACKNOWLEDGEMENTS

The authors extend their appreciation to Assist.Prof. Dr. Hassan Al-Karagoly for facilitate conducting the study in the college laboratories, and to Prof. Dr. Ali M. Ghazi for his assistance in statistical analysis.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, formal analysis, Abbas Fadhil Daham; investigation and data curation, Karar Yaser Hussain and Abbas Fadhil Daham; validation, Karar Yaser Hussain and Abbas Fadhil Daham; visualization, Karar Yaser Hussain and Abbas Fadhil Daham; writing—original draft preparation, Karar Yaser Hussain; writing—review and editing, Abbas Fadhil Daham; supervision, Abbas Fadhil Daham; project administration, Abbas Fadhil Daham. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abdulla, A.K., Daham, A.F., Hassooni, F.K., Al-Delemi, D.H.J. 2020. Early diagnosis of fetal sex in Arabian camel (Camelus dromedaries) by using polymerase chain reaction. Eurasia J. Biosci. 14(1), 2333-2337.

Ali A. 2004. Effect of gestational age and fetal position on the possibility and accuracy of ultra sonographic fetal gender determination in dairy cattle. Reprod. Domest. Anim. 39 (167), 190–194.

Allen Lun, F.M., Chiu, R.W., Chan, K.C., Yeung Leung, T., Kin Lau, T., Dennis Lo, Y.M. 2017. Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin. Chem. 54, 1664-72.

Aguiar Filho, C.R., Freitas Neto, L.M., Santos, M.H.B., Neves, J.P., Lima, P.F., Oliveira M.A.L. 2010. Transrectal ultrasound to identify the fetal sex of goats (In Portuguese). Ciência. Animal. 1(1), 125-130.

Amer, H. A. 2010. Ultrasonographic assessment of early pregnancy diagnosis, fetometry and sex determination in goats. Anim. Reprod. Sci. 117, 226–231.

Arashiro, E.K.N., Ungerfeld, R., Clariget, R.P., Pinto, P.H.N., Balaro, M.F.A., Bragança, G.M., Riberio, L.S.R., da Fonseca, J.F., Brandao, F.Z. 2018. Early pregnancy diagnosis in ewes by subjective assessment of luteal vasularization using colour Doppler ultrasonography. Theriogenology. 106, 247-252.

Asadpour, R., Asadi, M.H., Jafari –Joozani, R., Hamidian, G.H. 2015. Ovine fetal sex determination using circulating cell-free fetal DNA (ccffDNA) and cervical mucus secretions. Asian Pac. J. Reprod. 4(1), 65-69.

Bischoff, F.Z., Sinacori, M.K., Dang, D.D., Marquez-Do, D., Horne, C., Lewis, D.E. Simpson, J.L. 2002. Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for firrst and second trimester non-invasive prenatal diagnosis. Hum. Reprod. Update. 8(6), 493-500.

Dal, G.E., Enginler, S.O., Baykal, K. Sabunca, A. 2019. Early pregnancy diagnosis by semiquantitative evaluation of luteal vascularity using power Doppler ultrasonography in sheep. Acta Vet. Brno. 88, 19–23.

D'Aversa, E., Breveglieri, G., Pellegatti, P., Guerra, G., Gambari, R. Borgatt, M. 2018. Non-invasive fetal sex diagnosis in plasma of early weeks pregnants using droplet digital PCR. Mol. Med. 24, 14.

Dervishi, E., Sanchez, P., Alabart, J.L., Cocero, M.J, Folch, J., Calvo, J.H. 2011. A suitable duplex PCR for ovine embryo sex and genotype of PrnP gene determination for MOET based selection programmes E. Reprod. Domest. Anim. 46, 999–1003.

Drury, S., Hill, M., Chitty, L.S., 2016. Cell-free fetal DNA testing for prenatal diagnosis. Adv. Clin. Chem. 76, 1–35.

Erdogan, G. 2012. Ultrasonic assessment during pregnancy in goats – A review. Reprod. Domest. Anim. 47, 157–163.

Grzybowski, G., Prusak, B., Romaniuk, B. 2006. A novel variant of the amelogenin gene (AMEL-X) in cattle and its implications for sex determination. Anim. Sci. Pap. Rep. 24(2), 111-118.

Kadivar, A., Hassanpour, H., Mirshokraei, P., Azari, M., Hosseini, K.H., Karami, A. 2013. Detection and quantification of cell-free fetal DNA in ovine maternal plasma; use it to predict fetal sex. Theriogenology. 79, 995-1000.

Kashimada, K., Koopman, P. 2010. Sry: the master switch in mammalian sex determination. Development. 137, 3921-3930.

King, V., Goodfellow, P.N., Pearks, Wilkerson, A.J., Johnson, W.E., O'Brien, S.J., Pecon-Slattery, J. 2007.Evolution of the male-determining gene SRY within the cat family felidae. Genetics. 175, 1855–1867.

Lo, Y.M., Corbetta, N., Chamberlain, P.F., Rai, V., Sargetn, I. L., Redman, C.W. 1997. Presence of fetal DNA in maternal plasma and serum. Lancet. 350, 485–487.

Miura, K., Higashijima, A., Shimada, T., Shoko, M., Yamasaki, K., Abe, S., Jo, O., Kinoshita, A., Yoshida, A., Yoshimura, S., Niikawa, N., Yoshiura, K., Masuzaki, H., 2011. Clinlcal application of fetal sex determination using cell-free fetal DNA in pregnant carriers of X-linked genetic disorders. J. Hum. Genet. 56, 296-299.

Neto, L.M., Santos, M.H., Aguiar, Filho, C.R., Almeida Irmão, J.M., Cavalcanti Caldas, E.L., Neves, J.P., Lima, P.F., Lemos de Oliveira, M.A., 2010. Ultrasonographic fetal sex identification in pregnant sheep derived from natural mating and embryo transfer. J. Reprod. Dev. 56, 347–350.

Paula, N.R.O., Cruz, J.F.; Lopes Júnior, E.S., Teixeira, D.I.A., Lima-Verde, J.B., Rondina, D., Freitas, V.J.F. 2003. Pregnancy diagnosis in Saanen goats using the doppler effect and real-time ultrasound. Rev. Bras. Med. Vet.10(3),166-169. (In Portuguese)

Pfeiffer, I., Brenig, B. 2005. X- and Y-chromosome specific variants of amelogenin gene allow sex determination in sheep (Ovis arie) and European red deer (Cervus elaphus). BMC Genet. 16, 6.

Quirino, C.R., Leal, S.R., daSilva Fontes, R., Marques, V.C.L., Matos, L.F., Filho, G.A.D.S. 2010. In: 9th World Congresson Genetics Applied to Livestock Production (WCGALP), Leipzig, Germany; August, 1-6.

Ramos, E.S. 2006. Cell-free fetal DNA in maternal plasma and non-invasive prenatal diagnosis. Rev. Lat. Am. Enfermagem. 14(6), 964-967.

Reichenbach, H.D., Santos, M.H., Oliveira, M.A., Meinecke-Tillmann, S., Buerstel, D.M. 2004. Fetal sexing in goat and sheep by ultrasonography. In: Santos MH, Oliveira MAL, Lima PF (eds), Diagnosis of Pregnancy in Goat and Sheep. Varela, Sao Paulo. 117–136. (In Portuguese).

Saberivand, S., A., Maasoumeh, M., Parisa, A. 2015. Sex determination of ovine embryos by SRY and amelogenin (AMEL) genes using maternal circulating cell free DNA. Anim. Reprod. Sci. 164, 9-13

Santos, M. H. B., Aguiar Filho, C. R., Freitas Neto, L. M., Santos Junior, E. R., Freitas, V. J. F., Neves, J. P., Lima, P. F., and Oliveira, M. A. L. 2007. Sexing of Savana goat fetuses using transrectal ultrasonography. Medicina. Veterinária. 1, 50–55.

Santos, R.S. D., Rocha, L.F.; Guimarães, A.S.B., Jesus, R.D.L.D.; Santana, A.L.A., Biscarde, C.E.A.; Bittencourt, R.F., Barbosa, L.P. 2018. Fetal sexing in small ruminants through visualization of the genital tubercle. Rev. Bras. de Saude e Prod. Anim. 19(4), 360-370.

Schiefer, W.C. 1980. Statistics for the biological sciences. 2nd ed. Addison. Wesley puplCoomp, Clifornia, London. PP:123-131

Tavares, K.C.S., Carneiro, I.S., Rios, D.B., Feltrin, C., Ribeiro, A.K.C., Gaudêncio-Neto, S., Martins, L.T., Aguiar, L.H., Lazzarotto, C.R., Calderón, C.E.M., Lopes, F.E.M., Teixeira, L.P.R., Bertolini, M., Bertolini, L.R. 2016. A fast and simple method for the polymerase chain reaction-based sexing of livestock embryos. Genet. Mol. Res. 15,1. gmr.15017476.

Tein, M.S., Lau, T.K., Haines, C.J., Leung, T.N., Poon, P.M., Wainscoat, J.S., Johnson, P.J., Chang, A.M., Hjelm, N.M. 1998. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for non-invasive prenatal diagnosis. Am. J. Hum. Genet. 62, 768–75.

Wang, G., Cui, Q., Cheng, K., Zhang, X., Xing, G., and Wu, S. 2010. Prediction of fetal sex by amplification of fetal DNA present in cow plasma. J. Reprod. Dev. 56, 639-642.

Zhou, Y., Zhu, Z., Gao, Y.,Yuan, Y., Guo, Y., Zhou, L., Liao, K., Wang, J., Du, B., Hou, Y., Chen, Z., Chen, F., Zhang, H., Yu, C., Zhao, L., Lau, T.K., Jiang, F., Wang, W., 2015. Effects of maternal and fetal characteristics on cell-free fetal DNA fraction in maternal plasma. Reprod. Sci. 22, 1429–35.

How to cite this article;

Karar Yaser Hussain and Abbas Fadhil Daham. Early determination of fetal sex in singleton pregnant ewes by real-time polymerase chain reaction and ultrasonography, a comparative study. Veterinary Integrative Sciences. 2022; 20(2): 267 - 277.