IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 9, Issue 11 Ver. II (Nov. 2016), PP 38-45
www.iosrjournals.org

Prenatal determination of farm animal fetal sex using free fetal DNA in maternal plasma

Najmeh Davoodian[1*], Ali Kadivar[2]
[1]*Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran*
[2]*Department of Clinical Science, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran*

Abstract: *In many farm animal species, the preference of a specific sex is obvious mainly due to commercial implications; so for comprehensive and efficient results in the livestock management plans specially breedingand selection programs, it is necessary to include acost-effective and reliable strategy for early determination of fetal sex. Several invasive and non-invasive approaches have been established for fetal gender detection in farm animals at early gestational age. Non-invasive determination of fetal sex based on analysis of cffDNA in maternal plasma would be a useful tool for management decisions andprovides opportunities for prenatal genetic diagnosis. Among the numerous demonstrated applications forcffDNA, early fetal sexing appears to be promising area for immediate application in farm animal industry. Still there is great potential for the continued application of this genetic material for prenatal fetal genetic evaluation. The significant contributions of cffDNA in general and its practical application in horse, cattle and sheep fetal gender detection in particular are collected in this paper.*

Keywords: *prenatal, fetal sex, cffDNA, farm animal*

I. Introduction

Prenatal diagnosis and prenatal screening are aspects of prenatal care that focus on detecting anatomic and physiologic problems with the zygote, embryo or fetus as early as possible, either before gestation even starts or as early in gestation as practicable. Using medical tests, problems such as chromosome abnormalities and gene mutations that would lead to genetic disorders and birth defectswill be detected. In some cases, the tests are administered to determine if the fetus will be aborted. Screening can also be used for prenatal sex determination which is the prenatal testing for discerning the sex of a fetus before birth. This article reviews recent literatures which discuss prenatal sex determination in farm animals with special emphasis on analysis of cell free fetal DNA in maternal plasma which introducing a new approach for prenatalevaluation.

1.1, Sex determination and development

Sexual differentiation is the process of development of any male- or female-specific physical or behavioral characteristic. During this process, organisms are equipped with the necessary anatomy and physiology to allow sexual reproduction to occur. The sexual fate of the organism is castat fertilization but sex differences range greatly and is revealed during fetal development[1]. In mammals, sex is determined by an XY sex-determination system, where an XX chromosome combination determines a female, and an XY chromosome combination a male. Sex determination results from the interplay of genes in a complex network [2].Only a few genes on the Y-chromosome are required to make the indifferent fetus into a male; the lack of these genes results in a female. In most mammals, including humans and domestic animals, sexual fate is determined genetically by the presence of the SRY gene (sex-determining region on the Y chromosome), which encodes the testis-determining factor on the Y chromosome, inducing differentiation of cells derived from the genital ridges into testes[3, 4].

1.2, Importance of prenatal sex determination

Sex, male or female is a genetic trait, which cannot be manipulated effectively in breeding programs [5]. Control of the sex ratio of animals is desired in the livestock industry. Nowadays, determining the sex of an unborn bovine or equine fetus is increasingly requested in the marketplace. Precise diagnosis of fetal sex in domestic species has considerable research and commercial applications in the livestock industry[6-8].There are multiple phenotypic consequences of genetic sex that determine the future reproductive characteristics of the newborn in animal production industry. For example dairy calves might become a herd replacement, producing offspring and lactating or become a male byproduct and be sold for veal. Behavior, size and efficiency of growth are considered as other phenotypic outcomes of genetic sex that programmed during fetal life[9]. All sectors of the cattle industry would derive economicbenefits from the ability to determine the sex ofcalves in early gestation. As international orders are placed requesting heifers pregnant with heifer calves, so modern dairy production get benefits from the birth of female offspring[10]. Early fetal sex determination can change

the value of the pregnancy and could be used to direct salesof calves prior to their birth, increasing the commercialvalue[11].For meat production purposes, however, male offspring are generally advantageous due to faster growth and better feed efficiencies[10].In beef cattle breeds in United States, steers are worth about US$ 60 more than heifers at weaning[12]because male calves tend to gain higher weights and subsequent higher feed efficiency during fattening[5]. Sexing of twins is also advantageous in the case of unwanted pregnancies, such as those bearing freemartin[13].Determining of fetal sex is usefulin production systems and in artificial inseminationbreeding programs of large purebred herds, when combined withmanagement decisions[11]. Itwill also help dairy farmers in deciding whether or not to retain pregnant cows already assigned for culling[13].
Controlling the gender of offspring has considerable economic value in agriculture[14]. The control of sex ratio in livestock and poultry to increase efficiency of food production is potentially worth millions of dollars annually[15].

In contrast to dairy and beef cattle, that preference of a specific sex is due to commercial implications, in horses, individual considerations is the choice between desired and non-desired sex[16]. For example, the male thoroughbred is preferred for racing or classical riding while higher prices are paid for female yearling Quarter horses bred for racing [17, 18]. Therefore, Determination of equine fetal sex can provide a useful service to breeders as it allows commercial strategies to be implemented. Decision to sell a pregnant mare with a fetus of the desired or undesired sex and culling broodmare is easier when fetal gender is known[8].
These are few of the commercial uses for fetal sexing. Potential demand exists in other species, including exotics and camelids.Early prenatal determination of sex is required for prevention of sex-linked diseases in humans. There are more than 350 X-linked diseases known in the human. Sex selection is required for fetuses at risk of X-linked diseases as identification of a male fetus indicates hemizygosity for the X chromosome and thus potential disease if the mother is a carrier (e.g. haemophilia). It is also of use in the clinical management of fetuses at risk of congenital adrenal hyperplasia (CAH) [19-21]. Furthermore, there has always been an interest in the use of sexing methods for family balancing. In general, for comprehensive and efficient results in breedingand selection programs, it is necessary to include acost-effective and reliable strategy for early fetal sex determination. It can increase the genetic gain inthe herd and decrease the cost of progeny testing [22, 23].

1.3, Methods of fetal sex determination

several approaches have been established for genetic sex typing, such as karyotyping[24], H-Y antigen detection[25], X-linked enzymatic determination[26], DNA probes for embryos or cells obtained by amniocentesis[27], ultrasonic examination of fetal structures[13] and the polymerase chain reaction (PCR)[28-31].Karyotyping by amniocentesis has been employed for sexing of equine and bovine embryos[32-34]. The technique is a time consuming and invasive process carrying a small but significant risk of miscarriage[27, 35] as well as requiring too much labor and could notbe used routinely as a standard reproductive managementpractice. Immunological detection of the male specific H-Y antigen is another approach for fetal sex determination. Using this technique in the horse embryos, Wood et al. [36] obtained an overall accuracy of 82 % while in bovine embryos, the accuracy of sex determination was 81% [37]. This method does not prove successful in embryos that have already initiated blastocoel formation [37].On the other hand, equine embryos enter the uterus at the late morula or early blastocyst stage [38], so only a small percentage of equine embryos recovered from the uterus would be applicable for this method. This method is non-invasive and less time-consuming than karyotyping, but the results are less subjective and not accurate enough and the method has not been proven to be reproducible [39].

The other technique has been employed for embryo sexing in bovine species ismeasurement of testosterone level in fetal fluids [40].Itis an invasive process requiring fetal fluid sampling and has some risks of abortion. The X-linked enzymatic determination of glucose-6-phosphate dehydrogenase (G6PD) [26], is another technique could be named which requires embryos to be sexed before inactivation of X chromosome, thus limiting this approach to cleavage and morula stage embryos [41]. Another approach for animal fetal gender determination is transrectal ultrasonic diagnosis of fetal gender in utero.Ithas been done accurately in cattle [42] and horses [43] under farm conditions and appears to be a rapid and reliable technique. Muller and Wittkowski reported the visualization of the scrotal swelling in male and mammary glands in female bovine fetuses by real-time ultrasound for sex determination with an accuracy of 94% between days 70 and 120 post fertilization[44]. Reports of fetal gender determination based on the identification and location of the genital tubercle relative to surrounding structures are also available [45, 46]. This technique poses some risk for the operator and they require much experience for accurate determination of sex as well as specialized equipment. Another method, molecular sexing based on polymerase chain reaction (PCR), is a practical alternative method that can provide sensitive, precise, rapid, and accurate results to determine the fetal sex. In addition, PCR-based embryo sexing is becoming increasingly prevalent compared to other methods for commercial purposes [28-30, 47] due to its simplicity and cost-effectiveness. PCR had been established for gender determination of bovine [31, 48, 49], ovine[48] and porcine[50] pre implantation stage embryos.

It is already known that sexual differentiation is determined primarily by the presence or absence of the Y chromosome[51]. In the presence and expression of the sex determining region Y (SRY) gene, the male pathway prevails, otherwise the female pathway gains control of gonadal development [52]. The PCR method for gender determination is usually based on the presence or absence of Y-specific amplification [50]. Another approach is to amplify the ZFY gene (zinc-finger protein Y) located on Y-chromosome and its X-chromosomal homologue, ZFX [48, 49, 53]. Previously The ZFY gene was thought to be the testis determining factor in mammals, afterwards it was confirmed that this factor is a gene termed SRY [3] and amplification of these loci by PCR have been used successfully in sex determination of many mammalian species [53, 54]. ZFX, ZFY and SRY have successfully been used for equine embryo sexing [6, 55-58] by single cell PCR identification. Choi et al., performed successfully and more accurately duplex PCR of the male specific SRY gene because genes present in multiple copies could enhance the sensitivity of PCR for sex determination[56]. Sex determination in cattle embryos before transfer is routinely and successfully achieved by nested PCR using DNA extracted from as few as two to four blastomeres[53, 54], However, equine embryos are much more sensitive to cell biopsy [59, 60] due to the presence of acellularcapsul until day 21 pregnancy. Similarly analysis of fetal sex in cells collected via amniocentesis from fetal fluids was applied in cattle [61]. Sex determination was performed via chromosome analysis and detection of sex-specific DNA by PCR method. The practicability of this technique is limited because the collection of fetal fluid is challenging.

In 1971, the detection of fetal cells originating from regressing chorionic villi releasing into the lower uterine pole in the woman cervical mucus by Shettles, afforded a potential source of fetal material for non-invasive testing[62]. Polymerase chain reaction was amplified using bovine cervical mucus for gender determination and 7 out of 13 male fetuses were correctly sexed with the sensivity of 71.4% [63]. Female somatic cells are equipped with two X chromosomes and could thus theoretically produce twice as much X-specific transcripts as male cells [64]. However, one X chromosome is inactivated in females to avoid over-expression of X-linked genes. X-inactivation requires a specific RNA called X-inactivated specific transcript (Xist) RNA. Based on the knowledge on the presence of Xist, an approach for sex determination in equine embryos was published. Sexing was possible in 77% of embryos collected before day 7 and results are comparable to those of routine sex determination in bovine embryos [58, 65]. The technique requires embryo fixation and is not applicable for sexing of embryos with the aim to produce foals. Molecular determination of fetal gender followed from the discovery of significant amounts of fetally derived DNA in maternal plasma [66]. During pregnancy, maternal and fetal circulations are separated by placental membranes. However, cell lysis resulting from physical and immunologic damage and also developmentally regulated apoptosis of fetal tissues could allow fetal DNA to cross the placental membrane [66].

Non-invasive determination of fetal sex based on analysis of cffDNA (cell-free fetal DNA) in maternal plasma offers an alternative source of fetal genetic material [67]and would be a useful tool for prenatal genetic diagnosis.Lo et al used a nested polymerase chain reaction (PCR) system to detect the fetal DNA (cffDNA) in maternal plasma and serum and identified the Y-chromosome derived gene *TSPY*, on which is found the DYS14 marker sequence [66]. After that, they developed the more sensitive quantitative real-time PCR systems [68], and showed the concentrations of cffDNA between 3.4% and 6.2% of total DNA in maternal plasma and direct correlation between the amount of cffDNA in maternal plasma and the age of gestation. cffDNA, as its rapid clearance from the maternal circulation, is an ideal source for prenatal investigation of the fetal genotype [69].
However, little is known about the physical and biological characteristics of cffDNA in maternal plasma [70]and sensitivity of DNA extraction methods could play a crucial role in this kind of investigation.
Generally, being less invasive, quick, highly sensitive and reliable, cost effective are the main advantages of the PCR method for gender determination. Thus, considering all these items, fetal sexing using PCRand DNA isolated from the maternal plasma could become an important tool to predict fetal sex.

1.4, Determination of prenatal fetal sex using cell-free fetal DNA (cffDNA)

The passage of nucleated cells from fetal to maternal circulation was first noted by Walknowska et al in 1969[71]. Since then the potential applications and limitations of fetal cells for prenatal testing have been well characterized. In 1997, the discovery of fragmented, cell-free fetal DNA circulating in maternal plasma and serum by Lo et al afforded a potential alternative to isolation of rare fetal cells for non-invasive testing[66]. Fetal DNA fragments which originate in trophoblast cells lining the placental intervillous space, are released into maternal circulation after trophoblast degradation; the presence of placenta derived mRNA and DNA due to apoptosis of fetal cells in maternal blood were also observed as a minor sources of fetal genetic material in maternal circulation [72, 73]. It has been proved that the transfer of cell-free fetal DNA to maternal blood is detectable in all pregnancies [74]. Cell-free fetal DNA can be detected in maternal circulation as early as 5 weeks of gestational age and persists throughout pregnancy [75]. Fetal cells persist in the maternal circulation after delivery [76] but most studies report that, cell-free fetal DNA, due to its mean half-life of 16.3 minutes, is cleared from circulation within a matter of hours after delivery and does not persist in the maternal circulation

post partum, thus previous pregnancies do not confound identification and analysis of fetal DNA from a current pregnancy [77]. This makes it a potentially ideal source for prenatal investigation of the fetal genotype.
In the initial phase of gestation, the concentration of circulating fetal DNA in maternal plasma is very low but this figure increases gradually as the gestationprogresses [78]. The concentration of cell-free fetal DNA relative to total DNA ranges from 3.4% to 6.2%, or 25.4 to 292.2 genome equivalents per milliliter of maternal bloodduring pregnancy[68]. The detection of fetal DNA in maternal plasma does not require prior enrichment and is much simpler than detecting fetal nucleated cells in maternal blood. In human medicine, this approach has been shown to have application in the prenatal diagnosis of fetal paternally inherited genetic disorders (e.g fetal rhesus D status and sex-linked disorders) as well as common pregnancy-associated disorders such as preterm labor and preeclampsia [74]. One of the potential clinical applications is non-invasive identification of male fetuses at risk for X-linked disorders because identification of male DNA in maternal plasma is relatively easy. In this way fetal gender determination using DNA analysis of maternal serum has been suggested as a 'pre-test' to decide whether invasive testing should be performed on a fetus at risk to inherit an X-linked condition [79]. This test could also effectively be used to determine which pregnant women are carrying a potential fetus with congenital adrenal hyperplasia and should receive treatment [19, 80]. The success of this technology has resulted in the implementation of noninvasive fetal gender determination for women at risk of X-linked disorder into routine clinical practice in some units in the United Kingdom [81].

The initial discovery of cell-free fetal DNA in maternal plasma relied on polymerase chain reaction (PCR) amplification and electrophoresis of *DYS14*, a multi-copy sequence located within the *TSPY* gene on the Y chromosome.So fetal DNA was necessarily detected only in male-bearing pregnancies with the sensitivity of 80% [66]. More recently, prenatal sex determination has shifted to the detection of a single copy gene *SRY*, the sex-determining region on the Y chromosome, which may provide more reliable diagnostic capability than *DYS14 [79]*. Moreover, Laboratory techniques for sex detection have also been improved to quantitative real-time PCR, which increases throughput and improves accuracy to 97% to 100% in the first trimester of pregnancy [68, 82].The targeting of the multi-copy DYS14 sequence of *TSPY* appears more sensitive for confirmatory analysis of the presence of cffDNA in maternal-plasma samples[83]. *TSPY* is a multi-gene family containing both functional genes and pseudogenes and the effect of copy-number polymorphisms on quantitative analysis between different individuals has not been explored.
SRY as a single target represents the best choice. There are several articles reporting real-time PCR analysis of the *SRY* gene that achieve sensitivities in excess of 90%, many 100% [81]. Furthermore, the incidence of false-positive results appears to be extremely low with none described in series using real-time PCR for analysis of either the *SRY* or *DYS14*gene. Recently, a nested PCR approach to detect the Amelogenin gene in maternal plasma has been described [84]. Amelogenin is an intronic size polymorphism of *AMELXY* located on both the X and Y chromosomes [85] and sometimes used in maternal-plasma testing. It is a common target in QF-PCR when coupled with analysis of invasively sampled cffDNA[86, 87]. There are now a number of articles in the literature reporting the detection of fetal gender based on cffDNA in domestic animal maternal plasma (table 1). Utilization of cffDNA as a new approach for prenatal sex determination is getting more popular in the livestock industry. Hereunder, it will be reviewedemploying this approach for prenatal detection in horse, cattle and sheep.

1.4.1, Equine fetal sex detection using cffDNA

Non-invasive determination of fetal sex in mares based on analysis of fetal material collected from maternal plasma would be a useful tool providing opportunities for prenatal genetic diagnosis. In this way, a new promising method for sexing in horse was employed by De Leon et al in 2012. Using this technique, they identified cffDNA in maternal blood of 20 thoroughbred mares in the final three months of pregnancy. Sex determination was performed by PCR amplification of SRY gene resulting in a sensitivity of 72.7% and overall accuracy rate of 85%. Furthermore, using SRY/2nd PCR and qPCR techniques, sensitivity and accuracy went up to 90.9% and 95% respectively [88]. Afterward, Kadivar et al in 2016 developed a real-time PCR assay for detecting cffDNA in arabian pregnant mare plasma predicting fetal sex through identifying SRY gene. The cffDNA was extracted from blood plasma of 28 pregnant mares between 8 and 20 weeks of gestation and real-time PCR was performed for the SRY gene as well as GAPDH as the internal control. The accuracy of test was 88% whereas sensitivity and specificity were 85.7% and 90.9% respectively. There were a positive predictive value (PPV) of 92.3% (CI 70.7– 99.6), and a negative predictive value (NPV) of 83.3% (CI 59.9–91.2)[89].

1.4.2, Bovine fetal sex detection using cffDNA

Recently, there has been a great interest in the possibility of detecting minimal amounts of fetal DNA in the bovine maternal plasma in the attempt to establish reliable non-invasive techniques for prenatal diagnosis of fetal gender. In 1996, Kadokawa et al. believed, based on their previous research, that fetal cells were very rare or may be absent in bovine maternal blood and that they were not able to detect bovine fetal cell DNA in

pregnant maternal blood for prenatal sexing by a PCR method[90]. But then, some recent reports are published regarding the prediction of the bovine fetal sex by PCR using DNA isolated from maternal plasma and specific articles regarding the evidence of bovine transplacental fetal DNA passage. In the first study, Xi et al. reported in 2006 that they could successfully predict fetal sex by amplifying the SRY gene from pregnant cow blood with the overall accuracy rate of 60 % during 30–59 days of gestation[91]. In the second study, Nested PCR Was employed to detect the fetal SRY in the 110 tested plasma samples from the pregnant cows (Wang et al. 2010). the authors could successfully amplify the fetal DNA from maternal plasma and fetuses could be sexed with an overall accuracy rate of 100 % (43/43) for males and 91.0 % (61/ 67) for females[92]. In the third study, fetal sex was correctly determined by amplification of Y-specific sequences in the plasma of 35pregnant cows in 88.6% of cases. Regression analysis showeda strong relationship between the probability of correctly predicting fetalsex and the stage of gestation. It was estimated that the test performed at 55.0 days or later post fertilization would have 99.9 % accuracy [93]. Furthermore, in another study by Lemos et al. in 2011, PCR amplification results using TSPY gene primers matched the fetal phenotypic gender in all 47 male and 37 female fetuses studied [94]. Hitherto, the passage of cffDNA (a fetal specific and Y specific sequences) to maternal circulation had been shown throughout pregnancy of cattle [95].

1.4.3, Ovine fetal sex detection using cffDNA

It is obvious that prediction of fetal sex in the ovine species could be useful in the management decisions so set up a simple and accurate method for fetal gender determination in sheep seems necessary. In this regard, Kadivar et al. for the first time reported in 2013 that they had successfully predicted fetal sex by amplifying the SRY gene from 46 pregnant ewe blood plasma during the second to fifth months of gestation. They utilized real-time PCR assay to detect free fetal DNA in pregnant ewe plasma. The sensitivity and specificity of test were 100% with no false negative or positive results. The quantitative changes of fetal DNA during pregnancy were also investigated using quantitative real-time polymerase chain reaction and determined that fetal DNA levels are significantly increased during pregnancy, up to approximately 1.65-fold in the last 2 months of pregnancy[96]. Determination of ovine fetal gender was followed by amplification of a Y specific insertion in Amelogenin gene by real-time PCR assay[97]. Kadivar et al. employed a new approach in applying Amelogenin for fetal sex determination in ovine species. The test sensitivity, specificity and accuracy were 96.5%, 87.5% and 93.3% respectively for 45 pregnant ewes with age of pregnancy varying from 8 to 18 weeks of gestation. Furthermore, the result of relative quantification showed that the cell free fetal DNA values were significantly higher in ewes with more than 3 months of pregnancy relative to those with less than it [97].

II. Conclusion

It is concluded that non-invasive testing, using maternal peripheral blood as a source of fetal genetic material, is a progressive field for early prenatal diagnosis in human studies. It avoids the risk of miscarriage and provides the possibility of widespread use of these techniques. Use of these genetic source in domestic animals is in its infancy. All studies conducted so far, have been focused on sex prediction in farm animals. The emergence of next generation sequencing techniques, provides a new study area that makes it possible to use this valuable genetic source not only for fetal sex prediction but also for other genetic evaluations prenatally.

Table1. Studies reporting identifying male fetuses from ffDNA in the maternal circulation of farm animals.

Author	N	Gestation (weeks)	PCR technique	Gene	accuracy
De Leon (2012)	20 mares	3 months	PCR	SRY	85%
De Leon (2012)	20 mares	3 months	2nd PCR &qPCR	SRY	95%
Kadivar (2016)	28 mares	8-20 weeks	Real-time PCR	SRY & GAPDH	88%
Xi (2006)	30 cattle	30-59 days	PCR	SRY	60%
Wang (2010)	110 cattle	30-242 days	Nested PCR	Sry	100%
da Cruz (20120	35 cattle	55-270 days	PCR	Y specific amplicons	99.9%
Kadivar (2013)	46 ewes	8-17 weeks	Real-time PCR	SRY & GAPDH	100%
Kadivar (2014)	45 ewes	8-18 weeks	Real-time PCR	Amelogenin	93.3%

References

[1]. D. Wilhelm, S. Palmer, P. Koopman, Sex determination and gonadal development in mammals, *Physiological reviews, 87, 2007,* 1-28.

[2]. B.C. Morrish, A.H. Sinclair, Vertebrate sex determination: many means to an end, *Reproduction, 124, 2002,* 447-57.

[3]. P. Koopman, J. Gubbay, N. Vivian, P. Goodfellow, R. Lovell-Badge, Male development of chromosomally female mice transgenic for Sry, *Nature, 351, 1991,* 117-21.

[4]. B.L. Griffiths, M.J. Smith, J.W. Foster, A.-M. Frischauf, R. Lovell-Badge, P.N. Goodfellow, A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif, *Nature, 346, 1990,* 240244.

[5]. G. Seidel, Economics of selecting for sex: the most important genetic trait, *Theriogenology, 59, 2003,* 585-98.

[6]. J. Peippo, M. Huhtinen, T. Kotilainen, Sex diagnosis of equine preimplantation embryos using the polymerase chain reaction, *Theriogenology, 44, 1995,* 619-27.

[7]. S.-H. Han, B.-C. Yang, M.-S. Ko, H.-S. Oh, S.-S. Lee, Length difference between equine ZFX and ZFY genes and its application for molecular sex determination, *Journal of assisted reproduction and genetics, 27, 2010,* 725-8.

[8]. S. Bucca, Equine fetal gender determination from mid-to advanced-gestation by ultrasound, *Theriogenology, 64, 2005,* 568-71.

[9]. P. Farin, C. Farin, Perspectives on large calves following transfer of embryos produced in vitro, *Embryo Transfer Newsletter, 15, 1997,* 15-9.

[10]. D. Silversides, N. Pilon, R. Behdjani, A. Boyer, I. Daneau, J. Lussier, Genetic manipulation of sex differentiation and phenotype in domestic animals, *Theriogenology, 55, 2001,* 51-63.

[11]. M. Santos, R. Moura, R. Chaves, A. Soares, J. Neves, H. Reichenbach, et al., Sexing of Boer goat fetuses using transrectal ultrasonography, *Anim Reprod Sci, 3, 2006,* 359-63.

[12]. G. Seidel. Sexing sperm for beef and dairy cattle breeding. Factors affecting calf crop: Biotechnology of reproduction: CRC Press, Boca Raton, FL; 2002. p. 281-6.

[13]. R. Rajamahendran, D.J. Ambrose, B. Burton, Clinical and research applications of real-time ultrasonography in bovine reproduction: a review, *The Canadian Veterinary Journal, 35, 1994,* 563.

[14]. L.A. Johnson, G.R. Welch, K. Keyvanfar, A. Dorfmann, E.F. Fugger, J.D. Schulman, Gender Preselection in Humans? Flow Cytometric Separation of X and Y Spermatozoa for the Prevention of X-Linked Diseases, *Obstetrical & Gynecological Survey, 49, 1994,* 342-3.

[15]. R. Foote, Normal development of fetuses resulting from Holstein semen processed for sex separation, *Theriogenology, 24, 1985,* 197-202.

[16]. M. Panarace, R. Pellegrini, M. Basualdo, M. Belé, D. Ursino, R. Cisterna, et al., First field results on the use of stallion sex-sorted semen in a large-scale embryo transfer program, *Theriogenology, 81, 2014,* 520-5.

[17]. B. Chezum, B. Wimmer, Roses or lemons: adverse selection in the market for thoroughbred yearlings, *Review of Economics and Statistics, 79, 1997,* 521-6.

[18]. N. Lansford, D.W. Freeman, D.R. Topliff, O.L. Walker, Hedonic pricing of race-bred yearling Quarter Horses produced by Quarter Horse sires and dams, *Journal of Agribusiness, 16, 1998,* 169-86.

[19]. R.J. Rijnders, C.E. van der Schoot, B. Bossers, M.A. de Vroede, G.C. Christiaens, Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia, *Obstetrics & Gynecology, 98, 2001,* 374-8.

[20]. R.W. Chiu, T.K. Lau, P.T. Cheung, Z.Q. Gong, T.N. Leung, Y.D. Lo, Noninvasive prenatal exclusion of congenital adrenal hyperplasia by maternal plasma analysis: a feasibility study, *Clinical chemistry, 48, 2002,* 778-80.

[21]. J. Bartha, K. Finning, P. Soothill, Fetal sex determination from maternal blood at 6 weeks of gestation when at risk for 21- hydroxylase deficiency, *Obstetrics & Gynecology, 101, 2003,* 1135-6.

[22]. K.A. Chan, J. Zhang, A.B. Hui, N. Wong, T.K. Lau, T.N. Leung, et al., Size distributions of maternal and fetal DNA in maternal plasma, *Clinical Chemistry, 50, 2004,* 88-92.

[23]. M. Resende, C. Moreira-Filho, C. Leal, M. Ramalho, A. Almeida, R. Vantini, et al., Falha na sexagem por inibição do desenvolvimento de embriões bovinos produzidos in vitro com anticorpos anti HY, *Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 60, 2008,* 594-9.

[24]. W. King, Sexing embryos by cytological methods, *Theriogenology, 21, 1984,* 7-17.

[25]. G. Anderson, Identification of embryonic sex by detection of HY antigen, *Theriogenology, 27, 1987,* 81-97.

[26]. T. Williams, A technique for sexing mouse embryos by a visual colorimetric assay of the X-linked enzyme, glucose 6-phosphate dehydrogenase, *Theriogenology, 25, 1986,* 733-9.

[27]. S. Leibo, W. Rall, Prenatal diagnosis of sex in bovine fetuses by amniocentesis, *Theriogenology, 33, 1990,* 531-52.

[28]. P. Bredbacka, A. Kankaanpää, J. Peippo, PCR-sexing of bovine embryos: a simplified protocol, *Theriogenology, 44, 1995,* 167-76.

[29]. M. Thibier, M. Nibart, The sexing of bovine embryos in the field, *Theriogenology, 43, 1995,* 71-80.

[30]. B. Shea, Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study, *Theriogenology, 51, 1999,* 841-54.

[31]. T. Peura, J.-M. Hyttinen, M. Turunen, J. Jänne, Areliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction, *Theriogenology, 35, 1991,* 547-55.

[32]. M. Murer-Orlando, K. Betteridge, C. Richer, Cytogenetic sex determination in cultured cells of pre-attachment horse embryos, *Ric Sci Educaz Permanent, 24, 1982,* 372-8.

[33]. A. Romagnano, W.A. King, C.-L. Richer, M.-A. Perrone, A direct technique for the preparation of chromosomes from early equine embryos, *Canadian journal of genetics and cytology, 27, 1985,* 365-9.

[34]. v.L.A. Moustafa, J. Hahn, R. Roselius, Versuche zur Geschlechtsbestimmung an Tag 6 und 7 alten Rinderembryonen, *Berliner und Muenchener Tieraerztliche Wochenschrift, 1978,*

[35]. K. Makondo, G. Amiridis, I. Jeffcoate, P. O'Shaughnessy, J. Boyd, C. Paterson, et al., Use of the polymerase chain reaction to sex the bovine fetus using cells recovered by ultrasound-guided fetal fluid aspiration, *Animal reproduction science, 49, 1997,* 125-33.

[36]. T. Wood, K. White, D. Thompson, F. Garza, Evaluation of the expression of a male-specific antigen on cells of equine blastocysts, *Journal of reproductive immunology, 14, 1988,* 1-8.

[37]. M.-T. Ramalho, J. Garcia, C.R. Esper, R. Vantini, B. Alves, I.A. Junior, et al., Sexing of murine and bovine embryos by developmental arrest induced by high-titer HY antisera, *Theriogenology, 62, 2004,* 1569-76.

[38]. I. Battut, S. Colchen, F. Fieni, D. Tainturier, J.F. BRUYAS, Success rates when attempting to nonsurgically collect equine embryos at 144, 156 or 168 hours after ovulation, *Equine Veterinary Journal, 29, 1997,* 60-2.

[39]. R. Veerhuis, P. Hendriksen, A. Hengst, L. Kruijt, M. Tieman, P. Booman, The production of anti-HY monoclonal antibodies: their potential use in a sex test for bovine embryos, *Veterinary immunology and immunopathology, 42, 1994,* 317-30.

[40]. B. Akyüz, O. Ertuğrul, M. Kaymaz, H. Macun, D. Bayram, The effectiveness of gender determination using polymerase chain reaction and radioimmunoassay methods in cattle, *Theriogenology, 73, 2010,* 261-6.

[41]. M. Monk, A. Handyside, Sexing of preimplantation mouse embryos by measurement of X-linked gene dosage in a single blastomere, *Journal of reproduction and fertility, 82, 1988,* 365-8.

[42]. S. Curran, J. Kastelic, O. Ginther, Determining sex of the bovine fetus by ultrasonic assessment of the relative location of the genital tubercle, *Animal Reproduction Science, 19, 1989,* 217-27.

[43]. S. Curran, O. Ginther, Ultrasonic diagnosis of equine fetal sex by location of the genital tubercle, *Journal of Equine Veterinary Science, 9, 1989,* 77-83.

[44]. E. Müller, G. Wittkowski, Visualization of male and female characteristics of bovine fetuses by real-time ultrasonics, *Theriogenology, 25, 1986,* 571-4.

[45]. S. Curran, O. Ginther, Ultrasonic determination of fetal gender in horses and cattle under farm conditions, *Theriogenology, 36, 1991,* 809-14.

[46]. S. Curran, Fetal sex determination in cattle and horses by ultrasonography, *Theriogenology, 37, 1992,* 17-21.
[47]. J.F. Garcia, Practical considerations of embryo manipulation: preimplantation genetic typing, *Theriogenology, 56, 2001,* 1393-9.
[48]. P. Bredbacka, J. Peippo, Sex diagnosis of ovine and bovine embryos by enzymatic amplification and digestion of DNA from the ZFY/ZFX locus, *1992,*
[49]. G.D. Pollevick, S. Giambiagi, S. Mancardi, L. De Luca, O. Burrone, A.C. Frasch, et al., Sex determination of bovine embryos by restriction fragment polymorphisms of PCR amplified ZFX/ZFY loci, *Nature Biotechnology, 10, 1992,* 805-7.
[50]. C. Fajfar- Whetstone, A.L. Rayburn III, L.B. Schook, M. Wheeler, Sex determination of porcine preimplantation embryos via y- chromosome specific DNA sequences, *Animal Biotechnology, 4, 1993,* 183-93.
[51]. T.S. Painter, The Y-chromosome in mammals, *Science, 53, 1921,* 503-4.
[52]. R.P. Piprek, Molecular and cellular machinery of gonadal differentiation in mammals, *International Journal of Developmental Biology, 54, 2010,* 779-86.
[53]. B. Kirkpatrick, R. Monson, Sensitive sex determination assay applicable to bovine embryos derived from IVM and IVF, *Journal of reproduction and fertility, 98, 1993,* 335-40.
[54]. E. Aasen, J.F. Medrano, Amplification of the ZFY and ZFX genes for sex identification in humans, cattle, sheep and goats, *Nature Biotechnology, 8, 1990,* 1279-81.
[55]. M. Huhtinen, J. Peippo, P. Bredbacka, Successful transfer of biopsied equine embryos, *Theriogenology, 48, 1997,* 361-7.
[56]. Y.H. Choi, A. Gustafson-Seabury, I.C. Velez, D.L. Hartman, S. Bliss, F.L. Riera, et al., Viability of equine embryos after puncture of the capsule and biopsy for preimplantation genetic diagnosis, *Reproduction, 140, 2010,* 893-902.
[57]. J. Jarazo, A. Gambini, L. Muredas, R. Fernandez-Martin, D. Salamone, 151 horse embryo biopsy: effect on pregnancy rates and successful sex determination depending on the size of the embryo, *Reproduction, Fertility and Development, 25, 2012,* 224-.
[58]. J. Beckelmann, S. Budik, C. Bartel, C. Aurich, Evaluation of Xist expression in preattachment equine embryos, *Theriogenology, 78, 2012,* 1429-36.
[59]. T. Stout, S. Meadows, W. Allen, Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival in vivo, *Animal reproduction science, 87, 2005,* 269-81.
[60]. P. Flood, K. Betteridge, M. Diocee, Transmission electron microscopy of horse embryos 3-16 days after ovulation, *Journal of reproduction and fertility Supplement, 32, 1981,* 319-27.
[61]. T. Bongso, P. Basrur, Prenatal diagnosis of sex in cattle by amniocentesis, *The Veterinary Record, 96, 1975,* 124-6.
[62]. L.B. Shettles, Use of the Y chromosome in prenatal sex determination, *Nature, 230, 1971,* 52-3.
[63]. M.R. Divar, H. Sharifiyazdi, M. Kafi, Application of polymerase chain reaction for fetal gender determination using cervical mucous secretions in the cow, *Veterinary research communications, 36, 2012,* 215-20.
[64]. C.J. Epstein, S. Smith, B. Travis, G. Tucker, Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos, *1978,*
[65]. A. Romagnano, C. Richer, W. King, K. Betteridge, Analysis of X-chromosome inactivation in horse embryos, *Journal of reproduction and fertility Supplement, 35, 1986,* 353-61.
[66]. Y.D. Lo, N. Corbetta, P.F. Chamberlain, V. Rai, I.L. Sargent, C.W. Redman, et al., Presence of fetal DNA in maternal plasma and serum, *The Lancet, 350, 1997,* 485-7.
[67]. K.M. Finning, L.S. Chitty. Non-invasive fetal sex determination: impact on clinical practice. Seminars in Fetal and Neonatal Medicine: Elsevier; 2008. p. 69-75.
[68]. Y.D. Lo, M.S. Tein, T.K. Lau, C.J. Haines, T.N. Leung, P.M. Poon, et al., Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis, *The American Journal of Human Genetics, 62, 1998,* 768-75.
[69]. T.-W. Lau, T.N. Leung, L.Y. Chan, T.K. Lau, K.A. Chan, W.H. Tam, et al., Fetal DNA clearance from maternal plasma is impaired in preeclampsia, *Clinical chemistry, 48, 2002,* 2141-6.
[70]. Y.D. Lo, K.A. Chan, H. Sun, E.Z. Chen, P. Jiang, F.M. Lun, et al., Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus, *Science translational medicine, 2, 2010,* 61ra91-61ra91.
[71]. J. Walknowska, F. Conte, M. Grumbach, Practical and theoretical implications of fetal/maternal lymphocyte transfer, *The Lancet, 293, 1969,* 1119-22.
[72]. L.L. Poon, T.N. Leung, T.K. Lau, Y.D. Lo, Presence of fetal RNA in maternal plasma, *Clinical Chemistry, 46, 2000,* 1832-4.
[73]. M. Alberry, D. Maddocks, M. Jones, M. Abdel Hadi, S. Abdel- Fattah, N. Avent, et al., Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast, *Prenatal diagnosis, 27, 2007,* 415-8.
[74]. Y.D. Lo, T.K. Lau, L.Y. Chan, T.N. Leung, A.M. Chang, Quantitative analysis of the bidirectional fetomaternal transfer of nucleated cells and plasma DNA, *Clinical chemistry, 46, 2000,* 1301-9.
[75]. L. Birch, C.A. English, K. O'Donoghue, O. Barigye, N.M. Fisk, J.T. Keer, Accurate and robust quantification of circulating fetal and total DNA in maternal plasma from 5 to 41 weeks of gestation, *Clinical chemistry, 51, 2005,* 312-20.
[76]. D.W. Bianchi, G.K. Zickwolf, G.J. Weil, S. Sylvester, M.A. DeMaria, Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum, *Proceedings of the National Academy of Sciences, 93, 1996,* 705-8.
[77]. Y.D. Lo, J. Zhang, T.N. Leung, T.K. Lau, A.M. Chang, N.M. Hjelm, Rapid clearance of fetal DNA from maternal plasma, *The American Journal of Human Genetics, 64, 1999,* 218-24.
[78]. F.Z. Bischoff, M.K. Sinacori, D.D. Dang, D. Marquez-Do, C. Horne, D.E. Lewis, et al., Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for first and second trimester non-invasive prenatal diagnosis, *Human reproduction update, 8, 2002,* 493-500.
[79]. H. Honda, N. Miharu, Y. Ohashi, O. Samura, M. Kinutani, T. Hara, et al., Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum, *Human genetics, 110, 2002,* 75-9.
[80]. D.W. Bianchi, Prenatal exclusion of recessively inherited disorders: should maternal plasma analysis precede invasive techniques?, *Clinical chemistry, 48, 2002,* 689-90.
[81]. N.D. Avent, L.S. Chitty, Non- invasive diagnosis of fetal sex; utilisation of free fetal DNA in maternal plasma and ultrasound, *Prenatal diagnosis, 26, 2006,* 598-603.
[82]. A. Sekizawa, T. Kondo, M. Iwasaki, A. Watanabe, M. Jimbo, H. Saito, et al., Accuracy of fetal gender determination by analysis of DNA in maternal plasma, *Clinical chemistry, 47, 2001,* 1856-8.
[83]. B. Zimmermann, A. El-Sheikhah, K. Nicolaides, W. HOLzGREVE, S. Hahn, Optimized real-time quantitative PCR measurement of male fetal DNA in maternal plasma, *Clinical chemistry, 51, 2005,* 1598-604.
[84]. B. Zhu, Q.W. Sun, Y.C. Lu, M.M. Sun, L.J. Wang, X.H. Huang, Prenatal fetal sex diagnosis by detecting amelogenin gene in maternal plasma, *Prenatal diagnosis, 25, 2005,* 577-81.
[85]. S. Sasaki, H. Shimokawa, The amelogenin gene, *International Journal of Developmental Biology, 39, 2003,* 127-33.

[86]. V. Cirigliano, J. Sherlock, G. Conway, C. Quilter, C. Rodeck, M. Adinolfi, Rapid detection of chromosomes X and Y aneuploidies by quantitative fluorescent PCR, *Prenatal diagnosis, 19, 1999,* 1099-103.
[87]. B. Pertl, S. Kopp, P.M. Kroisel, L. Tului, B. Brambati, M. Adinolfi, Rapid detection of chromosome aneuploidies by quantitative fluorescence PCR: first application on 247 chorionic villus samples, *Journal of medical genetics, 36, 1999,* 300-3.
[88]. P.M.M. de Leon, V.F. Campos, O.A. Dellagostin, J.C. Deschamps, F.K. Seixas, T. Collares, Equine fetal sex determination using circulating cell-free fetal DNA (ccffDNA), *Theriogenology, 77, 2012,* 694-8.
[89]. A. Kadivar, R.D. Tafti, H.H. Khoei, M.H. Nasirabadi, N.S. Esfandabadi, N. Cheraghi, et al., Developing a Nested Real-Time PCR Assay for Determining Equine Fetal Sex Prenatally, *Journal of Equine Veterinary Science, 40, 2016,* 74-9.
[90]. H. Kadokawa, N. Takusari, M. Minezawa, H. TAKAHASHI, T. KARIYA, Absence of Fetal Cells in Bovine Jugular and Uterine Vein Blood at a Level of 1 in 10,000, *Journal of Reproduction and Development, 42, 1996,* 205-8.
[91]. J. Xi, B. Jia, R. Li, W. Chen, D. Wang, S. Yang, Rapid sex identification of embryos in different developmental stages by blood of pregnant dairy cattle, *J Shihezi Univ Natural Sci, 24, 2006,* 446-9.
[92]. G. Wang, Q. Cui, K. Cheng, X. Zhang, G. Xing, S. Wu, Prediction of fetal sex by amplification of fetal DNA present in cow plasma, *Journal of Reproduction and Development, 56, 2010,* 639-42.
[93]. A. da Cruz, D. Silva, E. Costa, P. De M-Jr, C. da Silva, D. Silva, et al., Cattle fetal sex determination by polymerase chain reaction using DNA isolated from maternal plasma, *Animal reproduction science, 131, 2012,* 49-53.
[94]. D. Lemos, P. Takeuchi, Á.F. Rios, A. Araujo, H. Lemos, E. Ramos, Bovine fetal DNA in the maternal circulation: applications and implications, *Placenta, 32, 2011,* 912-3.
[95]. L. Turin, P. Invernizzi, M. Woodcock, F.R. Grati, F. Riva, G. Tribbioli, et al., Bovine fetal microchimerism in normal and embryo transfer pregnancies and its implications for biotechnology applications in cattle, *Biotechnology journal, 2, 2007,* 486-91.
[96]. A. Kadivar, H. Hassanpour, P. Mirshokraei, M. Azari, K. Gholamhosseini, A. Karami, Detection and quantification of cell-free fetal DNA in ovine maternal plasma; use it to predict fetal sex, *Theriogenology, 79, 2013,* 995-1000.
[97]. A. Kadivar, H. Hassanpour, J.A. Dehcheshmeh, A novel approach to prenatal fetal sex diagnosis by detecting an insertion in the Y-chromosome of ovine Amelogenin gene, *Small Ruminant Research, 123, 2015,* 218-23.