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Comparison of two different DNA isolation methods in the detection of equine fetal cell-free DNA

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Table of Content

1.Introduction

The central aims of this thesis are to discuss and review- past findings, the current methodology and elements of modern day genetical intervention paired with what this could bring us in the future as to sex, paternity and even genetical health of the equine fetus. This investigation is a continuation of previous methods where the presence of cell free fetal DNA in selected pregnant mares was carried out using a PCR and electrophoresis techniques in order to potentially identify the sex- determining region (SRY) and therefore the Y-chromosome specific amelogenin gene indicating and therefore confirming the fetal in utero as being that of the male sex.

The success of previous studies regarding the ability to identify the fetal sex of the unborn foal is open to scrutinisation, where the accuracy of such must be of sufficient standard to warrant in future it's possible use as a common sex detection technique in commercial equine breeding practice. Preceding the amplification of fetal DNA using PCR cycles and essential stage of the methodology is the importance of the extraction of the fetal circulating cell free DNA itself and the greater amount extracted correlates directly with the amount of amplified sex determining regions of the DNA.

The aim of this thesis is therefore to determine the best possible extraction method available by comparing two methodologies and the results of such in order to indicate the protocol offering the higher concentration of extracted fetal DNA identified as having critical importance in the outcome of the determining PCR stages.

Importance of early detection of pregnancy in the modern day equine practice is widely acknowledged; furthering this, sex determination of the fetus is fast becoming of significant interest to both horse owners & breeders. As the informative and economical value of these detection methods evolve, there is a resulting upsurge in recent research and studies in the animal breeding and veterinary field surrounding this topic. There are several sectors of the equine industry where a colt or filly is favoured; breeding Thoroughbred bloodlines for instance, sportsbased preferences in show jumping or mares in Polo Argentino horses where the use of embryo transfer techniques are increasingly used.

In recent years, with the use of sex sorted semen and embryo transfer programs the shift and manipulation of the sex ratio of the fetus has been proven feasible, however with varying success rates there is scope for development with further research and advances. In modern day veterinary medicine ultrasound is currently used as the prevailing method for sexing fetuses in utero, as the gold standard. There are some shortcomings of this technique where, in future and with greater research we hope genetic sexing will provide an alternative with early, non-invasive, accurate results. This combined advancement in techniques of embryo transfer with invitro fertilisation means the early detection of fetal sex of such a pregnancy is warranted as a way to confirm success of other breeding manipulations giving breeders important and perhaps critical information about the unborn foal.

2. Literature Review

2.1 Importance of sex determination in equine

In large animal farming the desire of pre- determination of an offspring's sex is well established and evidently correlated to production and economic value but when considering the value in the equine field the reasons are often broader depending on the breed, intended use and ultimately the eventual owner's personal preference (Aurich and Schneider 2014).

The direct correlation between the popularity of a colt or filly and the intended use of such is evident. For breeders there are various scenarios where knowing the sex of the fetus is hugely beneficial allowing decisions to be made, for instance whether to keep or sell the mare in foal or plan for future breeding and mating lists. When looking for a new horse, potential buyers consider gender alongside confirmation and bloodlines. Chezum and Wimmer, 1997 highlight at yearling auctions in breeds such as Thoroughbreds, typically intended for a racing future, young colts are seen to go for a higher price than fillies. When considering stud fees this too can be the case, many breeders look for attractive breeding lines and a foals winning potential, a colt who can go on to achieve and with the genetic quality become of financial value through future stud fees (Wilson and Rambaut 2008).

Preference towards mares is widely known in the Argentine Polo horses, the majority of these sport horses are mares due to several traditional reasons including that mares are said to be better 'built' with softer temperament- critical to a sport requiring precision under pressure, furthermore breeding filly foals from successful mares is economically beneficial. Argentina is acknowledged as being at the forefront of equine reproductive techniques being the first to produce a healthy equine clone (Gambini, A and Maserati, M 2018). Where other breeding federations worldwide are cautionary or exclude assisted reproduction in official breeding registrations or studbooks, the Argentine Polo Breeders Association promoted the research and application of techniques such as Artificial insemination and embryo transfer.

In addition to breeding for athletic success, sex determination could also be very valuable when considering future desire to preserve rare or even endangered breeds or bloodlines. Ultimately the demand for sex determination of foals is to some degree controversial, up to individual preference

and it is unknown whether the need for such would meet the financial investment necessary (Samper and Morris 2012)

2.2 Sex linked diseases in horses

With advancements in the development of techniques for sex determination, the ability to observe abnormalities and defects within the fetal genome is technically a possibility using the extraction of cffDNA and the completion of the equine genome (Wade et al 2009). Sex linked diseases are defects in genes either on the X or Y chromosome resulting in unwanted traits or disease. X linked recessive traits appear more frequently in males, with females being carriers. Haemophilia A is one example where affected foals will suffer from prolonged bleeding times.

The most important sex chromosomal abnormalities reported in horses are deemed to be Chimerism, Turner's Syndrome and Sex Reversal Syndrome. Disorders from the Y linked chromosome are fairly rare as a result of the Y chromosome having few actual genes, those that are present are associated with male fertility and therefore commonly result in infertility of the affected males. Abnormalities with chromosome pairs ECAX and ECAY are commonly associated with reproductive problems in horses, many being under diagnosed through the lack of an efficient, affordable diagnostic tool (Anaya et al 2017).

In humans there has been extensive research in to the usage of cffDNA as a non-invasive diagnostic tool for pre-natal genetic diagnostics from maternal blood; this is invaluable as a safer method of testing as the previous methodology of testing was not without risk of the viability of the pregnancy. Further implications of these methodologies have proven very successful, now being used for Preimplantation genetic diagnosis of embryos for uterine transfers (BustamanteAragones et al 2014). Whilst the current priority of equine research regarding ccffDNA is the improvement and refinement of the accuracy of methodology for prenatal sex determination, there is significant future prospects for its use in genetic disease diagnosis considering advancements in human research.

2.3 Factors affecting the sex ratio

Various studies have shown that in the past that the theoretical sex ratio of 1:1 in horses is something that can be manipulated or shown to alter as a result of different factors, often associated with parental influence. Age for instance plays a significant role as highlighted in studies carried out on the breeding of mares and stallions- each of varying age; it is widely known that whilst the age of the stallion seems uncorrelated, in mares of increasing age there is a shift in sex ratio towards fillies (Santos et al 2015).

In addition to the age, nutritional status and condition of the mare at the time of conception is also proven to have an impact on the sex ratio. Trivers-Willard hypothesis (Trivers-Willard 1973) has been illustrated in various studies, where mares in poorer condition are more likely to have a female foal. Essentially a mare in an enriched nutritional environment will aid the uterine environment for the development of a healthy foal where often male embryos are seen to thrive (Cameron and Linklater 2007).

In addition to age and condition, sex dependent insulin like growth factor (IGF-1) is another element central to sex ratio in the early stages of pregnancy, IGF aids embryonic development by increasing cell proliferation and decreasing apoptosis. In high glucose conditions sex ratio is shifted towards male offspring; this is a result of elevated glucose being seen to be detrimental to female embryo development (Beckelmann and Budik 2013).

2.4 Current fetal sexing determination of horses

Gestation length of the mare lies between 320-362 days with most mares tending to have individual lengths and favouring a particular sex, this becomes increasingly apparent the more foals they have, in addition to this there are a vast range of factors affecting this time period as well as the sex of the foal (Davies Morel et al, 2002). Ideally sex determination is desired as early in pregnancy as possible thus allowing planning for the outcome of the pregnancy, even to the extent of whether to proceed with the pregnancy or sell the mare in foal. The use of sex sorted semen and embryo transfer offers the possibility of the sex to be pre-determined however; this must still be verified once the pregnancy is viable, these techniques although proven to work are not irrefutable, need refinements and ultimately clients may request sex determination without the use of these techniques.

Ultrasound techniques are well established in practice and the development of molecular techniques are ongoing and proving successful, detection of circulating cell free fetal DNA in maternal plasma to detect the fetal sex has been performed in later pregnancy but further research and adjustments of the methods are needed for early detection. (Aurich and Schneider 2014). Cytogenetic study is central to these current techniques allowing the determination of X, Y chromosomes, enzymes linkages and most vitally the sex specific DNA fragments of which the SRY gene fragment is essential (de Leon et al 2012).

2.4.1 Equine embryo transfer programmes

With the progression of reproductive biotechnologies such as artificial insemination and embryo transfer came huge opportunities for breeders and the advancement of genetics in breeding by the possibility of not only refining desired characteristics and manipulating bloodlines but now sex ratios (Crisan et al 2015). Embryo transfer is particularly advantageous as it allows the continuation of genetics from mares who are too mature to endure pregnancy and competing mares to continue whilst producing a foal or even multiple foals in a breeding season from the donor mare.

Sex determination of equine embryos has been practiced by various methodologies classed as either invasive or non-invasive, the later aiming to avoid any damage to the embryos integrity and viability in the recipient uterus; methods include the monitoring of X linked enzymes and the detection of the sex linked H-Y antigen on the Y chromosome (Crisan et al 2015). It was however discovered (Aurich and Schnneider 2014) that false negatives were likely in embryos post blastocoel formation and as the equine embryo arrives to uterus at a later morula or early blastocyst stage not all recovered embryos would be viable. The invasive methodology is at the risk of destroying the embryo; by taking a biopsy of cells from the zona pellucida the harvested genetic material can undergo cytogenic analysis using PCR method to identify and target a DNA segment specific to Y chromosome, this was first carried out in an equine embryo by Hubtinnen et al 1997. The equine karyotype in horses has 31 pairs of autosomal chromosomes and a pair of sex chromosomes (Richer et al 1990). Shown to be highly accurate this invasive technique is valuable however the nature of such an invasive methodology significantly affects viable pregnancy rates, it is hoped that in future this shall be improved and that this technique may also be used to identify genetically linked diseases.

2.4.2 Fetal sexing by using sex sorted semen in horses

Sex sorted semen is very well established in the bovine species at a commercial level, enabling the skew of the sex ratio towards the financially beneficial female sex with the methodology of flow cytometry having been refined since discovery in 1989 (Johnson LA 2000). In equine and porcine species a larger quantity of sperm is required to inseminate the mare and therefore this highlighted a constraint of this technique as only a certain volume of sperm can be sex sorted per hour using flow cytometry (Pannarace et al 2014). Additional to the lacking volume, equine sorted sperm are also typically less viable with both a decreased survival time and increased intolerance to the freezing process. This intolerance to freezing is a large logistical problem as semen is sold worldwide often as frozen samples. Semen is sorted using very high-speed flow cytometry methodology based on amounts of DNA within the X and Y chromosomes, shown to be stable in healthy sperm (Garner, D.L 2006).

Spermatozoa sexing technology involves various essential phases to ensure specificity of results whilst preserving the fertilisation capability of the spermatozoa. First is the preparation and marking of the semen, this is done using the Hoechst 33342 fluorochrome dye allowing identification of nucleic acids whilst ensuring vitality of the spermatozoa (Garner, D.L 2006). Addition of FD-C40- a red food dye-identifies any dead and damaged protozoa initiating their removal from the sample. Differentiation and sorting of cells is based on their size and content, identified by the amounts of fluorescence emitted by marked material; validity and efficiency are monitored continuously where both the quality of the sperm sample and the shape of the head of the spermatozoa play critical roles (Pindaru et al 2016).

Sex sorted stallion semen technologies must be combined with both embryo transfer and artificial insemination; the use of deep AI has proven to aid with equine (and porcine) insemination with lower the amount of sorted semen than the natural amount usually required for fertilisation (Johnson LA 2000).

2.5. Applied fetal sexing determination of horses

2.5.1 Fetal sexing using ultrasound

The use of ultrasonographic technique to detect pregnancy, embryonic development and fetal sex, like with many domesticated mammals is the most widely used methodology among veterinary technicians in equine medicine. Both transrectal and transabdominal techniques are used depending on the stage of embryonic development. As the pregnancy develops it is possible for the operator to visualise the maturation of the fetus and determine the sex.

Early detection of equine fetal sex is most successful between 59-68 days of gestation using real time linear ultrasound with a transrectal technique and with the correct plane and positioning (Curran and Ginther 1989, A.Tonisen et al 2015). Detection of sex is based on the anatomical differentiation of the location of the genital tubercle, appearing as a hyperechoic equal sign on the ultrasound. Initially located between the hind limbs, the genital tubercle migrates closer to the umbilicus in the male as the precursor of the penis and towards the rectum in the female as the precursor of the clitoris. (S. Bucca 2005, Aurich 2014).

As gestation progresses after day 70 this initial transrectal technique is inadequate around 140 days as the fetus descends lower and more ventrally into the pelvic cavity. At this point a combination of both transrectal and transabdominal ultrasonic views can be used to detect the fetal sex with the determination of external genitalia of the fetus; this can be done within a wider range of time up to 8 months gestation, the optimum time being between Day 120-210 (S. Bucca 2005). Transabdominal technique provides a non- invasive, low risk procedure essential in advanced gestation for fetal determination (A.Tonissen et al 2015).

With the technological advancement of Doppler ultrasonography this may also be used as an effective technique at the advanced gestation stage; as seen in *Figure 1.* In males the Doppler allows the detection of the blood flow along the mediastinum testis and within the plexus pampiniformis and in females, although less accurate, the oval shape of the gonads can be recognised (H. Resende et al 2014)

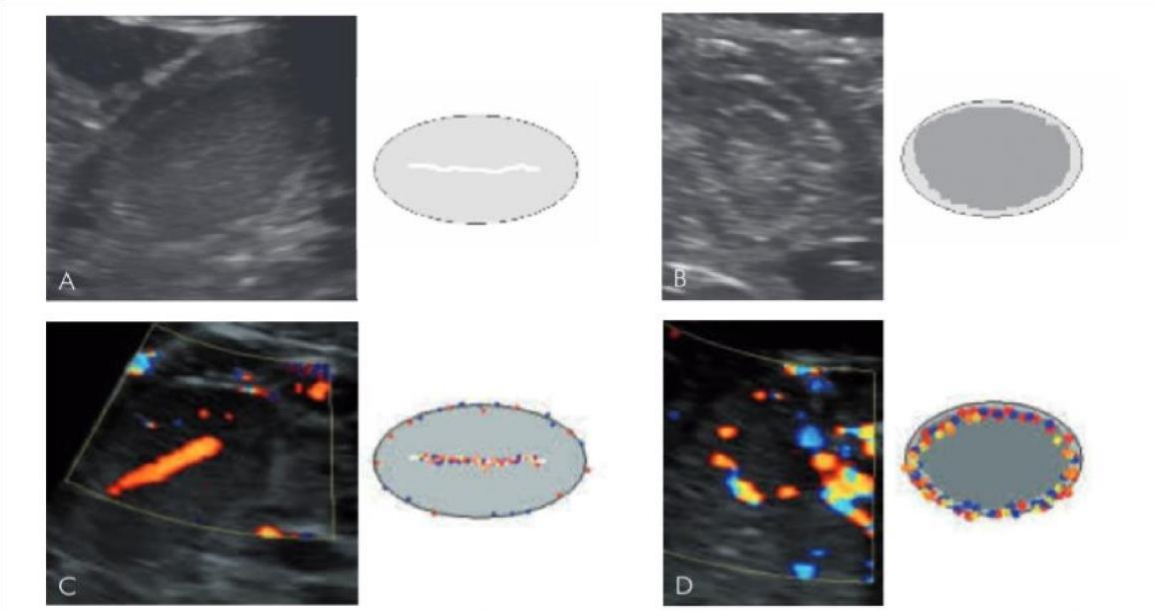

Figure 1- Contour drawings and real B-mode Doppler sonographic transabdominal images of advanced stage gestation.

A- Fetal male gonad -typical oval form and hyperechogennic longitudinal line of mediastinum testis.

B- Fetal female gonad- typical oval form, hyperechoic circle around an echodense centre showing the cortex and medulla of the ovary

C- Fetal male gonad- oval shape with intense signal along the longitudinal axis, representing theblood flow along the testis and a signal of the lateral contour representing the plexus pampiniformis.

D- Fetal female with typical oval shape and a strong circular signal representing the corticomedullary border.

The reliability of the ultrasonographic technique is however entirely dependable on the proficiency of the operator carrying out the examination signifying that though this technique is highly regarded- equipment and expertise are vital to provide accurate results and to ensure the safety of both the operator and the mare (Holder 2002). Advancement in PCR technology aims to produce a method allowing earlier detection than ~55 days where the veterinary technician need only provide an adequate sample for DNA extraction.

2.6 Molecular fetal sex diagnosis

Moving from traditional methods of transrectal or transabdominal ultrasonography, determination of the equine fetus has now been identified as possible using molecular techniques such as Polymerase Chain Reaction used to amplify DNA, fast becoming a sensitive, accurate, reliable and in future practical alternative (de Leon et al 2012). Molecular sex diagnostics in mammals is based upon the DNA variations between the two sexes; various methods and protocols are used for the process involving the extraction of the DNA and PCR techniques where fetal DNA extraction is critical to accuracy of results.

2.6.1 Molecular diagnostics used in fetal sex determination in various mammals

With the discovery and implication of using molecular techniques alongside the sex determining region (SRY) gene (Sinclair et al 1990) in subsequent years the methodology has been adapted and used globally for various species. PCR sequencing and amplification of various mammalian taxa including primates, carnivores, Asian elephants and the Indian rhino where investigated, demonstrating that this could open a valuable door to new conservation projects and gene lines (Fernando and Melnick 2001). Richard and Carrey (1994) also demonstrated the value of molecular sexing in *Physeter macrocephalus*, the sperm whale, as a species where sexual dimorphism lead to difficulty in sexing visually- this was then explored in species such a birds and other aquatic mammalians.

Sexing in livestock is of significant benefit to the farming industry with regard to breeding, and within production lines of products such as meat and milk; molecular sexing therefore quickly provided a non-invasive, reliable method for sexing bovine fetuses, favouring female offspring as an addition to technologies such as sexed semen and artificial insemination. Davoudi et al (2012) developed and evaluated a method using PCR of fetal DNA extracted from maternal plasma of pregnant cows; two DNA primers, amelogenin gene (bAML) and BC1.2 were designed and amplified to show on the X/Y chromosome of the bovine (Davoudi et al, 2012).

In order to determine the sex of the fetus from a maternal sample the amplification of the Y chromosome specific fragment of the DNA is needed, this is based on the sex determining SRY gene indicating with its presence the male sex of the fetus. (Aurich and Schneider 2014). Fetal sex

determination can also be carried out with the amplification of homologous gene fragments based upon the chromosomes being of different lengths allowing the identification of either the X or Y chromosome for diagnosis (Fernamdo and Melnick 2001). Sex typing with PCR methodology using SRY, the amelogenin gene of the Y and X chromosomes (AMELY/ AMELX) and the Zinc-Finger genes (ZFX/ZFY) can be analysed through PCR and gel electrophoresis protocols (Hasegawa et al 2000). Both methodologies are relevant as the detection of homologous gene fragments allows the confirmation of a female chromosome as opposed to the detection of the SRY gene which is solely based on its absence to confirm the female sex, where the possibility of a failed PCR could easily lead to a false male sex detection (Fernando and Melnick 2001)

2.6.2 Origin of cell free fetal DNA

It is known that during gestation, maternal and fetal circulations are separated by the fetalplacental unit, in equine the diffuse epitheliochorial placenta creates an essential barrier and communication between the mare and foal. With the discovery of substantial fetal derived DNA in maternal plasma, placental crossing deemed to be the result of physical, immunologic damage and developmental apoptosis.

This finding of circulating free fetal DNA gives feasible means for the molecular determination of fetal gender from maternal plasma and from then on, this possibility was explored (Redman, Lo et al 1997, Davoodian et al 2016). The realisation that cell lysis of fetal tissues results in free fetal DNA reaching maternal circulation immediately offered new possibilities of non-invasive techniques for prenatal testing and sex determination, where previously methods such as cell biopsy's in embryo transfer and amniocentesis procedures, whilst providing DNA, often required putting the viability of the fetus at risk (Davoodian and Kadivar 2016). The isolation of Y specific fetal DNA fragments in maternal blood is therefore now know to be able to identify the gender based upon the absence or presence of the male Y chromosome sex determining gene. All molecular sexing is based upon this through the usage of extraction and amplification with PCR as with the determination of the SRY gene or lengths of homologous XY chromosome fragments. The methodology using ccffDNA was initially explored and sequenced for human maternal plasma. From this, new technology and protocols are being developed and further researched for equine reproductive manipulation- as with other mammals.

From research in humans the amounts of circulating cffDNA in maternal blood plasma has shown to be directly correlated with stage of gestation and fetal development, using quantitative real time PCR it showed concentrations of cffDNA to be between 3.4% and 6.2% throughout gestation (Lau et al 2002). Lo et al (2002) discovered that another benefit of ccffDNA was the clearance rate of maternal plasma being very rapid meaning that any previous pregnancies do not compromise the detection of the current prenatal sex determination as well as any testing for prenatal genetic disease. (Lo et al 1997). Furthermore, it was also proved that the transfer of fetal DNA to maternal blood occurs in all pregnancies, thus confirming that the detection of sex by using circulating free

fetal DNA is feasible for all pregnant mares as ffDNA may be extracted from all maternal blood samples if handled correctly (Lo et al 2000).

2.6.3 Use of ccffDNA (cell-free fetal DNA) in the detection of fetal sex

Initially (Lo et al) used a nested PCR system to detect the cffDNA in the maternal serum and plasma in order to identify the Y chromosome gene; as with previous molecular diagnostic techniques, TSPY (Testis Specific Protein Y encoded) gene from which the DYS14 marker sequence was detected. The amplification and electrophoresis of the DYS14 which is within the TSPY gene allowed for the prenatal detection of a male fetus only and with a sensitivity of 80% (Lo et al 1997). Progression from this initial technique has provided more reliable results than via the detection of DYS14 by using the detection of the SRY sex determining region instead as a single target gene as opposed to multi copy DYS14 (Honda et al 2002). Furthermore, with the current use of quantitative real time PCR, sensitivities in excess of 97% have been established with an overwhelmingly low to nil cases of false positive results..

Prenatal sex diagnosis through isolation of the amelogenin gene in maternal plasma has more recently been described as an alternative methodology that, as previously highlighted, allows for the simultaneous amplification of not only the Y but X chromosome. There by offering a methodology that could determine a female fetus based not solely on the absence of a Y chromosome derived gene (Zhu et al 2005). Amelogenin gene is a size polymorphism of AMELXY located on both chromosomes, recently nested PCR techniques are being researched to use this gene as a marker for detection of fetal gender. This methodology has been successfully carried out in livestock species, Kadivar et al (2015) used the Amelogenin gene with a relative sensitivity and accuracy of 96.5%, this study also ascertained that the relative quantification of cell free fetal DNA values were significantly higher in ewes past 3 months of gestation.

There have now been various studies enquiring into various methodologies of fetal sex determination and each carried out at various stages of gestation (Table 1). Current and future research projects aim to meet accuracy in standards and methodology in order for ccffDNA to become a frequently used, sensitive and importantly a non- invasive prenatal sex diagnostic tool with the potential to be used for other prenatal testing such as for genetic diseases.

Author	No. of animals	Gestation	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 (2012)	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	Amelogennin	93.3%

Table 1. Studies reporting identifying male fetuses from ffDNA in maternal circulation (Davoodian and Kadivar 2016)

2.6.4 Fetal sex detection using cell free DNA in horses

When adapting the methodology from human reproductive research there is a basis of understanding and techniques that can be directly transferred and used in developing the same test in Equids; there are however differences and as such resulting challenges became evident.

Initially there is the isolation of equine cffDNA from maternal DNA through the extraction process, studies carried out highlight that maternal DNA, as expected, decisively overpowers fetal DNA, which has been shown to represent a mere 3-6% of total DNA present (Lo et al 1998). Humans possess a haemochorial placenta which of the various species is a type of placenta which is made up of the least feto-maternal histological layers allowing passage of various nutrients and immunological exchange. Horses however have a epitheliochorial placenta consisting of 6 layers from the fetal and maternal membranes: this means that there is a much more restricted interface between fetus and mare highlighting the fact that equine maternal plasma will likely contain significantly less amount of cffDNA (Kadivar et al 2016).

The Federal University of Pelotas in Brazil developed and carried out a technique to determine sex of the fetus using ccffDNA in equine species, before this the methodology had only been known and reliable in human medicine and some livestock species, this study aimed to aid early sex determination. This research project consisted of blood from 20 Thoroughbred mares, all in the final 3 months of gestation where ccffDNA was isolated from maternal plasma and a PCR was undertaken to determine the fetal sex by isolating and confirming the absence or presence of the Y chromosome. Two non-pregnant mares and two virgin mares were used to control the molecular technique. Amplification of the fetal DNA was carried out using the polymerase chain reaction technique, a total of two PCR stages were carried out, a PCR and then a 2nd PCR and a quantitative PCR to determine the sex of the fetus using primers designed to target the SRY gene, a target primer designed for GAPDH was also used as a control for the PCR accuracy. The first SRY/PCR had an accuracy of 85% and a sensitivity off 72% correctly identifying 8/11 males, specificity was 100% identifying all females. It was seen that in the second expanded reamplification PCR and qPCR the number of accurately identified male sexed fetuses increased, showing an even greater percentage of 95% accuracy and 90% sensitivity. (DeLeon et al 2012).

This study also demonstrated that by using larger volumes of plasma and highly sensitive molecular techniques there was a reduction of possible test failures. These failures may be the result of undetectable concentrations of ccffDNA leading to false fetal female determination as a result of the absence of a Y chromosome sequence. The importance of the extraction phase of the protocol is emphasised here as the accuracy of determination of the female sex is so critically linked to the concentrations of extracted ccffDNA.

In more recent studies Kadivar et al carried out a further investigation using similar methods of PCR to detect the SRY gene in 28 pregnant Arabian mares between 8 and 20 weeks of gestation, an earlier stage than the De Leon study. Real time PCR was performed to identify both the SRY gene and GAPDH as a control. Initially the results were inconclusive for SRY gene but the 2nd round of PCR proved successful with an accuracy of 88%, sensitivity of 85.7% and a specificity of 90.9%, and in general correctly identifying 12/13 males and 10/12 females, proving that the detection of circulating cffDNA can be used to detect the fetal sex very early on in gestation (Kadivar et al 2016).

When comparing different species protocols and the methodology used to date in equids there are many similarities within these and development off such. While in humans using ccffDNA is a fairly well-known concept and researched topic, in most other species it is still to be explored and methodology refined. Similarities in controls however are evident as an essential element in measuring accuracy and efficiency whilst methods are developed; glyceraldehyde-3 phosphatedehydrogenase (GAPDH) gene is used is as an internal control for efficiency of cffDNA extraction which is vital to ensure there is reduced false negatives (De Leon et al 2012, Kadivar et al 2016). In each of the experiments there were also additional controls, De Leon used nonpregnant and virgin mares and Kadivar DNA from a virgin mare and male genomic DNA to demonstrate accuracy. For instance amplification of Y chromosome specific sequences in male blood was used as positive controls to ensure the primers amplified the correct sequence. In future studies it is essential to note control, measure accuracy, sensitivity and efficiency and compare to previous research with their methodology of control in mind.

2.7 Conclusions from Literature Review

Equine fetal sex determination is currently carried out using transrectal ultrasonographic techniques as a standard practice, historically ultrasound has offered a safe, efficient and inexpensive way to diagnose the sex of a fetus by identifying the genital tubercle at around 55 days of pregnancy. Ultrasound is not without its limitations, later in pregnancy transrectal diagnosis becomes increasingly challenging to impossible and alternate techniques such as transabdominal and Doppler ultrasound are required, and in addition to this any locating and identification of the genital tubercle requires a very skilled operator and the right equipment. Embryo transfer and sex selected sperm offer pre determination of sex before implantation however molecular sexing proposes potential as a new methodology for the prenatal fetus, based on the detection of cell free fetal DNA, using PCR there is potential for this to become a routine test with advances in future for genetic abnormalities.

To the best of our knowledge these are the only two studies on the detection of equine fetal sex determination to date, using methodologies adapted from the human molecular protocols. They share various similarities and whilst both studies conclude that the use of fetal circulating cell free DNA is a non-invasive diagnostic tool to detect prenatal fetal sex both too highlight that the method must be developed further in order to have better accuracy and sensitivity.

Many aspects of the methodologies including extraction methods and primer design still vary and the accuracy, sensitivity and specificity of the results are not yet refined and supported enough through research for these to currently be viable routine laboratory tests. Although accuracy in previous studies proves the value of cffDNA, false negative and positive results are a significant area of concern. False negatives resulting from a failure to detect the Y chromosome, possibly due to the yield of ccffDNA through different extraction methods and at varying stages of gestation (Kadivar et al 2016).

Several kits for isolation off ccffDNA have been introduced however they are all financially cost inefficient, especially with the intention of the methods becoming routine tests (Clausen et al 2007). False negatives are of high concern as the basis of molecular fetal sex detection is that female fetuses are not detected directly and instead identified by the absence of the Y chromosome.

A possible method to reduce this would be to use multiple loci as DYS14 is used in human studies, this is something that could be applied to the equine protocols (Clausen et al 2007).

The goal is to develop a methodology to obtain the highest amount of cell free DNA that is circulating within the maternal plasma, in order for this to happen there must be a strategy to increase sensitivity. The extraction method for obtaining the ccffDNA must be optimised to ensure a high yield, concurrently the time of gestation should also be considered as standard time period to ensure accuracy. Many studies based upon PCR amplification of ccffDNA have highlighted the significance of an increase in yield of ccffDNA with samples taken later in gestation and therefore an increase of accuracy (Kadivar et al 2013).

So far in the research into use of ccffDNA in equine species there has not been exploration of the use of homologous X and Y fragments for targeting primers which would potentially offer more accurate results and specificity regarding the determination of the female prenatal fetus. Kadivar et al carried out a study of such in ewes with a 93% accuracy, highlighting the significance of such methodology.

With the completion of the equine genome (Wade et al 2009) cffDNA has the potential to be of use in the detection of genetic diseases. To our knowledge there has been no research into the diagnosis of genetic defects prenatally using circulating cell free fetal DNA, this highlights how significant the current research is into ccffDNA and how compared to human methodologies and knowledge, the exploration in to prenatal sexing and sexing using this molecular methodology is still very much in the primary stages.

The initial aim of this study is to investigate a methodology which yields a high amount of fetal cell free DNA from a maternal blood sample, comparing the performance and yield from two DNA extraction techniques. The methodology and results will then be scrutinised in order to determine the accuracy and sensitivity of the test as an indication into whether this could in future be used in clinical practice diagnostics.

3. Materials and Methods

3.1 Sampling and preparations

Pregnant mares' samples

In order for this investigation to take place samples were obtained from twelve mixed genotype Hungarian bred mares from Lipizzaner, Hungarian Sport Horses to be used within the study. Samples were obtained from various breeding stables. All mares have progressed as healthy natural gestations and were monitored as being asymptomatic of any abnormal signs during the study; each were carrying a single foal fetus confirmed by the private veterinarian. Each of the mares were sampled once in November 2018; as they were not all bred at the same time and pregnancy timings were unknown to us the 12 blood samples have therefore been collected from various stages of pregnancy ranging from 125-300 days of gestation.

Blood sampling and preparation of plasma samples

Peripheral blood was collected from the *vena jugularis* on the left side of the neck into tubes containing gel clotting activator (Vacutainer® Serum Tube, BD Medical, USA) using a Nr 21 gauge vacuum needle (Vacutainer® Needle, BD Medical, USA). The area of venipuncture was cleaned with ethanol prior to sampling to disinfect the skin surface. In each case 9ml of blood was taken. In order to avoid possible circadian changes in blood values, blood sampling was performed between 9 and 12 am in all cases. Once obtained samples were then transported in a cooling box into the laboratory within 6 hours of being taken. On arrival to the lab samples were centrifuged for 10 minutes at approximately 1000 × *g*. Serum samples were then stored in Eppendorf tubes at –80 °C until analysis to avoid loss of bioactivity and contamination. There were no repeated freeze/thaw circles in order to avoid any alterations to samples.

3.2 DNA extraction from plasma samples

The isolation of DNA is essentially the extraction of the nucleic acid from the rest of the cell organelle from the given sample; the cell membrane and nuclear envelope must be broken down and the DNA extracted must be precipitated and purified from other cell debris.

As illustrated two extraction methods; ZYMO ccffDNA vacuum kit and the Phenol-chloroformisoamyl alcohol method were applied and compared by measuring the quantity of the extracted cell free DNA with Qubit Fluorometer. The yield of the purified DNA extracted is a critical step in the process of determining the fetal sex as it is directly related to the accuracy of the results with the amplification of the extracted ccffDNA through the PCR system giving the result of the determined sex.

3.2.2 Phenol-chloroform-isoamyl alcohol method

The PCI extraction method is based upon a liquid-liquid DNA isolation method; solubility separation using a preparation of three chemicals Phenol, Chloroform and Isoamyl alcohol is central to this methodology. Proteins are denatured and collected in organic phase and nucleic acid stay in the aqueous phase (UltraPure, Thermo Fisher Scientific, Bioscience Ltd).

The twelve plasma samples were purified and concentrated using a modified "organic/dialysis" method (Comey et al. 1994, Pádár et al. 2001). A total of 600 μL of plasma from each sample and a TE buffer of equal volume were combined with 60 μL of proteinase K solution (PCR grade, 20 mg/mL, ThermoFisher Scientific, Bioscience Ltd, Budapest, Hungary) in a 2 mL Eppendorf tube and digested overnight in a 56°C thermo block. The following day, 780 μL of UltrapureTM phenol:chloroform:isoamyl alcohol (ThermoFisher Scientific, Bioscience Ltd, Budapest, Hungary) was added to the digested solution. After vortexing for 30 seconds and centrifugation for a duration of 10 minutes at 13.000 rpm, the supernatant was transferred into a sterile 2 mL Eppendorf tube and the previous extraction process was repeated. Microcon®-30 centrifugal filter units (Merck Millipore, Merck Ltd., Budapest, Hungary) were used for purification and concentration of the extracted serum solution. Purified DNA was then recovered in 50 μL of TE buffer and concentration was measured by a Qubit 2.0 Fluorometer (Life Technologies Corporation, Biocenter Ltd., Szeged, Hungary).

3.2.1 ZYMO cfDNA Vacuum kit

For the second extraction method a Quick cfDNA Serum and Plasma kit was used (ZYMO Research corp, D4076) with a vacuum manifold intended to provide a simple yet reliable protocol to obtain high quality extracted circulating cell free DNA from blood serum or plasma ready for analysis via PCR. This protocol included various stages, Figure 2 below indicates a section of the procedure and the kit used.

Figure 2- Procedure overview of 12 samples loaded to vacuum vials attached to the vacuum pump. (Department of Animal Breeding. University of Veterinary Medicine Budapest)

Initially the reagent is prepared; 20mg Proteinase K added to 1040 µL Proteinase K Storage buffer, vortexed to dissolve and stored at -20 ^{oC}. 48ml of 100% ethanol must be added to 12ml RNA Wash Buffer.

The same samples used for the Phenol-chloroform-isoamyl alcohol method were used for this purification protocol. First centrifuge samples >12000 x g for 15 minutes to remove any debris and

precipitate whilst in a fresh tube 200 µL Quick cfDNA/cfRNA Digestion Buffer per 200 µL of sample was mixed via a pipette and 10 µL Proteinase K per 200 µL sample was mixed via vortexing for 10 seconds. This was then incubated at 37^{oC} for 2 hours.

1 volume Quick-cfDNA/cfRNA™ Binding buffer was added to the digested sample and thoroughly mixed with a 10 second vortex, 1.5 volume of 100% isopropanol was then added and the sample again mixed for 10 seconds. As with the Phenol-chloroform-isoamyl alcohol method the concentration of extracted DNA was measured using a Qubit 2.0 Fluorometer.

3.3 Measurement of DNA quantity with Qubit fluorometer

Following the above described extraction methods, a Qubit Fluorometer was used to measure the quantity of extracted fetal DNA in order to obtain results and compare the efficiency of both methods. Qubit Fluorometers provide an 'accurate and sensitive biomolecule quantification' through quantitation assays (ThermoFisher Scientific, Bioscience Ltd, Budapest, Hungary). In order to obtain an accurate quantification, the fluorometer detects a florescent dye that is specifically bound to the target molecules of DNA. This use of fluorometers provides a much more sensitive and accurate measurement of even low concentrations of DNA than with previously used UV spectrophotometry. For experiments such as this it is critical to know the methods providing the most amounts of extracted DNA as this will go on to determine the reliability of the sex determination PCR. (Invitrogen by life technologies, Qubit® 2.0 Fluorometer Catalog no. Q32866).

3.4 Statistical analysis

The possible association of the measured DNA quantities and the effect of gestational age on DNA quantities have been evaluated using regression analysis, normality testing in the R program (R Core Team (2016); R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/).

4. Results

The measured DNA quantities and other collected data is illustrated below in *Table 2*.
Because of the small sample size (n=12) we could not count the means and standard deviations of cell-free DNA in most of the cases. After determination of gestational months (Day 0-30: 1st gestational month, Day 31-60: 2nd and so on), we could determine mean ±SD for months 7 and 8. Mean (±SD) DNA quantity was 16,95±6,01 ng/µL for Month 7 and 11,36±9,28 ng/µL for Month 8. There was no association detected between DNA quantity and gestation months in this analysis.

Sample nr	Gestational days	Phenol-Chloroform method DNA quantity (nanogram/microlitre)	Zymo Plasma cell-free kit DNA quantity (nanogram/microlitre)
1	300	1,2	11
2	240	1,3	1,15
3	120	3,5	20
4	270	7,68	16,5
5	240	1,8	6,2
6	210	10,6	21,2
7	240	6,6	21,2
8	240	1,68	16,9
9	270	6,56	20,4
10	205	0,796	12,7
11	128	2,36	8,52
12	175	0,271	0,436

Table 2. Samples (1-12.), gestational days, and DNA-quantity according to the two different DNA isolation methods.

Results are shown in Figures 3-5. As it can be seen, there were significant differences between DNA quantities measured with the 2 different methods. Although the quantities of isolated DNA showed a significant correlation **P=0,00719, r=0,728, r^2 = 0,53** between the two different methods, they differed significantly **(P=0,0003).**

Evaluating via regression analysis**, P=** signified that there was a substantial difference in isolation of DNA despite methods being performed in the same conditions with the same operators and from the same samples.

Significantly more DNA could be isolated with the Zymo cell-free vacuum isolation kit as illustrated in Figure 4.

As Figure 5 indicates, there was no correlation between isolated cell-free DNA and gestational days of the pregnant mares.

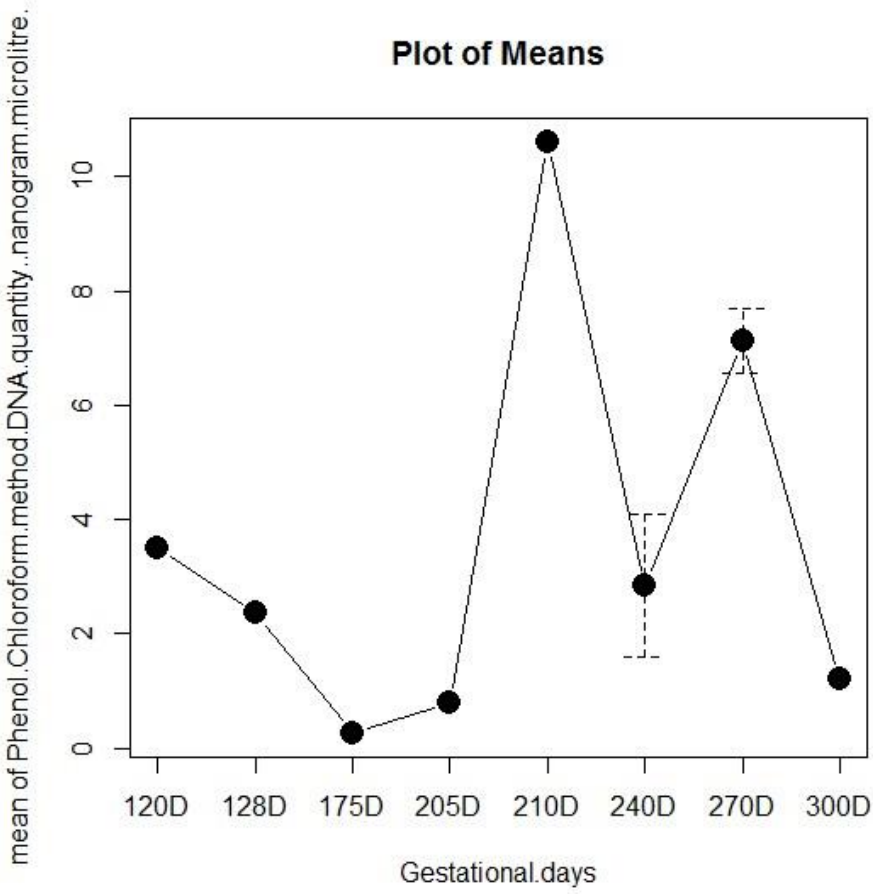

Figure 3. Plot of the means (isolated DNA quantity in nanogram/microlitre) in case the Phenol-chloroform-isoamyl-alcohol method.

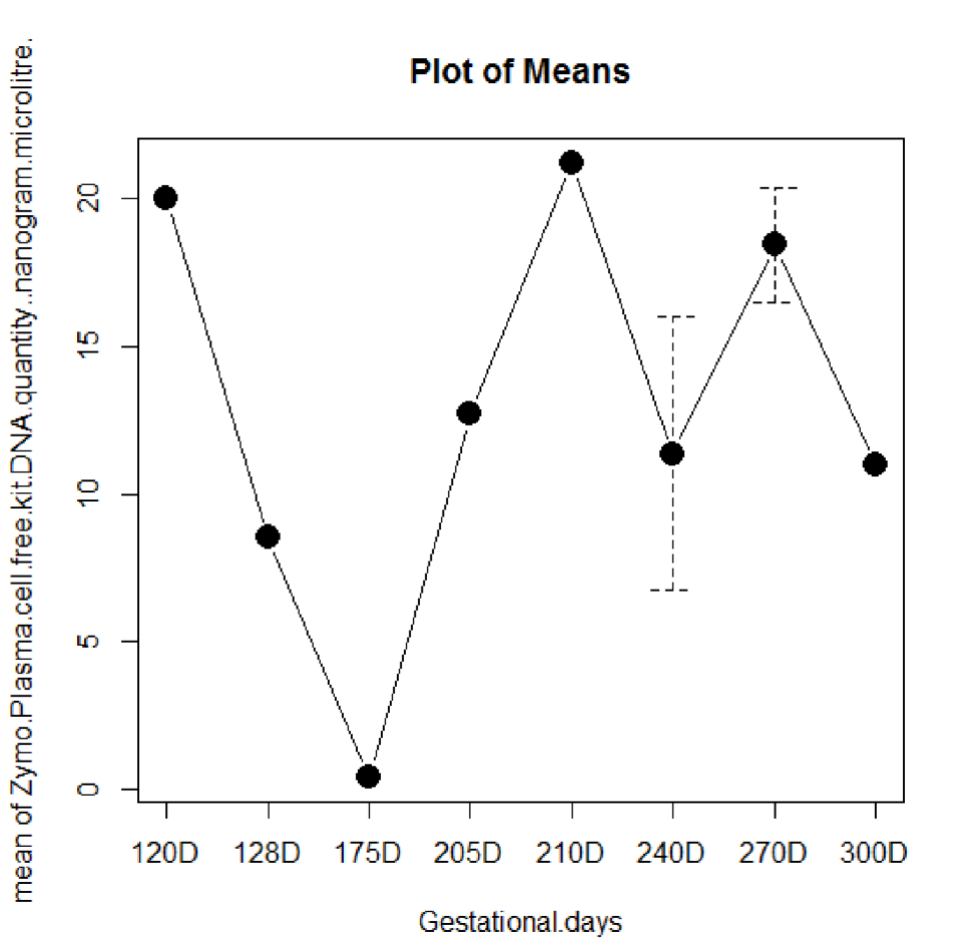

Figure 4. Plot of means (isolated DNA quantity in nanogram/microliter) in case the Zymo cell free DNA vacuum kit method.

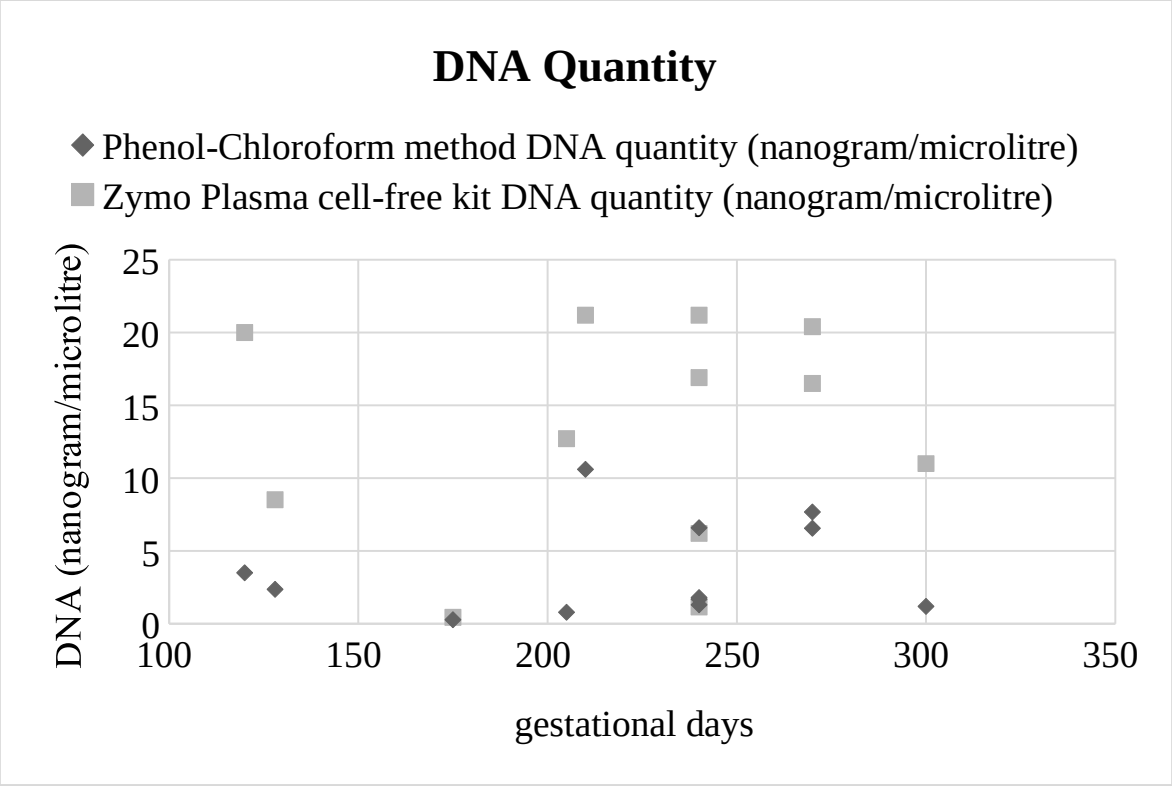

Figure 5. Comparison of isolated cell-free DNA quantities with the 2 different methods in relation of gestational days.

5. Discussion

The use of molecular techniques such as isolation of circulating ccffDNA from maternal blood has already shown a range of benefits spanning throughout many species; from human genetical fetal abnormalities, livestock production advancements, to aiding conservation projects for populations of endangered animals. Within the maternal blood plasma, circulating cell free fetal DNA is present; this is made up of short nucleotide fragments around 200bp that when isolated can be amplified to determine fetal sex (Ristanic, M. *et al*. 2018) as a continuation of technologies such as artificial insemination, embryo transfer and sex sorted semen.

Early sex determination is valuable to the equine breeding industry as it offers owners the opportunity to plan the outcome of the pregnancy depending on the fetal gender, it also allows for the insurance that sex sorted semen or artificial insemination techniques have been successful. Molecular sexing also offers an alternative to the traditionally used ultrasound where despite being a highly useful diagnostic tool does have disadvantages such as gestational age being only optimal for a window of time and operators must be skilled. This measurement of fetal DNA would allow an efficient way to safely and accurately determine fetal gender throughout the gestation with fetal DNA being present in maternal plasma at all stages of the pregnancy.

As the discovery of ccffDNA came about, Non-invasive prenatal Testing (NIPT) is now an efficient and accurate way to detect fetal risk for genetical disorders such as Downs Syndrome in women without the risk of potentially causing harm or stress to the fetus (Ondrej Pos et al 2019). In the future it is therefore hoped that a similar laboratory test may become available to investigate the genetical health of a prenatal foal without endangering the viability of the pregnancy by instead only sampling the mare's blood.

Previous studies have highlighted that the measurable amount of ccffDNA fluctuates with various factors including species, gestational age, the initial volume of maternal blood taken and critically the ability of the chosen protocol to accurately extract the fetal material. Equine maternal plasma has been discovered to posses lower ccffDNA concentrations than other species; thought to be associated with the strong barrier that is the equine placental interface.

The importance of the DNA isolation and extraction methods is essential to any investigation into the genetics of the fetus through maternal plasma, it is known that the quantity and the quality of the methodology used to obtain the fetal DNA samples will determine the sensitivity and accuracy of any subsequent PCR methods (Nolan et al., 2006). When associated with the sex determination of the foal the extraction concentration of ccffDNA is fundamental as the extracted nucleotides contain the Y chromosome specific fragment; the sex determining SRY gene which indicates the male sex. Low or unsuccessfully extracted levels of fetal DNA could therefore result in false female sex determination. In order to achieve full accurate results for sex determination the extraction method must be efficient, accurate and follow a refined protocol.

The options for DNA extraction are very diverse with a vast array of methodologies and protocols available. All extraction methods contain the same basic steps, cell lysis and removal of the membrane lipids, protein denaturation then removal of cellular components in order to isolate the DNA (Anandika Dhaliwal, 2013). With the advancement in technology, the identification of ccffDNA and the discovery of its use after amplification with PCR, methods of DNA extraction range from traditional organic methods to commercially designed kits. Organic extraction methods include the Phenol-chloroform-isoamyl alcohol method investigated in this study; although a frequently used and traditional method this extraction protocol does involve using some hazardous organic solvents that may alter any subsequent method and is also generally a time-consuming option (Schiebelhut et al 2016). Other organic extraction methods include Silica based technology, Magnetic separation and Anion exchange technology. These alternative extraction methods do offer efficient extraction, potentially with more ease and speed however do range in cost. Commercial kits are also available, for example the investigated ZYMO cfDNA Vacuum kit method, which by using lysis buffer reagent followed by organic extraction are known to have high yields of DNA whilst providing an efficient, quick alternative method.

When comparing both extraction methods the Qubit fluorometer was used to quantify the fetal DNA extraction efficiency; results showed that there was a significant difference in DNA yield depending on the extraction method applied and to a lesser degree, stage of gestation. Considering Figure 3 and Figure 4 both are able to illustrate that disregarding which extraction protocol was followed the yield of DNA was seen to fluctuate throughout the gestational times of sampling. The

isolated DNA quantity was measured in nanogram/microlitre in both methods; Figure 5 illustrates the respective differences in extracted DNA concentration comparing the two methods. From this figure the ZYMO plasma cell free kit clearly extracted a significantly larger amount than the Phenol-chloroform-isoamyl alcohol method. These results suggest that for future DNA extraction protocols intending to be used for subsequent PCR amplification the ZYMO kit should be chosen as it is indicated to provide the highest DNA yield as the most sensitive method for isolating the DNA, whilst concurrently being more efficient and using less hazardous materials.

In this study, samples were collected from the mares ranging from 4^{th} – 11^{th} months (Days 120330) of pregnancy but in the majority of months only 1 sample was available for evaluation therefore only limited conclusions can be stated. The limited number of samples could not be used to see trends in DNA quantity in different phases of gestation in the horse; further studies needed to provide data of DNA amounts during gestation. It seems to us that cell-free DNA quantity is variable during the gestation and conclusively there is likely many factors influencing DNA quantity in maternal plasma.

6. Summary

Circulating equine cell free fetal DNA must be isolated and extracted in order to carry out further diagnostics such as equine fetal sex determination. This study compared two isolation methods, a more traditional Phenol-chloroform-isoamyl alcohol method with a ZYMO cfDNA Vacuum kit to conclude which protocol may obtain the highest concentration of ccffDNA whilst taking into consideration stage of gestation which must be optimised to ensure high yields. Indicated from past studies it is assumed that the later into gestation the more circulating free fetal DNA would be present; this investigation showed otherwise that in fact different concentrations fluctuated throughout the various gestational ages. Our investigation showed that using a ZYMO quick vacuum kit provided significantly more extracted DNA. In order to complete this investigation a PCR will be run with the extracted fetal DNA to analyse and determine the sex of the twelve foals prenatally. In future studies there should be continued investigation into gestational stage concentrations and within the field future investigations in to the possibility of detection of genetical abnormalities from the equine genome.

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Phenol-chloroform-isoamyl alcohol method

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Qubit Fluorometer

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Figures

Figure 1-

A.Tönissen, G. Martinsson, H. Otzen, K. Schürmann, S. Schützel, F. Ertmer, A Kassens, J. Sielhorst, R. Brehm and H. Sieme. To perform fetal gender determination in the mare by ultrasound during early and advanced gestation (2015). Pferdeheilkunde 31 page 153-158

Figure 2-

Procedure overview of 12 samples loaded to vacuum vials attached to the vacuum pump. (Department of Animal Breeding. University of Veterinary Medicine Budapest)

Tables

Table 1-

N. Davoodian, Ali Kadivar. Prenatal determination of farm animal fetal sex using free fetal DNA in maternal plasma (Nov. 2016). Journal of Agriculture and Veterinary Science. Volume 9, Issue 11 Ver. II Page 38-45

Table 2

Samples (1-12.), gestational days, and DNA-quantity according to the two different DNA isolation methods.

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which I deem suitable for submission and defence.

Date: Budapest, 2 day 12 month 2019 year

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