FEASIBILITY STUDY ON BOVINE FETAL SEXING UTILIZING CIRCULATORY CELL-FREE FETAL DNA IN MATERNAL PERIPHERAL BLOOD

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By

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ABSTRACT

The studies conferred in this thesis were performed to establish an appropriate PCR methodology to target sex-chromosome specific genes for prenatal fetal sex determination in pregnant cows. The first study determined the effect of frozen and fresh plasma on cell-free fetal DNA (cffDNA) extraction for PCR-based fetal sexing and compared different DNA extraction methods on downstream PCR for fetal sexing in cows by detecting sex-chromosome-specific gene products. We found that none of the DNA extracts obtained from frozen plasma samples of cows (120-150 days pregnant) yielded any male fetus-specific PCR products, although all cows were carrying male fetii. In contrast, male fetus-specific amplicons were successfully amplified in 5/6 DNA extracts obtained by the phenol-chloroform method when fresh plasma samples were used. Although the smaller sample size was a limitation, we further found that DNA extraction methodologies compared in the present study (DNeasy Blood & Tissue Kit, QIAamp DSP Virus Kit, QIAamp DNA Blood Midi Kit, NucleoMag cfDNA Isolation Kit, MagMAX cfDNA Isolation Kit, KAPA Express Extract Kit, Salting-out protocol, and Phenol-chloroform method) appeared to show variability in their ability to isolate fetal DNA from freshly harvested maternal plasma/blood of pregnant cows.

The second study determined the minimum concentrations of cffDNA that must be present per milliliter of maternal plasma for successful extraction and PCR. In addition, we also compared Y-specific sequence PCR-based fetal sexing results in the cows divided into two gestational stages. We concluded that the spiked serum and cellular DNA from aborted male fetii could be successfully re-extracted (Blood & Tissue Kit) from maternal plasma at all dilution rates (0.5-100%). Furthermore, the lowest concentration of spiked fetal DNA for successful extraction (from maternal plasma) followed by Y-specific PCR and bAML PCR was found to be >31.25 pg/ml and >2ng/ml, respectively. Besides, we also found that the proportion of cows with a positive Y-specific PCR outcome were higher ($P<0.05$) in 151-240 days pregnant cows (90%; CI: 55.5-99.75) than 60-150 days pregnant cows (33%; CI: 7.5-70.1). Lastly, we found that the lower limit (35 pg/ml) of spiked male fetal DNA could be successfully recovered from maternal plasma of advanced pregnant cows (using Blood & Tissue Kit, DSP Virus Kit, and NucleoMag cfDNA Isolation Kit) for Y-specific PCR. However, there was variability in the Y-specific PCR results

when neat plasma samples from the same pregnant cows were processed for DNA extraction using these kits.

In conclusion, fresh but not frozen plasma could be used for PCR-based prenatal fetal sexing. Moreover, due to variability between different DNA extraction methodologies, the PCR assay for fetal sex determination was unreliable for fetal sexing in pregnant cows. Besides, cffDNA could be consistently extracted from maternal plasma for Y-specific PCR when present at amounts ≥31.25 pg/ml. Although a significantly higher proportion of samples from advanced pregnant cows yielded Y-specific fetal DNA amplicons, the study needs to be replicated on a large dataset to confirm our observations.

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LIST OF ABBREVIATIONS

AACC	American Association of Clinical Chemistry
ag	Attograms
AI	Artificial Insemination
bAML	Bovine Amelogenin
BCRC	Beef Cattle Research Council
bp	Base pairs
CAD$	Canadian Dollars
CCA	Canadian Cattlemen's Association
cfDNA	Cell-free DNA
cffDNA	Cell-free Fetal DNA
CI	Confidence Interval
CRS	CanFax Research Services
CVS	Chorionic Villus Sampling
d	Day(s)
EC	Extraction Control
eCG	Equine Chorionic Gonadotropin
EDTA	Ethylene Diamine Tetra-acetic Acid
FAO	Food and Agriculture Organization
fg	Femtograms
FISH	Fluorescent In-situ Hybridization
g	Gravitational Units
GDP	Gross Domestic Product
h	Hour(s)
hCG	Human Chorionic Gonadotropin

min	Minutes
ng	Nanograms
NIPD	Non-Invasive Prenatal Diagnosis
NTC	Non-Template Control
OCCS	Ontario Cow-Calf Survey
OECD	Organization for Economic Co-operation and Development
PAGs	Pregnancy Associated Glycoproteins
PCR	Polymerase Chain Reaction
pg	Picograms
PUBS	Percutaneous Umbilical Blood Sampling
RhD	Rhesus D
SDS	Sodium Dodecyl Sulphate
SRY	Sex-Determining Region Y
TSPY	Testis-Specific Protein Y
WCCCS	Western Canadian Cow-Calf Survey
wk	Week(s)

1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Beef industry: current status in Canada

Based on the 2020-29 agricultural outlook by the Organization for Economic Co-operation and Development (OECD) and Food and Agriculture Organization (FAO), global meat production is expected to reach 366 million tonnes (carcass weight) by 2029. Compared to 2020, the herds from developed and developing countries are expected to account for approximately 19% and 81% of additional meat produced, respectively. Amongst the developed countries, Canada and the US are projected to produce 4% more meat in 2029 than in 2020 (OECD-FAO, 2020). As per the 2020 industrial statistics of the Canadian Cattlemen's Association (CCA), Canada has been producing nearly 1.55 million tonnes of beef every year, out of which 409,967 tonnes was exported in 2019, thereby making Canada one of the leading exporters of meat at a global level. These beef exports added 3.1 billion CAD$ to the country's economy (CCA, 2020). Furthermore, the country's beef industry has been the second-highest provider of farm cash receipts (as per the latest data from 2015-19), thereby generating an 18 billion CAD$ annually for the Gross Domestic Product (GDP) (CCA, 2020). As per the 2021 data of CanFax Research Services (CRS), there are 3.53 million beef cows present in 59,784 ranches and farms (CRS, 2021a). According to the CCA, these ranches/farms harbor 84,740 operators who rely on beef production for more than 50% of their income (CCA, 2020). The CRS further reports that the western Canadian provinces of Alberta and Saskatchewan are home to more than half of the beef cattle, with approximately 1.5 and 1.1 million cattle, respectively (CRS, 2021a). The data from CCA suggests that the average number of beef cattle per farm in Alberta and Saskatchewan are 255 and 191, respectively, which is much higher than the national average of 169 (CCA, 2020).

As per 2019 data of Beef Cattle Research Council (BCRC), 53,236 of 59,784 (89%) farms of Canada were involved in cow-calf operations. The commercial cow-calf producers mainly focus on raising steers for the feedlots, with some growing their own replacement heifers. Whereas, the seedstock breeders provide replacement bulls and heifers with better genetics for breeding purposes (BCRC, 2019a).

According to the CRS, beef production in Canada increased by 25% from 2015-2019 due to herd expansion decisions based on more profitability (CRS, 2021b). Since 2016, the continuous positive

revenue that ranged from 97-287 CAD$/head has put the cow-calf sector in a really strong position (CRS, 2020). As per the 2020 industrial statistics of CCA, the calves have earned the most profit to the beef herds. Since the backgrounding and feedlot sectors are also dependent upon the cow-calf operations for their functioning, the cow-calf sector is central to the success of the beef industry. In 2019, around 9.4 billion $(Canadian) have been generated in farm cash income by the beef cattle industry that is chiefly dependent on cow-calf operations (CCA, 2020).

1.1.1. Cow-calf operations: status of reproductive management strategies in Canada

The success and profitability of every cow-calf operation primarily rely on the birth of a calf/per cow each. Contracting the length of the breeding season is the predominant determinant of a shorter calving season which ultimately ensures uniformity in the calf crop, more pounds weaned, and enhanced reproductive performance of both heifers and cows. The western Canadian Cow-Calf Survey (WCCCS) of 2018 reported that only 20% of the western Canadian cow-calf producers have a breeding season of < 63 days (BCRC, 2019c). Furthermore, the Atlantic and central Canadian cow-calf producers have even more longer breeding and calving seasons than the western Canadian beef producers on average. However, adhering to the breeding season of 63 days could result in a more contracted calving season, with more economic benefits, decreased labor, time, and resources involved (BCRC, 2019c). As per the WCCCS (2018), March-May and February-April were the most common calving seasons for mature cows and heifers, respectively, in western Canada. However, the Ontario Cow-Calf Survey (OCCS) of 2018 reported the most common calving seasons for mature cows and heifers being March-June and March-May, respectively (BCRC, 2019b). In another study, northern Quebec and northern Ontario were observed to have concentrated calving in late May for mature cows, and heifers usually started calving a couple of weeks before cows (Lamothe, 2018). In western Canada and Ontario, an almost similar proportion of cows calved during the first 21 days of the calving season, with the respective figures being 55% and 54% (BCRC, 2019a).

Producer decisions regarding the timing of calving during a year depend primarily on the marketability of calf crops. However, other essential factors such as the cost of feed, availability of grazing land, calf management and survivability, labor availability, and farming activities could influence calving seasons. Relatively higher demand for calves and more attractive prices during the fall had earlier influenced the cow-calf producers to choose late winter calving season (i.e.,

early during the year) so that calves can attain more weight by the time of marketing. In contrast, some advocated the benefits of early spring calving during high pasture productivity, which provides lactating cows access to abundant, nutritious forage (Adams et al., 1994; Fontes et al., 2020; Kruse et al., 2004; Lamb et al., 2016). Additionally, a defined breeding season facilitates planning for concentrating labor resources for their strategic use, especially in calving season (to observe cows for dystocia and post-partum reproductive problems), thereby controlling the non-essential investments and decreasing the input costs in the production system. Moreover, spring calving also ensures better survivability of the newborn calves owing to favorable climatic conditions. This practice is the same as the other wild and domestic ruminants, who breed naturally and give birth to offspring in spring.

Furthermore, when the breeding and calving seasons are confined to a defined window of time in a herd, the calves born in a particular time frame are more uniform in terms of their size and weight, which may fetch a relatively higher price in the market at the time of sale (Leupp et al., 2009; Rodgers et al., 2012).

1.1.2. Cow-calf operations: fall pregnancy diagnosis

Cattle pregnancy diagnosis is a crucial management tool for beef herds. It allows the producers to make informed decisions about winter feeding and marketing of animals depending on their pregnancy status. In a beef herd, the approximate cost of feeding a cow during 120 days of the winter period is US$ 220 (Prevatt, 2019), which constitutes one of the largest expenses of maintaining a cow in a beef cow herd. Therefore, pregnancy checking might allow producers to make decisions to potentially cull open cows so that they do not have to feed them throughout the winter or put them into a different feeding program leading to a different marketing strategy. Additionally, producers can also assess the reproductive performance of the breeding programs and determine if there is a need to identify potential factors/diseases for poor pregnancy rates in a year. Given these benefits and recommendations from veterinarians, the acceptance of pregnancy diagnosis as a routine farm practice appears to have gradually increased over the last four decades. Based on the producer surveys, about one-third of the Alberta beef herds in the late eighties used pregnancy checks, which grew to one-half in the late 90s (BCRC, 2019a). More recently, about two-thirds of the respondent herds in western Canada reportedly used pregnancy checks as one of the management practices as per WCCCS (2017) data (BCRC, 2019a). The remainder of the

producers who do not routinely check the pregnancy status of their cattle typically sell them in spring when the price for cattle is usually better than the previous fall. Based on the economic modelling (Muzzin & Ben-Ezra, 2015), pregnancy testing allows the producers to plan for the management of pregnant cows during the winter. The open cows can be culled or kept for overwintering to benefit from better spring prices next year, respectively. Such a decision should be based on the fall cull cattle prices, overwintering feed costs, and, more importantly, the expected average daily gain (ADG) during the winter. The ADG varies with the management system and dry lot feeding allows better ADG than swath or bale grazing (Muzzin & Ben-Ezra, 2015). Within the dry lot management systems, expected ADG of <1.2lb/day would favor a decision to cull the cattle in the fall at the time of pregnancy check (Muzzin & Ben-Ezra, 2015).

Various methods of pregnancy diagnosis in cattle include rectal palpation, ultrasonography, and detection of pregnancy-associated glycoproteins (PAGs) in the blood of the cows (Fricke et al., 2016; Northrop et al., 2019). As per 2019 data from BCRC, rectal palpation and ultrasonography are the most common methods used for pregnancy diagnosis in Canadian beef herds (BCRC, 2019a). Earlier, 50% of the western Canadian beef producers checked their heifers and cows for pregnancy (Waldner et al., 2010). A similar figure (49%) was reported for the beef producers in Saskatchewan (Jelinski, 2012). According to WCCCS of 2017, approximately 62 and 17% of producers had their cows and heifers, respectively, checked for pregnancy status. Rectal palpation was the most commonly used method for pregnancy diagnosis; however, ultrasound is largely replacing rectal palpation for the vast majority of veterinarians and producers (BCRC, 2019a). Rectal palpation is a rapid and cost-effective method, and an experienced palpator can accurately diagnose pregnancy as early as 30-35 d. Real time brightness mode ultrasonographic exam could provide an accurate diagnosis as early as 25-28 d, however, it needs an experienced personnel, proper handling facility, and involves costly equipment (Hylan et al., 2009; Romano et al., 2006). Nevertheless, in western Canadian cow-calf herds, pregnancy diagnosis is usually carried out annually in the fall season, particularly from September to November, generally, 50-90 days after the end of the breeding season (Mortimer & Hansen, 2006; Waldner, 2005). The use of PAGs may be limited to remote areas where veterinarian visits are infrequent to justify taking blood samples from cows and submitted to a laboratory for pregnancy diagnosis.

1.1.3. Importance of fetal sexing at pregnancy diagnosis

The objective for prenatal sex determination varies in sexually dimorphic species, from social issues or sex-related diseases in humans to management aspects in domesticated animals. Knowledge of the fetal gender before birth offers many potential benefits to purebred and commercial cow-calf operations. Knowing the fetal sex at the time of pregnancy diagnosis could be used to market pregnant cows carrying a fetus with specific gender to potential buyers. Cows carrying female fetii could be kept or sold for replacement heifer development programs within or outside herds. Cows carrying a male fetus would benefit purebred producers hoping to raise and sell future bull studs. Also, knowing the gender proportion of the future calf crop could help producers plan to sell steers for backgrounding or feedlot operations. Despite these advantages, prenatal fetal sexing has found limited use in beef herds compared to dairy cattle.

1.1.4. Methods to determine the sex of future progeny

Multiple tools have been developed for gamete (sperm), embryo, and fetus to select the future progeny of a particular sex that may be utilized for different breeding programs. For example, the producers that use artificial insemination (AI) may opt for frozen sex-sorted semen (primarily X-bearing sperm) if they need additional replacement heifers in the next year. On the other hand, the use of sexed embryos would be suited for producers applying embryo transfer programs for genetic improvement of their herds. Beyond the embryonic stage, techniques for the prenatal sex determination of fetus would be appropriate for the producers engaged in natural breeding programs.

Available methods for prenatal fetal sexing can be broadly categorized into genetic and non-genetic or invasive and non-invasive procedures. Genetic methods would involve assessing and evaluating fetal chromosomal material obtained directly (Invasive: amniocentesis, chorionic villus sampling, fetal blood sampling, coelocentesis, and embryoscopy) or indirectly (Non-invasive: fetal DNA in maternal circulation). Non-genetic methods include the use of ultrasonography for fetal sex determination. The first documented use of these techniques in both humans and cattle has been presented in Table 1.1.

The following subsections will discuss different techniques to determine the sex of the future progeny, their potential uses, and applications for the cattle industry.

Table 1.1: The first reported use of various prenatal diagnostic tools in humans and cattle.

Technique	Year of first reported use in humans	Reference	Year of first reported use in cattle	Reference
Amniocentesis	1877	(Kelley, 2010)	1975	(Bongso & Basrur, 1975)
Chorionic villus sampling (CVS)	1983	(Brambati & Simoni, 1983)	2005	(Teksen et al., 2005)
Fetal blood sampling	1964	(Freda & Adamsons Jr, 1964)	1974	(Comline et al., 1974)
Coelocentesis	1993	(Jurkovic et al., 1993)	Not used	---
Embryoscopy	1954	(Westin, 1954)	Not used	---
Ultrasonography	1958	(Donald et al., 1958)	1987	(Hanzen & Delsaux, 1987)
Fetal DNA from maternal blood	1997	(Dennis Lo et al., 1997)	1996	(Kadokawa et al., 1996)

1.1.4.1. Preconception selection by sperm sexing

One of the rapid and easily adaptable means to select the sex ratio of calf-crop is by using sex-sorted semen for AI. However, the requirements for a particular sex of progeny may vary for the dairy and beef cattle industry. Specifically, dairy farmers would opt for female calves to support the replacement heifer program whereas; beef producers would prefer male calves to have more steers available to enter the feedlots (Naniwa et al., 2019). However, sex-sorted semen is more commonly used in the dairy industry due to the broader utilization of AI for breeding in dairy than beef cattle. As per the 2014 data of the WCCCS, AI was used by only 18% of the western Canadian beef producers (BCRC, 2018).

The sperm-sexing technology was first utilized in rabbits (Johnson et al., 1989) and then later adapted to other species (De Graaf et al., 2014; Seidel Jr, 2012). Sexing of mammalian sperm on a commercial scale is based on the differences in the DNA content between X- and Y-

chromosome-bearing sperm. Across different farm animal species, the X-chromosome-bearing sperm has 3.6% to 4.45% more DNA content than the Y-chromosome-bearing sperm (Seidel Jr, 2012). Cattle, in general, have 4% additional DNA in the X-sperm than Y-sperm; however, there are some variations between different breeds (Garner et al., 2013). The sperm sexing technology involved combining fluorescent staining (Hoechst 33342) of sperm with flow cytometry. The Hoechst 33342 is a cell-permeable stain that would bind to the minor groove of the DNA helix in the sperm (Garner, 2009). Upon excitation with laser, the differences in the intensity of stain would facilitate identifying X- and Y-sperm by the fluorescent detectors (Garner, 2009). Critical aspects of efficient sperm sorting include cell permeating dye, the flow of sperm in a single file in droplets, the orientation of sperm to the lasers and photodetectors while maintaining sperm viability throughout the process (Garner, 2009; Garner & Seidel, 2008). Studies have demonstrated that using sexed semen for in-vitro fertilization (Cran et al., 1993) and AI (Seidel et al., 1997) results in the birth of desired sex offsprings with a likelihood of nearly 90%.

Since the initiation of SexedULTRA™ technology, there has been a tremendous rise in the fertility of sexed semen (Vishwanath & Moreno, 2018). Therefore, the use of sexed semen has increased from 9.4% to 30.7% from 2007-2015 in the case of dairy heifers (Hutchison et al., 2016). While sexed semen has overtaken conventional semen in the case of Jersey cows, its use is increasing commonly in Holstein cows (Vishwanath & Moreno, 2018). By using SexedULTRA™ semen, conception rates equalling 90% of the conventional semen have been observed (Vishwanath & Moreno, 2018). Presently, the fertility rates of both conventional and sexed semen have become almost equivalent (Heuer et al., 2017). Practically, the use of sexed semen in commercial cow-calf herds of Western Canada may be limited to the seedstock producers who use AI and embryo transfer programs for breeding (Hall & Glaze, 2014).

1.1.4.2. Preimplantation embryo sexing

Sex-ratio of the future progeny can also be selected by testing embryonic cells for Y-specific DNA probes (van Vliet et al., 1989), immunological assays of male-specific antigens such as H-Y antigen (Flores-Foxworth, 2007), cytogenetic analysis (Hirayama et al., 2013), and quantification of the X-linked enzymes (Vijaya Lakshmy et al., 2018). Immunological assay of H-Y antigen and quantification of X-linked enzymes are non-invasive but lack accuracy. In contrast, Y-specific DNA probes and cytogenetic analysis are highly accurate but require biopsy (Sharma et al., 2017).

With recent progress made in the field of molecular biology, relatively more quick and authentic techniques of embryo sexing such as fluorescent in-situ hybridization (FISH) using Y-specific DNA probes (Sharma et al., 2017) and polymerase chain reaction (PCR) (Vijaya Lakshmy et al., 2018) have been introduced.

Even though much progress has been made in bovine embryo sexing, relatively lower pregnancy rates following embryo transfer, along with the high costs of superstimulation, limits the use of this reproductive technique. Furthermore, like sperm sexing, this technique lacks practicality in commercial beef herds because of the non-intensive management of cows in pastures where they remain inaccessible during breeding. According to the data of Mississippi State University, embryo transfer was used by only 1.6% of the beef producers in the United States (Larson & Parish, 2015).

1.1.4.3. Invasive methods of genetic fetal sexing

Various diagnostic methods that involve breaching tissue layers allow visualization of fetus or acquisition of fetal cellular material were primarily developed for humans and later adapted for pregnant animals (Shulman et al., 2008). These procedures include amniocentesis, CVS, fetal blood sampling, coelocentesis, and embryoscopy/fetoscopy and have been briefly discussed individually in the subheadings below.

1.1.4.3.1. Amniocentesis

Amniocentesis refers to the extraction of amniotic fluid that surrounds the fetus in pregnant women and animals. The technique has been reportedly used since the late 1800s for aspirating excess amniotic fluid in the cases of women affected with hydramnios and was later adapted for amniography (Menees et al., 1930). The initial approach of transabdominal amniotic tap in women improved with the introduction of ultrasound in the 1970s. Currently, ultrasound-guided transabdominal amniocentesis is performed in humans from 16-week of pregnancy for early detection of chromosomal abnormalities, genetic disorders, and fetal maturity as well as diseases (Cruz-Lemini et al., 2014).

The inactivated X-chromosome (Barr bodies) was discovered in the late 1940s (Barr & Bertram, 1949). A decade later, amniotic cellular material was obtained from mid-pregnancy (vaginally/laparoscopically) that was used for fetal sex determination in pregnant women (Fuchs

& Riis, 1956). The Barr body represents inactivated condensed X-chromosome present in the nucleus, which could be detected using Cresyl violet staining in amniotic samples from women carrying a female fetus (Barr & Bertram, 1949). However, the accuracy of Barr bodies detection may be limited by the contamination with maternal cells in all species and similarities between prominent chromocenters (not linked to sex) observed in some mammals such as rodents (Nayyar & Barr, 1966). Alternatively, the amniotic cells can be cultured for karyotyping or processed for DNA isolation to assess the presence of sex-chromosome-specific genes using PCR.

In cattle, amniocentesis has been carried out using needle insertion through vaginal fornix (Bongso & Basrur, 1975), sacrosciatic ligament (Bongso et al., 1978), ischiorectal approach (Eaglesome & Mitchell, 1977), flank surgery (Leibo & Rall, 1990) and transvaginal ultrasound (Garcia & Salaheddine, 1997). One of the first reports on amniocentesis in cattle described the vaginal approach on 70-100 d pregnant cows. It involved using a 12", 18-gauge needle introduced into the dorsal fornix to aspirate amniotic fluid followed by in-vitro culture of fetal cells for cytogenetic evaluation (Bongso & Basrur, 1975). A subsequent study used right flank and/or vaginal approaches in six pregnant (4-8 months) dairy cows to determine the sex of the fetus that resulted in two abortions and one preterm birth (Inoue et al., 1982). In a later study, amniotic samples from >1000 pregnant (7-22 wk) cows in an embryo transfer program were collected using a left flank surgical approach under sedation and local anesthesia (Leibo & Rall, 1990). Following the collection, amniotic cells needed 10-13 d of in-vitro culture to reach concentrations sufficient for metaphase spread analyses. Amongst the 216 cows that continued the pregnancy, approximately 13% abortion rate was observed. Compared to other approaches, ultrasound-guided transvaginal amniocentesis using fine needle (22-gauge) puncture was more efficient as the operator can visualize the needle path and is unlikely to cause abortions (Kamimura et al., 1997). Nevertheless, ultrasound-guided transvaginal amniocentesis would require caudal epidural anesthesia, well-trained personnel, took on average 20 min/animal and might be associated with sample collection failure in some cows (Kamimura et al., 1997). Although amniocentesis is a great tool to obtain valuable fetal cells during pregnancy for prenatal diagnostic applications, the procedure poses a significant risk to the fetal viability in-utero, requires a high degree of skill and expertise, and additional turnaround time for the results of cytogenetics or PCR.

1.1.4.3.2. Chorionic villus sampling (CVS)

Besides amniocentesis, CVS remains the commonly used technique for prenatal screening and diagnosing chromosomal and genetic disorders in humans (Carlson & Vora, 2017). As the name implies, CVS involves collecting fetal components of the placenta (chorionic villi). This technique was first used in pregnant women in the late 1960s using endoscopes through a transcervical approach (Mohr, 1968). Since the chorionic villi originate from the trophoblast layer of an embryo, the CVS can be used to determine fetal sex and the fetus's genetic makeup. Currently, the CVS is carried out in 10-13 wk of pregnancy in women through either transcervical or ultrasound-guided transabdominal approaches. Over the last six decades, the equipment and technique have evolved to minimize the risk of pregnancy losses due to bleeding, amniotic leakage, and limb reduction associated with CVS (Carlson & Vora, 2017). Instead of using biopsy forceps during the early years, specialized catheters or fine gauge needles are used to take the villus sample (Carlson & Vora, 2017). In a recent metanalysis, the pregnancy loss associated with the CVS sampling in twin pregnancy in women is similar to those observed in women that did not undergo CVS (Mascio et al., 2020). Despite continued use in humans, the CVS has not been adapted for cattle, likely due to the differences in the type of placentation. The chorionic villi in cattle interdigitate into the maternal crypts only at the specialized placentation sites (caruncles) in the uterus. Only available literature in cattle describes the collection of fluid using CVS from the placenta obtained after slaughter and microdissection of cotyledons to obtain cells for a short culture followed by karyotyping of mitotic cells (Teksen et al., 2005). Although not impossible, the collection of fluid by CVS would require an ultrasound-guided method (vaginally or transabdominal), sedation, or regional anesthesia and trained personnel.

1.1.4.3.3. Fetal blood sampling

Fetal blood sampling evolved from the initial work on using hysteroscopy (transcervical) or an endoscope (transabdominal) in an attempt to direct blood transfusion to the fetus affected with hemolytic diseases (Westin, 1954). Two decades later, the fetal blood sample was collected for the first time using a pediatric cystoscope to diagnose blood disorders (Valenti, 1973). In the early 1980s, the technique of ultrasound-guided pure fetal blood sampling in-utero was developed that involved blood aspiration from the umbilical vein by inserting a fine needle at the base of the umbilical cord (Daffos et al., 1983, 1985). The new technique was called ultrasound-directed

percutaneous umbilical blood sampling (PUBS) and replaced the fetoscopy for cordocentesis (Shulman et al., 2008). Initially, the primary focus of fetal blood sampling was to prenatally assess fetal hematologic abnormalities (Ryan & Rodeck, 1993). The PUBS, an invasive technique, has been associated with blood loss, lowered fetal heart rate, and abortion and therefore is recommended mainly for the diagnosis and management of hematological disorders of the fetus in human medicine (Berry et al., 2013; Peddi et al., 2021).

In veterinary medicine, fetal catheterization initially emerged as a tool providing access to fetal blood for in-vivo research on acid-base balance, oxygenation, and endocrinology of the fetii. Various surgical techniques for the introduction and subsequent maintenance of catheters in the uterine and umbilical blood vessels have been established for sheep and goats (Meschia et al., 1965), cattle (Comline et al., 1974), mare (Comline et al., 1975), and pig (Randall, 1977). On the other hand, a few follow-up studies reported that the installation of catheters had a high failure rate in cattle (Aoki et al., 2002; Schmidt et al., 2004). The fetal blood sampling has a low potential for fetal sex determination in cattle due to the invasive nature of the procedure, risk of complications such as septicemia, peritonitis, and abortions, and the availability of non-invasive methods.

1.1.4.3.4. Coelocentesis

As the name indicates, coelocentesis involves aspiration (centesis) of fluid from the extraembryonic fluid-filled cavity (coelom) that forms between two mesodermal layers and surrounds the amnion and yolk sac. The coelom appears by the end of the fourth week of gestation in humans (Hamilton & Boyd, 1960; Jones & Jauniaux, 1995) and by the third week in cattle (Greenstein & Foley, 1958). In humans, coelocentesis is carried out between six and nine weeks (coelom is larger than amnion during this period) of pregnancy using transabdominal or transvaginal ultrasound-guided approaches as an early prenatal diagnostic tool for fetal diseases (Aiello et al., 2018; Hamilton & Boyd, 1960; Jauniaux et al., 2003). The cells harvested from coelocentesis could be used for chromosomal analysis, molecular diagnosis, and potentially sex determination (Jurkovic et al., 1993; Pietropolli et al., 2014). Quantitative PCR (Findlay et al., 1996) and fluorescence in-situ hybridization (Pandya et al., 1995) following coelocentesis have proved to be helpful in fetal sex determination in humans (Jurkovic et al., 1993). The transvaginal coelocentesis in women has been associated with a higher risk of abortions (25%) (Ross et al., 1997), whereas the transabdominal approach requires highly skilled operators (Pietropolli et al.,

2014). Current literature does not provide evidence on coelocentesis in cattle to collect cells of fetal origin.

1.1.4.3.5. Embryoscopy

Embryoscopy refers to the procedure of visualization of embryo in-utero following the insertion of an endoscope into the extracoelomic cavity through either the transabdominal route or transcervical route (Cullen et al., 1990). This procedure is sometimes called embryofetoscopy and is used in pregnant women between five to 11 weeks of pregnancy to evaluate missed abortions, high-risk pregnancies, and fetal syndromes (Paschopoulos et al., 2006; Vicente et al., 2020). This technique can potentially be used for fetal sex determination in women >9 weeks pregnant; however, miscarriage has been reported in 12% of the women undergoing this procedure (Ville et al., 1997). Additionally, a routine transabdominal B-mode ultrasound examination would provide information about the fetal sex without risking the pregnancy in women, although at a later gestational age. In cattle, none of the available literature describes the use of embryoscopy for fetal sex determination.

1.1.4.4. Ultrasonography

Since the first report of two-dimensional ultrasound images of a human fetus in an abdominal scan in 1958 (Donald et al., 1958), the brightness mode real-time ultrasonography has improved image resolution substantially for use in both human and animal gynecology and obstetrics. About two decades later, Stocker and his colleagues recorded the fetal sex in pregnant women (third trimester) using ultrasound (Stocker & Evens, 1977). While visualizing the perineal region of the fetus in transverse or sagittal planes, the authors used the presence of external genitalia indicative of a male, while its absence indicated a female. These ultrasound observations were confirmed against the gender of the baby at birth. The observed accuracy in this study was 95% and 91.5% in the diagnoses of a male and female singleton fetus, respectively. A subsequent study on women (10-20 weeks pregnant) described the differences in angulation of external genitalia of the male and female fetus when viewed in the sagittal plane for fetal sex determination (Emerson et al., 1989). The external genitalia of the male fetus (fetal phallus) is angled vertically or cranially, while that of a female fetus (representing clitoris) is characteristically angled caudally (Emerson et al., 1989). Later studies indicated that the accuracy of fetal sex determination during the first trimester in

women would vary with fetal age and sonographer skills (Colmant et al., 2013; Kearin et al., 2014; Manzanares et al., 2016).

Transrectal ultrasonography can be considered a minimally invasive method to evaluate the bovine female genital tract (its contents) and ovaries. The initial study in cattle reported an accuracy of 94% (42/54) to determine fetal sex between 73 to 120 days post-conception (Müller & Wittkowski, 1986). The sex of the fetus was assessed by the presence of the external feature of mammary buds in a female or scrotal (genital) swelling in a male (Müller & Wittkowski, 1986). The researchers did not observe genital tubercle in the male or female fetus, likely due to the low resolution of the ultrasound machines available during that period. Subsequently, with the improved resolution of ultrasound machines, the bovine fetal sex determination at early stages in dairy cows was reported based on the location of echogenic bilobed genital tubercle in relation to other embryonic structures (Curran et al., 1989). The genital tubercle in an embryo represents the prime structure of mesodermal origin (Noden & Lahunta, 1985) and is present between the two fetal hindlimbs at day 48-49 of gestation (Curran et al., 1989). Based on daily transrectal ultrasound observations, the genital tubercle was observed to translocate cranially towards the umbilical cord in a male fetus and caudally towards the base of the tail in a female fetus (Curran et al., 1989). By day 55, the genital tubercle was located caudal to the umbilicus in the male fetus or cranial to the tail base in the female fetus (Curran et al., 1989), likely under the influence of sex hormones secreted by fetal gonads. The sex of fetii in this study was confirmed with the recovery of the aborted fetus at day 60. Another study confirmed these observations and revealed 93% and 91% accuracy for male and female fetus detection, respectively, between days 56-80 (Kamimura et al., 1994). After day 70, the genital tubercle has reduced echogenicity due to the bilaterally present genital folds, which form prepuce and vulva in males and females, respectively (DesCoteaux et al., 2010). Field trials on a large dataset have reported accuracy of >98% for the fetal sex determination with an average time of 2 minutes per cow (Curran, 1992; Quintela et al., 2011). Consequently, fetal sexing using ultrasound has been adopted mainly by dairy herds. However, the technique has not been widely utilized by the beef cattle industry. Usually, a significant number of beef cattle, expectedly between 3-5 months of gestation, are presented for the fall pregnancy checks, sometimes under limited handling facilities. Thus, there is less available time per cow for the ultrasound examination and a larger fetus along with reduced echogenic contrast between the embryonic structures reduce the

accuracy of fetal sex prediction. These factors contribute to a decreased likelihood of using ultrasound for prenatal sex determination in routine veterinary practice.

1.1.4.5. Cell-free fetal DNA (cffDNA) in maternal peripheral blood

The methods mentioned so far have several limitations for their broader use in the beef industry due to the level of skill and training required, invasiveness, and associated cost. As a result, there have been persistent efforts to identify novel strategies to aid in the sex-determination of future progeny. The discovery of cffDNA in the maternal circulation of pregnant women at the end of the 20th century provided a unique opportunity to perform non-invasive prenatal diagnosis (NIPD) (Dennis Lo et al., 1997). The NIPD is based on analyzing fetal DNA obtained by routine processing of a single peripheral blood sample drawn from a pregnant female without posing any risk to the developing fetus and mother (D'Aversa et al., 2018). The isolated fetal DNA can then be used to target sex-chromosome specific and other genetic markers (Dennis Lo et al., 1997; Devaney et al., 2011; Fernández-Martínez et al., 2012). Since the inception of this technique, cffDNA has been used for prenatal fetal sex determination in various mammals viz. cattle (Da Cruz et al., 2012; Lemos et al., 2011; Ristanic et al., 2018), sheep (Asadpour et al., 2015; Kadivar et al., 2013), mare (de Leon et al., 2012; Tonekaboni et al., 2020), elephant (Vincze et al., 2019), and rhinoceros (Stoops et al., 2018) with varying degree of accuracy. The more detailed information on cffDNA and its use in non-invasive genetic fetal sexing will be discussed in the upcoming section.

1.2. Cell-free DNA (cfDNA)

The presence of cfDNA was first documented when the circulating free nucleic acids were discovered in the plasma of clinically healthy and diseased individuals (Mandel, 1948). Subsequent research indicated that cfDNA commonly exists as short double-stranded deoxynucleic acid fragments (as low as 150 bp) and in low concentrations (on average <100 ng/ml) (Cicchillitti et al., 2017; Fleischhacker & Schmidt, 2007). Evidence suggests that the cfDNA is present predominantly in the cell-free fraction (plasma and serum) of the whole blood and other body fluids (Jahr et al., 2001; Stroun et al., 2000).

1.2.1. Source and clinical implications of cfDNA

Even though the exact origins of cfDNA are debatable, the most likely sources of cfDNA include cell death (apoptosis, necrosis) and active release from cells (Grabuschnig et al., 2020). Cell death by apoptosis and necrosis would lead to cfDNA of different base pair lengths. Programmed cell death or apoptosis generally leads to cfDNA of ~150 bp or length in its multiple, similar to the size of a single or multiple nucleosomes (Stephanie et al., 2014). However, cfDNA smaller or intermediate base pair size can also be observed in various studies, possibly due to cleavage nucleases and technical differences in extraction or DNA library preparation (Ma et al., 2017). It is less likely that cfDNA would primarily originate from necrotic cell death, as necrosis in cancer patients was associated with larger DNA fragments (10Kbp) (Jahr et al., 2001). The various characteristics of cfDNA that support the apoptotic pathways being its key source include rapid turnover (Tsumita & Iwanaga, 1963) and short half-life of cfDNA in the circulation (Dennis Lo et al., 1999) through yet poorly understood mechanisms.

Additional less studied processes that could potentially release cfDNA include phagocytosis, autophagy, and neutrophil extracellular trap formations (Brinkmann et al., 2004). Examples of the active release of cfDNA include mitochondrial DNA release through cell reparative changes (Mair et al., 2019) and extrachromosomal circular DNA through cellular excretory mechanisms (Anker et al., 1975; Zhu et al., 2017). Lymphocytes and several other living cells of the body have been observed to spontaneously liberate some of their newly manufactured DNA (Stroun et al., 2001; Van Der Vaart & Pretorius, 2008). These observations indicate a possible role of cfDNA in performing the vital functions of the cells or its active participation in an intercellular signaling pathway to control the active intercommunication among the cells (Gardiner et al., 2015).

While the source and function of the cfDNA may differ between physiological and pathological conditions, the predominant factor contributing to the release of cfDNA in circulation is pathological (Ranucci, 2019). The high concentrations of cfDNA have been reported in the plasma/serum of the patients with several disease conditions viz. trauma and myocardial infarction (Chang et al., 2003), stroke (Tsai et al., 2011), and various types of tumors, which suggest that cfDNA may be involved in a feedback association with inflammation and its liberation tends to initiate a proinflammatory cytokine response (Frank, 2016). Hence, a simple peripheral blood sample collection provides a practically feasible diagnostic option with an extensive scope of

clinical applications such as non-invasive early diagnosis to prognosis, foreseeing the response to anti-cancer therapy, and detecting minimal residual disease (Lewis et al., 2016; Ulrich & Paweletz, 2017). Similarly, cffDNA that circulates in the blood of pregnant mammals also carries a great potential of being used as a prenatal diagnostic marker which is discussed in more detail in the upcoming section.

1.2.2. Cell-free fetal DNA (cffDNA)

The presence of intact fetal cells in the maternal blood has been debated since the first study indicated the presence of intact fetal cells (likely DNA) in the maternal blood of pregnant women using lymphocyte culture and karyotyping (Walknowska et al., 1969). A subsequent study reported fetal cells in blood samples obtained from pregnant women with flow cytometry (Herzenberg et al., 1979) and fluorescence in-situ hybridization (Poon et al., 2000). However, a later study could not confirm the findings of intact cells being present in maternal circulation (Bischoff et al., 2003). It has been estimated that each millilitre of maternal blood from pregnant women contains on average one fetal DNA equivalent (Bianchi, 1999; Sekizawa et al., 2000).

The discovery of nucleic acids of fetal origin in the plasma of clinically healthy pregnant women by Dennis Lo and colleagues (Dennis Lo et al., 1997) provided the opportunity to develop a non-invasive tool for prenatal screening and testing in human medicine. In a follow-up study, it was observed that the maternal plasma contains a higher proportion of maternal (94-97%) rather than fetal cfDNA (3-6%) (Dennis Lo, Tein, et al., 1998). A more recent study on prenatal testing of aneuploidy recorded a high variability (0.6-50%, mean 10.2%) in the cffDNA fraction in plasma of pregnant women (Curnow et al., 2014). The cffDNA in pregnant women was first detectable at 4-5 weeks in gestation and was observed to undergo a rapid clearance following the delivery, suggesting that it is specific to pregnancy (Dennis Lo et al., 1999; Dennis Lo, Tein, et al., 1998). Further, the cffDNA in maternal plasma increased with the advancement of the gestational age of the fetus, whereas it decreased with increased body mass index and maternal age (Dar et al., 2016; Dennis Lo, Tein, et al., 1998). It has been estimated that the cffDNA content in maternal plasma increased at the rate of 0.1% per week between 9-20 weeks of gestation and then at 0.6% per week after 21 weeks of pregnancy in women (Dar et al., 2016). However, no such studies analyzing the relative proportion of fetal DNA compared to maternal DNA have been performed in domestic mammalian species to the best of our knowledge.

1.2.2.1. Source of cffDNA

An older study provided evidence that cells from either side would cross over the placental interface in humans throughout the pregnancy (Desai et al., 1963). Initially, the cffDNA in the maternal blood was thought to be contributed by fetal nucleated erythrocytes and leukocytes (Bianchi, 1999; Walknowska et al., 1969), a hypothesis that was disproved by a later study (Angert et al., 2003). Another similar phenomenon responsible for the transfer of fetal cells to maternal circulation could be 'microchimerism' as it has been well documented that there is an occasional bi-directional exchange of cells between mother and the fetus during pregnancy in women (Shrivastava et al., 2019). Observations that cffDNA was higher in the amniotic fluid than maternal blood and was present in maternal body fluids (peritoneal, cerebrospinal) and urine, led to the proposition that cffDNA might be transported by concentration gradient (Al-Yatama et al., 2001; Angert et al., 2004; Bianchi et al., 2001).

Fetal trophoblast cells that enter the maternal side would likely be targeted by the maternal immune cells, thus releasing their DNA in the process. A study has demonstrated that around 80% of pregnant women may have a detectable amount of fetal DNA in their blood by 28 days of gestation. However, it takes 28-30 days to establish fetoplacental circulation post-conception (Guibert et al., 2003). This finding along with an extremely rare occurrence of fetal hematopoietic cells in the maternal circulation strongly suggested that the predominant source of cffDNA was the trophoblast of the placenta rather than hematopoietic cells. Additionally, a strong positive correlation between human chorionic gonadotropin (hCG) concentrations and fetal DNA in maternal blood was found (Ohashi et al., 2002; Sekizawa, Sugito, et al., 2001). Furthermore, the presence of placenta-specific mRNA molecules in the plasma of pregnant women provided more convincing evidence that the placenta was the chief source of cffDNA (Ng et al., 2003). Subsequent studies confirmed that fetal trophoblast cells undergoing apoptosis are considered the most likely source of cffDNA in the peripheral circulation of the pregnant woman (Alberry et al., 2007; Bischoff et al., 2005). These observations were further confirmed in mice through studies on placental explants as humans and rodents have similar placentation (Phillippe & Adeli, 2017).

1.2.2.2. Placental (fetal-maternal interface) classification

The placenta is a transient organ that is only present during pregnancy to facilitate the growth and development of the fetus in-utero. Although considered a feature of eutherian mammals, there has

been an ongoing discussion about the placenta being present in the late gestation of marsupials (Guernsey et al., 2017). Across different species, the placenta performs multiple functions that include nutrient and metabolite exchange between the mother and the fetus; hormonal secretion, physical and immunological protection of the fetus from pathogens and manage fetal excretions (Bauer et al., 1998; Burton & Fowden, 2015; Carter, 2012). Nonetheless, the placental organization varies between different species of animals (Enders & Blankenship, 1999). So, it is essential to consider the placental diversity before generalizing the endocrinological, physiological, immunological, and other data from animals to humans, especially in the transfer of biomolecules, drugs, and chemicals (Burton et al., 2006).

During the early developmental stages, the yolk sac placenta (choriovitelline) provides the crucial support to the embryo till 11.5d of pregnancy (19-20d gestation) in mice (Malassiné et al., 2003), 21d of pregnancy in dogs and humans (Freyer & Renfree, 2009; Malassiné et al., 2003), 30d in pigs (Friess et al., 1980) and up to 40-50 d in horses (Enders & Liu, 1991; P. Wooding & Burton, 2008). In ruminant, the choriovitelline placentation may be absent or short-lived (few days) as the vascular part of the yolk sac is not observed to make contact with chorion (Carter & Mess, 2018). Except for rodents, the chorioallantois (vascular supply of chorion provided by vessels originating from allantois) is the primary form of the placenta through a significant part of pregnancy in humans and domesticated animals. The chorioallantois placentae have been classified primarily based on the gross morphological shape of the attachment site and histological structure at the feto-maternal interface (Burton et al., 2006; Telugu & Green, 2007; P. Wooding & Burton, 2008). These classification systems and each category of the placenta are being discussed in the following subsections.

Placental classification based on the gross shape

This classification scheme for the placental type is based upon the organization of the villi or outgrowths of the surface of the outermost fetal membrane, i.e., chorion (King, 1992). Four types of placentae that have been recognized under this classification include diffuse, cotyledonary, zonary, and discoid. However, variations in different shapes have been observed and will be discussed along with each major category.

Diffuse placenta

Equine (singleton and twins most common) and swine (polytocus species) have diffused placenta, characterized by the uniform distribution of chorionic villi across the placental surface (Allen & Stewart, 2001; Friess et al., 1980). Even though the equine placenta appeared overtly smooth, it had microvilli with secondary and tertiary branching patterns to form the "microcotyledons" that would attach to complementary invaginations in the uterus surface (Pozor, 2016). These microvilli connections would not be seen at the chorionic surfaces opposite the cervix and oviductal end of the uterine horns and in the folds that carry placental vessels (Pozor, 2016). In the case of swine, there is a complex folding of allantochorion forming ridges that get positioned over their respective crypts or grooves in the maternal endometrium (Friess et al., 1980).

Cotyledonary placenta

Both domesticated and wild ruminants of the Bovidae family have been observed to have the chorionic villi arranged in localized round or oval areas known as "cotyledon" (Carter, 2019; King, 1992). The maternal sites for the attachment of cotyledons were identified as caruncles and the placental units were collectively called placentomes (Carter, 2019; King, 1992). Cows and small ruminants have dome or concave-shaped and cup or convex-shaped caruncles, respectively, usually arranged in rows (P. Wooding & Burton, 2008). The number of placentomes varies between species, 3-8 in deer and 20-150 in domesticated ruminants (Carter, 2019; P. Wooding & Burton, 2008). The localized placentation sites along with secondary and tertiary branching of the chorionic villi (that interdigitates into maternal crypts) in ruminants have been proposed to provide a concentrated vascular exchange per gram of fetal tissue (Baur, 1977). The average number of placentomes in cattle has been observed to increase from 10 to 80 between 35 d to 70 d of gestation (Assis Neto et al., 2010). The placentomes in growing heifers have been reported to be greater in number but have less surface area per cotyledon than the mature cows (Eetvelde et al., 2016). Additionally, the same study observed a seasonal variation in the cotyledon size, although the reason and physiological significance were not apparent. There are likely some compensatory mechanisms for placentation to adapt to the fetal needs, maternal status, and other yet to be determined factors.

Zonary placenta

Mainly elephants and carnivores (cats, dogs, wild canids, and felids) have the zonary or ring or belt-shaped placentation (Favaron et al., 2014; Leiser & Koob, 1993; Miglino et al., 2006; Ramsey, 1975); however, a rare case of similar placentation has been reported in humans (Steemers et al., 1995). Observed characteristics of the zonary placenta were an organization of the chorionic villi as a girdle in the center of the chorioallantoic sac, which was complete in dogs but incomplete about 2/3rd in the cats (Miglino et al., 2006). The chorionic girdle has a central broad transfer zone around the conceptus that serves as an important region of exchange of nutrients from the dam to a fetus, whereas the pigmented zone surrounding it is involved in the transport of iron. Another transparent zone presents distally on both the chorionic ends is responsible for absorbing substances directly from the uterus (Senger, 2003).

Discoid placenta

A single circular or disk-shaped placenta was observed in primates, rabbits, and rodents; however, variations have been reported in rhesus monkeys with a bidiscoidal placenta (King, 1992). The chorionic villi attachment was only observed in the localized circular zone on the chorioallantois and a corresponding region of the maternal placenta. The location of the placenta could be anywhere in the uterus except for the fundus, which might vary with the presence of multiple fetii (P. Wooding & Burton, 2008).

Placental classification based on the histological structure

The classification of mammalian placentae based on the number of layers (interhemal membrane) between the maternal and fetal blood was first proposed by Grosser in 1909 and has been considered the most informative regarding placental functionality (Grosser, 1909). The three primary forms of placenta have been recognized under this criterion of classification, which includes epitheliochorial, endotheliochorial, and hemochorial (Carter & Martin, 2010; Enders & Carter, 2006).

Epitheliochorial placenta

The epitheliochorial placenta has been considered the most superficial in terms of the contact between the maternal and fetal vascular components as all the six layers viz. uterine vascular endothelial lining, uterine connective tissue, uterine epithelial lining, chorionic cell layer, fetal connective tissue, and fetal endothelial cell lining, are intact. This type of placenta is found in mare, sow, and ruminants (although with some modifications).

The equine epitheliochorial placenta is characterized by the temporary appearance of specialised structures called 'endometrial cups' that collectively arise from maternal endometrium and fetal trophoblast. These cups develop between 35-60 days post-conception and are 5-10 in number over the entire placental surface. These structures produce equine chorionic gonadotropin (eCG) hormone that forms accessory corpora lutea to maintain pregnancy and start disappearing after 60 days post-conception (Senger, 2003).

The swine placenta has a velvety surface with numerous tightly packed chorionic villi uniformly distributed above the entire chorion (Senger, 2003). The interhemal region of the placenta consists of fetal and maternal blood vessels that are present under the basement membrane of the trophectoderm and endometrium without any loss of the endometrial tissue (Leiser et al., 1998; Perry, 1981). The thin interhemal layer and rich supply of blood vessels reduce the distance for diffusion of substances between dam and fetus (Enders & Blankenship, 1999).

Ruminant placentation was initially called syndesmochorial based on initial observations that the endometrial lining undergoes degeneration, and the chorionic epithelium and uterine connective tissue layers were in direct contact (King, 1992). Based on ultrastructural studies using electron microscopy in sheep, the ruminant placenta was observed to have synepitheliochorial placentation (F. B. P. Wooding, 1980, 1984). Based on these studies, the fetal trophoblast layer consisted of mononuclear and binuclear cells. The binucleate cells that first appear at the time of implantation would represent up to 20% of the trophectoderm cell population and secrete proteins and hormones (Nakano et al., 2001). Some fetal binuclear cells would translocate and undergo apical fusion with the uterine epithelial cells to form a syncytial layer, hence the name synepitheliochorium (Cornelis et al., 2013; F. B. P. Wooding, 1980, 1984). The syncytium consisted of mononuclear uterine epithelial cells along with trinucleate and multinucleate cells.

Endotheliochorial placenta

The endotheliochorial placentation of carnivores featured a loss of uterine epithelium and connective tissue layers, leaving a four-layer thick interhemal membrane bringing maternal and fetal vascular supply closer (Barrau et al., 1975; Leiser & Koob, 1993; P. Wooding & Burton, 2008). The trophectoderm cells were observed to fuse and invade the uterine epithelial layer, followed by necrosis of uterine epithelial cells and their phagocytosis by trophoblast cells (Barrau et al., 1975). As the chorionic villi syncytium expanded, the hematoma was observed at the margins of the placental girdle, and the maternal capillaries system also reorganized to adapt to fetal nutrient requirements (Leiser & Koob, 1993; P. Wooding & Burton, 2008). In the case of elephants, the placenta is predominantly endotheliochorial with some scattered hemochorial regions (Ramsey, 1975).

Hemochorial placenta

Hemochorial placenta represents the most invasive type of placentation and has been observed in primates (hemomonochorial), rabbits (hemodichorial), rats and mice (hemotrichorial), with single, double, and triple trophoblastic layers, respectively (Steven, 1975; Takata et al., 1997). This type of placenta is characterized by the disappearance of all tissue layers on the maternal side and the fetal trophectoderm is in direct contact with the maternal blood (King, 1992).

In primates, the placental villi are directly in contact with maternal blood. The outer surface of placental villi that bathes in maternal blood vessels is called syncytiotrophoblast, whereas the layer just beneath it is called cytotrophoblast (Enders & Blankenship, 1999). The stromal region of placental villi harbours fetal blood vessels.

1.2.2.3. Comparative permeability of placenta between different animal species

The primary purpose of the placenta is to transfer the nutrients from the dam to the fetus while simultaneously performing several secretory and regulatory tasks necessary for pregnancy maintenance. Most of the biomolecules traverse the placenta through the phenomenon of simple diffusion, active transport, and/or facilitated transport (Furukawa et al., 2014). In the instance of simple diffusion, the thickness of the cellular/tissue barrier between fetal and maternal blood (interhemal distance) would be the predominant factor influencing the ability of the molecules to

pass through the placenta. The biomolecules will diffuse through the placenta more efficiently if the interhemal membrane is less thick. Similarly, the rate of active and facilitated transport would also be influenced by the thickness of interhemal distance. Hence, the histological structure of the placenta (i.e., the layers separating maternal and fetal blood) strongly determines the transfer of biomolecules through the feto-maternal interface (Mihaly & Morgan, 1983; Miller et al., 1976; Père, 2003). As a result, the biomolecules (such as fatty acids and keto acids) might move rapidly from mother to fetus and vice-versa in primates, rabbits, and rodents (hemochorial placenta); however, their transfer across the placenta may be limited in ruminants, mare, and sow (Père, 2003).

1.2.2.4. Diagnostic importance of cffDNA

Since its discovery, the cffDNA has been used for prenatal screening for fetal sex, fetal blood-group antigens, and chromosomal abnormalities. Initial studies described the usefulness of cffDNA as a non-invasive tool for fetal sex determination in pregnant women (Dennis Lo, Tein, et al., 1998; Sekizawa, Kondo, et al., 2001). The sex determination was based upon the detection of Y chromosome-specific sequences where the presence of the Y sequence corresponds to the male fetus while its absence corresponds to the female fetus. Most commonly targeted Y-chromosome sequences using PCR included a single locus *DYS14* gene (Dennis Lo et al., 1990), a single locus sex-determining region of Y-chromosome (SRY) gene (Shah & Smart, 1996), and a multilocus deleted in azoospermia (DAZ) genes (Stanghellini et al., 2006). Further, the cffDNA was used to screen the fetus for X-linked disorders viz hemophilia and Duchenne muscular hypertrophy in pregnant women that were a carrier for these diseases (Breveglieri et al., 2019). Additionally, the extracted cffDNA has been applied to detect fetal Rhesus D (RhD) blood type in pregnant women that were RhD negative (Dennis Lo, Hjelm, et al., 1998); aneuploidies viz. trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), and trisomy 21 (Down syndrome) (Mennuti et al., 2015; Skrzypek & Hui, 2017); microduplications, microdeletions, and genetic diseases occurring due to mutations that followed paternal-specific inheritance (Bianchi, 2012; Ferrari et al., 2015).

Although much research on cffDNA has focused on prenatal diagnostics in women, this technology is still in the nascent stages in the case of bovine. Few studies have suggested that

cffDNA could be used for prenatal fetal sex determination in pregnant cattle (Da Cruz et al., 2012; Ristanic et al., 2018).

1.2.2.5. Challenges in fetal sex determination using cffDNA

Since the maternal cfDNA would exceed the fetal fraction in maternal blood samples, amplifying cffDNA has been challenging (Wright & Burton, 2009). In humans, a minimum of 2-4% fetal fraction of cfDNA has been suggested in the maternal blood that would be required for obtaining reliable results following prenatal assays (Ashoor et al., 2013; Canick et al., 2013; Fan & Quake, 2010; Hui & Bianchi, 2020). The lower concentrations of cffDNA in maternal circulation would potentially increase the false-negative or no-call results (Wataganara et al., 2016). However, methods such as high depth sequencing would likely compensate for the low yield of cffDNA, but at added costs. Various factors have been observed to be associated with changes in the fetal fraction of the cfDNA in the maternal blood of pregnant women, which could affect the results of prenatal testing. For instance, fetal fraction rises with an increase in gestational age (Kinnings et al., 2015), crown-rump length (Ashoor et al., 2013), multiple pregnancies (Hedriana et al., 2020), and serum-free hCG concentration (Ashoor et al., 2013; T. J. Lee et al., 2018). In contrast, fetal fraction decreases in case of mosaicism (Brison et al., 2018), triploidy (Nicolaides et al., 2014), increase in maternal weight (Ashoor et al., 2013), maternal autoimmune disease (Hui et al., 2014), animals bred by assisted reproductive techniques (T. J. Lee et al., 2018).

Additionally, we can expect even further lower quantities of circulating cffDNA in the plasma of pregnant cows than pregnant women, considering the presence of more tissue layers at the placental interface in bovines.

1.2.2.6. Size of fetal versus maternal cfDNA sequences

Several studies using Y-chromosome have confirmed that cffDNA has a shorter base-pair length than maternal cfDNA. The first study comparing fetal and maternal cfDNA sequences using fluorescent PCR reported that the size of cffDNA sequences was usually < 300 bp, whereas the maternal cfDNA was >1000 bp (Li et al., 2004). The authors further proposed that cffDNA might be enriched based on the size selection with a 300 bp length threshold. Other reports indicated that the median length of maternal cfDNA was approximately 500 bp, while the significant proportion of the cffDNA was <300 bp (K. C. A. Chan et al., 2004; Li et al., 2004). In contrast, a subsequent

study showed that the length distribution of cffDNA differs from that of maternal cfDNA as an enhanced proportion of cffDNA fragments shorter than 150 bp are found in maternal plasma, which possibly exists because of the differential nucleosomal packaging during the complex process of apoptosis or the difference in the nucleosome binding force (Fan et al., 2010). Although the cffDNA is significantly shorter than maternal cfDNA, cffDNA increases in size as the pregnancy advances (K. C. A. Chan et al., 2004).

1.2.3. Assessment of cfDNA

Although much research has been conducted in cfDNA diagnostics in the past few decades, only a few cfDNA based clinical tests have found their way even in human medical practice. One of the main reasons behind the slow adoption of research to the lab is the lack of standard operating procedures, as cfDNA extraction and quantification protocols have evolved and significantly differ between laboratories. Additionally, there has been a lack of consensus on the sample type, sample handling, storage conditions, and their effects on concentration and fragmentation of cfDNA (El Messaoudi et al., 2013). The following subsections will discuss different factors that could affect the yield of cfDNA from the peripheral circulation.

1.2.3.1. Sample type, anticoagulants, and collection vials

Several studies on the paired serum and plasma samples have demonstrated significantly lower cfDNA concentrations in plasma than serum (T. H. Lee et al., 2001; Umetani et al., 2006). The serum cfDNA concentrations were reportedly 1.63 to 11-fold higher than the plasma in healthy individuals (K. A. Chan et al., 2005; Ginkel et al., 2017). Similarly, the serum samples from cancer patients had an 11-fold higher concentration of cfDNA than their plasma samples (Park et al., 2012). Based on the ratio of 201/105 bp amplicons following qPCR for leptin, higher integrity of the cfDNA was noted in serum (50%) than plasma (33%) (K. A. Chan et al., 2005; Holdenrieder et al., 2008). These remarkably higher cfDNA concentrations with better integrity in serum were attributed to the release of genomic DNA from leukocytes undergoing lysis during the clotting process (K. A. Chan et al., 2005; Lam et al., 2004; T. H. Lee et al., 2001). In order to avoid further contamination and dilution of the fetal fraction of cfDNA, plasma samples have been preferred over the serum samples for the extraction of cffDNA for downstream diagnostic assays in humans (Fernando et al., 2010; Meddeb et al., 2019).

As multiple anticoagulants are available to harvest plasma from blood samples, it is essential to determine the anticoagulant suitable for the stability of cfDNA. Several studies that compared citrate, ethylenediaminetetraacetic acid (EDTA), and heparin as anticoagulants identified EDTA as the preferred anticoagulant, while heparin was observed to affect downstream processing by causing PCR inhibition (Beutler et al., 1990; Jung et al., 1997). Further, plasma collected using EDTA vials could still be used, even if the processing of blood samples was delayed up to six hours (Lam et al., 2004). Storage of blood samples in potassium EDTA vials beyond six hours under refrigeration had been associated with a gradual increase in total cfDNA due to the release of longer DNA fragments from cell lysis, while the cffDNA content did not change (Barrett et al., 2011; Page et al., 2011). As a result, there was a reduction in the relative proportion of cffDNA (Barrett et al., 2011; Page et al., 2011).

Nevertheless, to avoid the immediate need for blood processing, special tubes for blood collection have been introduced containing specific preservatives to stabilize the blood leukocytes for extended periods. Blood samples taken from pregnant women and stored at room temperature for one week in the cell stabilizing tubes showed no change in the concentration of total cfDNA over time, while the samples stored in EDTA tubes under the same conditions showed an increase in cfDNA after 24 h (Fernando et al., 2010; Wong et al., 2013). During the storage period, the fetal copy number remained stable between the type of tubes; however, the fetal fraction decreased after 24 h in the EDTA tubes due to an increase in long maternal DNA fragments (Wong et al., 2013). Based on the American Association for Clinical Chemistry (AACC) recommendations, the samples collected in EDTA tubes kept at room temperature need to be processed within four h or 24 h of collection if kept under refrigeration. In contrast, the samples collected in specialized tubes might be kept for 5 d at room temperature before processing (Meddeb et al., 2019).

1.2.3.2. Handling, storage, and processing of samples before DNA extraction

The AACC recommends proper mixing of blood samples in collection tubes, screening blood samples for hemolysis and avoiding their use if the hemolysis is present (Meddeb et al., 2019). Some studies have indicated an increase in cfDNA following shaking of blood samples or repeat passaging of blood samples through small gauge needle (El Messaoudi et al., 2013; Nishimura et al., 2019). However, a recent study on a larger number of human samples found no correlation between the observed hemolysis and relative proportions of cfDNA (Stokowski et al., 2020). Since

adult RBC are non-nucleated, their lysis may only release RNA, not affecting cfDNA concentrations. However, the hemolyzed samples may be associated with the release of PCR inhibitors.

One of the critical preanalytical steps is centrifugation of the collected blood to separate cells from plasma. Centrifugation of blood at low speed (800×*g*) for 10 min resulted in increased cfDNA compared to a higher rate (16000×*g*) for the same time (Chiu et al., 2001). Further, two-step centrifugation was found to be more efficient for downstream processing of plasma for cfDNA extraction (Chiu et al., 2001). Some studies have argued against the need for double centrifugation (Trigg et al., 2018). However, the currently recommended procedure of plasma separation for human samples by the AACC includes centrifugation under refrigeration either at 800-1200×*g* (10 min at 4°C) followed by 16000×*g* (10 min at 4°C) or two serial centrifugation at 1600×*g* and 16000×*g* (10 min each at 4°C), respectively (Meddeb et al., 2019).

1.2.3.3. Repeated plasma freeze-thaw cycles and cfDNA yield

A significant decline in the integrity of cfDNA was observed every time plasma was subjected to freeze-thaw cycles, which showed that cfDNA was sensitive to temperature variations and got fragmented when plasma was repeatedly frozen and thawed (K. A. Chan et al., 2005). The same findings have also been replicated by El Messaoudi and co-workers, who have confirmed the sensitive nature of plasma cfDNA to the variations in temperature (El Messaoudi et al., 2013). As per the guidelines of AACC, plasma should not be subjected to more than two freeze-thaw cycles before DNA extraction to avoid the deterioration of cfDNA integrity (Meddeb et al., 2019).

1.2.4. Extraction protocols for cfDNA from plasma

Beyond the conventional protocols, a wide range of DNA extraction methodologies could be used for cffDNA isolation and downstream assays. The conventional protocols are cost-effective and could be modified as per the available laboratory facilities; however, they would be labor-intensive with increased turnaround times and variability (Bronkhorst et al., 2015; McKiernan & Danielson, 2017). The new commercial specialized kits are costly but have standardized protocols to enhance repeatability, facilitate automation and increase cfDNA yield (Trigg et al., 2018). The standard DNA extraction protocols are being discussed in upcoming subsections.

1.2.4.1. Silica based spin-column protocols

These commercial kits are based on the salt-dependent binding of DNA in the sample to the silica resin present in the spin columns and involve the following steps. The plasma/serum samples are treated with a buffer to lyse cells and proteinase-K for protein digestion. The buffer would also assist in attaching DNA to the silica-gel membrane in the spin column and remove enzyme inhibitors and other contaminants. Furthermore, the PCR inhibitory substances viz. proteins and divalent cations are eliminated in highly efficacious two-three washing steps. As a result, the pure nucleic acids left behind with the silica membrane are ultimately eluted in the elution buffer. A study comparing the cffDNA extraction efficiencies of QIAamp® Circulating Nucleic Acid Kit (Qiagen, Valencia, CA) and QIAamp® DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) found no differences in the cffDNA yields between these two kits (Manokhina et al., 2014). A recent study comparing QIAamp® DSP Virus Kit (Cat. No. 60704; Qiagen, Germany) and QIAamp® Circulating Nucleic Acid Kit (Cat. No. 1017647; Qiagen, Germany) obtained significantly higher cffDNA yields from the DNA extracts of QIAamp® Circulating Nucleic Acid Kit (Jain et al., 2019).

1.2.4.2. Magnetic beads-based protocols

As the name suggests, these kits are based on the selective binding of DNA to magnetic beads, which provide more surface area to make it more efficient in terms of DNA yield with minimum variations (Jorgez et al., 2006). Most magnetic beads-based DNA extraction kits comprise four extraction steps: lysis, binding (to magnetic beads), washing (2-3x), and elution. Examples of magnetic kits include the NucleoMag® Cell-Free DNA Kit (Cat. No. 744550.1; Macherey-Nagel, Germany) and MagMAX™ Cell-Free DNA Isolation Kit (Cat. No. A29319; Applied Biosystems, USA) suitable for both manual and automated platforms. Studies have reported that magnetic beads provide higher yields of plasma cfDNA in human lung and colon cancer patients (Stemmer et al., 2003) and cffDNA in pregnant women compared to other column-based and conventional DNA extraction methods (Jorgez et al., 2006).

1.2.4.3. Other protocols

Phenol chloroform-based methods and salting-out protocols are the most common traditional techniques for cfDNA extraction from plasma/serum samples (Bronkhorst et al., 2015). Phenol

chloroform-based DNA extraction involves using proteinase K and sodium dodecyl sulfate (SDS) for the enzymatic dissolution of cellular and protein components of the sample. After this, the sample is treated with a combination of equilibrated phenol: chloroform: isoamyl alcohol (25:24:1) which promotes the separation of the organic phase (containing cellular debris and lipids) from the aqueous phase (containing nucleic acids). The aqueous phase that contains the purified nucleic acids is then transferred to a new centrifuge tube for further analysis following centrifugation. Alternatively, the nucleic acids in the aqueous phase can also be recovered by ethanol precipitation or concentrated through centrifugal filter units (Comey et al., 1994; McKiernan & Danielson, 2017). However, the phenol-chloroform extraction method uses hazardous chemicals, and involves a substantial manual effort. In addition, the chances of sample mishandling and contamination are significantly increased because of the involvement of multiple tube transfer steps (Köchl et al., 2005). Another fundamental limitation of the phenol-chloroform method is the carry-over of phenol and other PCR inhibitors in the isolated DNA sample that significantly compromise the downstream processing (Lemarchand et al., 2005), thereby increasing the chances of false-negative results in the PCR. Many variations of phenol-chloroform protocol have been used for extraction of cfDNA in several research studies in humans involving cancer diagnostics (Elshimali et al., 2013; Hufnagl et al., 2013; Xue et al., 2009), parasitic diagnosis (Wichmann et al., 2009), and evaluation of ageing biomarkers (Jylhävä et al., 2011). Furthermore, the phenol-chloroform protocol has also been used to extract cffDNA in the studies focusing on PCR-based prenatal fetal sex determination in cattle (Davoudi et al., 2011; Ristanic et al., 2018) and sheep (Kadivar et al., 2013).

Another conventional method, the salting-out protocol, was used to extract cfDNA for fetal sex determination in cattle (Lemos et al., 2011). In this method, lysis of the blood cells is performed using lysis buffer until a clean pellet is obtained. Then, proteinase K and SDS are added for the enzymatic dissolution of cellular and protein components of the sample. Subsequently, the sample is treated with a saturated solution of NaCl for the precipitation of proteins. The supernatant is then treated with absolute ethanol to precipitate DNA which is eluted after washing it with 70% ethanol (Shokrzadeh & Mohammadpour, 2018). As this protocol uses whole blood to extract cfDNA, the chances of contamination with genomic DNA from maternal blood cells are high. A different throughput method involves using a novel buffer system and a thermostable protease (KAPA

Express Extract Kit®, Cat No. KK7100; KAPA Biosystems, South Africa) to extract genomic DNA from blood and tissue samples.

The majority of these methods have been developed for human samples; however, some studies have used different kits to assess cffDNA for fetal sex determination in cattle (Da Cruz et al., 2012; Ristanic et al., 2018; G. Wang et al., 2010). Various methodologies that have been used for cffDNA extraction for prenatal fetal sex determination have been summarized in Table 1.2.

1.2.5. Storage of extracted cfDNA and freeze-thaw cycles

For human samples, the AACC suggested that cfDNA extracts may be stored for 3 h at room temperature, 24 h at 4°C, and 3 months to 10 years at -20°C to -80°C (Meddeb et al., 2019). Furthermore, it had also been shown that cffDNA in the plasma would deteriorate after the third freeze-thaw cycle (K. A. Chan et al., 2005; Meddeb et al., 2019).

1.3. Status of prenatal fetal sex determination in domestic and wild animals using cffDNA

Various studies have targeted cffDNA using molecular methods in the maternal plasma of pregnant wild and domestic animals to ascertain the sex of the fetus before birth. The following subsections summarize the successes and limitations of using PCR for prenatal fetal sexing in animals with endotheliochorial and epitheliochorial placentation. We have excluded primates and rodents, with hemochorial placentation, from the discussion.

1.3.1. Animals with endotheliochorial placenta

Since both dogs and cats carry multiple fetii during each pregnancy, the use of PCR for fetal sexing would be the least practical due to the challenge of differentiating fetii of different sexes based on PCR. As a result, ultrasound seems to be the feasible approach to determine fetal sex if at all the breeders would be interested (Prugnard et al., 2016). Elephants that share the placental morphology and histoarchitecture with carnivores have been studied recently to detect fetal sex using PCR (Vincze et al., 2019). Two sets of primers utilized in this study were to target SRY and AMEL (Amelogenin), while *PlpX (*proteolipid protein) specific for X-chromosome was used as a control. Amongst the pregnant elephants, one carried a male fetus and the other had a female fetus.

Table 1.2: Summary of PCR-based prenatal fetal sex determination using cffDNA in pregnant females of various mammalian species.

Species (n)	Gestational stage	cfDNA extraction method	Target Genes	Sample type	Accuracy of fetal sexing by PCR/salient findings	Reference
Elephant (n=2)	80-86 wk	Phenol-chloroform	SRY Amelogenin	Plasma	PCR predicted fetal sex matched fetal sex at birth	(Vincze et al., 2019)
Mares (n=20)	Last 3 months of gestation	QIAamp DNA Blood Mini Kit	SRY GAPDH	Plasma	1st round PCR: 85% 2nd round PCR: 95%	(de Leon et al., 2012)
Mares (n=28)	8-20 wk	DNP Kit	SRY GAPDH	Plasma	1st round PCR: Inconclusive 2nd round PCR: 88%	(Kadivar et al., 2016)
Mares (n=32)	First, second, and third trimester of pregnancy	*Prime Prep* Genomic DNA Kit	SRY TKY GAPDH	Plasma	1st trimester: 48.75% 2nd trimester: 68.75 3rd trimester: 75%	(Tonekaboni et al., 2020)
Mares (n=50)	8-20 wk	DNP Kit	SRY TSPY GAPDH	Plasma	93%	(Kadivar et al., 2021)
Rhinoceros (n=14)	61-490 d	Phenol-chloroform	SRY Amelogenin	Serum	1st round PCR: 71% 2nd round PCR: 100%	(Stoops et al., 2018)
Ewes (n=46)	2-5 mo	Phenol-chloroform	SRY GAPDH	Plasma	100%	(Kadivar et al., 2013)
Ewes (n=45)	8-18 wk	Phenol-chloroform	AMELY GAPDH	Plasma	93.3%	(Kadivar et al., 2015)
Ewes (n=32)	6-21 wk	Phenol-chloroform	AMLX AMLY	Plasma	78%	(Asadpour et al., 2015)
Cows (n=36)	10-40 wk	Isopropanol fractionation	btDYZ β-actin	Blood	No fetal DNA exists in maternal circulation	(Kadokawa et al., 1996)
Cows (n=16)	Mid-gestation Pre-calving Calving Post-calving	Qiagen Blood Kit	Y-specific β-actin	Blood	Predominant transfer of fetal genetic material to the maternal circulation occurs during parturition	(Turin et al., 2007)
Cows (n=110)	30-242 d	PDfast 100 DNA Plasma Kit	SRY	Plasma	Male fetii: 100% Female fetii: 91%	(G. Wang et al., 2010)

Cows (n=84)	30-270 d	Salting-out protocol	TSPY	Blood	100%	(Lemos et al., 2011)
Cows (n=38)	8-38 wk	Phenol-chloroform	bAML TSPY	Plasma	100%	(Davoudi et al., 2011)
Cows (n=35)	5-35 wk	Illustra GenomicPrep Blood Mini Spin Kit	Y-specific	Plasma	99.9% accuracy > 55 days post-conception	(Da Cruz et al., 2012)
Cows (n=40)	55-90 d 240-270 d	GeneJET Isolation Kit KAPA Express Extract Kit Phenol-chloroform	bAML TSPY	Blood	KAPA kit worked better than other two methods qPCR was better than conventional PCR	(Ristanic et al., 2018)
Women* (n=81)	5-10 wk	QIAamp DNA Blood Mini and Midi Kits	DYS14 SRY B-globin	Serum	30.55 copies of fetal DNA exist/ml of maternal serum 100% sensitivity of male fetus detection at 7th week of gestation	(Honda et al., 2001)
Women* (n=31)	7-32 wk	QIAamp DNA Blood Mini Kit	SRY ALT1	Plasma	100%	(Tungwiwat et al., 2003)
Women* (n=236)	11-13 wk	QIAamp DNA Blood Mini Kit	SRY DBY TTTY2	Plasma	99.1%	(Akolekar et al., 2010)
Women* (n=101)	8-20 wk	NucliSENS easyMAG system	DYS14	Plasma	Fetal sex can be predicted by taking maternal capillary blood from fingertip puncture	(Rita et al., 2018)
Women* (n=24)	5-10 wk	Quick-cfDNA Serum and Plasma Kit	SRY GAPDH	Blood	100%	(Kazachkova et al., 2019)

*These are a few representative studies out of overwhelming evidence on pregnant women available in existing literature.

Elephants were sampled four times between 80-86 weeks of gestation and plasma was processed for conventional PCR, the results of which matched with the fetal sex (summarized in Table 1.2).

1.3.2. Animals with epitheliochorial placenta

The horses are another species that has diffused epitheliochorial placenta and usually have singleton or rarely twin births. Recently, a couple of studies on a smaller number of mares have aimed at evaluating the usefulness of determining fetal sex in pregnant mares. In an initial study, plasma samples from thoroughbred mares in the last trimester of pregnancy were tested for SRY using two rounds of conventional PCR (de Leon et al., 2012). Based on the first PCR, the sex was accurately determined in 17/20 mares, whereas the accuracy was 95% (19/20) following the second round of PCR. In a subsequent study, blood samples were taken from 32 pregnant mares during each trimester of gestation, PCR was performed to target the SRY gene and results were compared with the sex of foals at birth (Tonekaboni et al., 2020). A gradual increase in accuracy of PCR for fetal sexing was observed as the gestation advanced (49%, 69% and 75% for 1st, 2nd, and 3rd trimester of pregnancy, respectively). Another study on Arabian mares (n=50) with a single fetus reported a higher accuracy (93%) using a duplex real-time PCR targeting SRY and TSPY sequences (Kadivar et al., 2021). The sensitivity and specificity of duplex PCR were 90% and 96%, respectively. A summary of all these studies in mares has been presented in Table 1.2.

Rhinoceros, another species with diffused epitheliochorial placenta, has also been studied regarding prenatal fetal sexing using PCR (Stoops et al., 2018). Serum from pregnant rhinoceros (n=14) in varying stages of gestation (61-490 days range) was tested using primers targeting SRY and equine AMEL (Amelogenin) genes. The authors reported that both DNA concentrations and PCR amplification were significantly reduced when frozen-thawed serum samples were used to determine fetal sex. In contrast, when they used fresh serum samples for DNA extraction, the first round of PCR provided a 71% accuracy of SRY gene amplification for pregnancies carrying male fetii. This accuracy increased to 100% when the first-round PCR products were used for the second round of PCR. However, no SRY products were obtained in either round of PCR when samples from female pregnancies were used (summarized in Table 1.2).

Amongst the ruminants, which have cotyledonary and synepitheliochorial placenta, sheep and cattle are the most studied species for prenatal sex determination using PCR. The findings of different studies have been briefly discussed below and summarized in Table 1.2.

Kadivar and co-workers performed the first study on PCR-based prenatal fetal sex determination in pregnant ewes (n=46) in 60-150 days of gestation. A pair of primers targeting SRY sequence and yielding a 286 bp PCR product from the male fetal genome was used to determine fetal sex. At the same time, another set of primers targeting the GAPDH gene and yielding a 467 bp PCR product was used as an internal control. The PCR-based fetal sex results were compared with the sex of the offsprings at birth. The qPCR methodology used in this study correctly predicted 31/31 male and 15/15 female fetuses with an overall accuracy of 100%. Furthermore, the authors also showed a significant rise in the levels of fetal DNA with advancement in gestation, and fetal DNA concentrations were found to be 1.65-fold higher in the last two months of pregnancy compared to the first three months (Kadivar et al., 2013).

Kadivar and co-workers conducted another experiment to determine ovine fetal sex prenatally using AMELY (Amelogenin-Y) primers. They included 45 pregnant Lori-Bakhtiari sheep 56-126 days in gestation in their study. A pair of AMELY primers were designed to amplify a 111 bp product out of the Y-chromosome of male fetuses while simultaneously incorporating an internal control that amplified a 467 bp sequence out of the GAPDH gene irrespective of the fetal sex. The PCR-based fetal sex results were verified with the sex of the offsprings at birth. They reported that the qPCR methodology used in their study correctly predicted 27/28 male and 15/17 female fetuses while 1/28 and 2/17 fetuses were wrongly predicted as female and male, respectively. The overall accuracy of this PCR-based ovine fetal sexing methodology was 93.3%, whereas the sensitivity and specificity of the test were 96.5% and 87.5%, respectively (Kadivar et al., 2015).

Asadpour and co-workers also performed PCR-based prenatal fetal sex determination in 32 pregnant ewes (42-147 days in gestation), with 15/32 carrying male and 17/32 carrying female fetuses confirmed at parturition. The primer set utilized in this study amplified a 217 bp and a 280 bp amelogenin gene sequence from the Y and X chromosomes, respectively. When PCR-based fetal sexing outcome was compared with the sex of the offsprings at birth, the PCR methodology was found to yield an overall accuracy of 78% with 12/15 and 13/17 correct male and female fetus predictions, respectively. However, 3/15 male fetuses and 4/17 female fetuses were wrongly predicted female and male, respectively, by the PCR assay (Asadpour et al., 2015).

Kadokawa and co-workers conducted a research study to evaluate the presence of fetal nucleic acids in bovine maternal circulation. For this purpose, they collected blood samples from the

jugular and uterine veins of 3 pregnant cows (2 carrying male fetuses; 1 carrying female fetus) in 84-140 days of gestation. In addition, they also collected blood samples from the jugular vein of 33 pregnant cows (18 carrying male fetuses; 15 carrying female fetuses) in 70-280 days of gestation. They performed DNA extraction from all these blood samples and carried out a PCR targeting Y-specific repeat sequence (btDYZ). Simultaneously, another primer set was also used to target the β-actin gene that served as an internal control. The PCR result showed no amplification of male-specific DNA sequence in any of the samples irrespective of the site of blood sampling. The authors thus concluded that fetal DNA might not be present in the adequate levels in maternal circulation. Therefore, blood from the pregnant cows could not be used for PCR-based prenatal fetal sexing (Kadokawa et al., 1996).

Xi collected blood samples from pregnant dairy cows (n=32) in 30-240 days of gestation for PCR-based prenatal fetal sex determination. Their PCR methodology involved targeting Y-chromosome-specific SRY gene sequences to identify male fetuses. In addition, they used a primer set to amplify the β-actin gene sequence irrespective of the fetal sex to serve as an internal control. When the DNA isolated from the plasma of these pregnant cows was used as a PCR template, an 80% overall accuracy of correctly identifying the fetal sex was reported. The author also noted that the accuracy of fetal sex prediction increases with advancement in gestation. The accuracy rates of 60%, 85.7%, and 92.3% were observed respectively for cows that were 30-60 days, 61-120 days, and 121-240 days pregnant (Xi, 2006).

Turin and co-workers conducted an experiment to determine the transplacental leakage of fetal genetic material to the maternal circulation in pregnant cows. The study was based upon the hypothesis that the epitheliochorial placenta of cows is least invasive and thus poses a barrier to the transfer of fetal cells/nucleic acids to the maternal circulation, unlike in primates. In this study, 16 pregnant cows carrying male fetuses were included, and the blood samples were collected at four different time points viz. mid-gestation (150 days of pregnancy), pre-calving (260 days of pregnancy), calving (within 2 days following calving), and post-calving (4 months after calving). DNA was extracted from these blood samples followed by qPCR targeting Y-chromosome-specific gene sequences to identify the male fetuses. Simultaneously, a set of primers targeting the b-actin gene sequence was also used in PCR as an internal control. Y-specific sequences were successfully amplified in 25% of cows during mid-gestation, 40% of cows at pre-calving, 73% cows at calving, and 40% of cows at post-calving. The authors thus concluded that although the

transplacental leakage of fetal genetic material to the maternal circulation occurs variably to a certain degree during pregnancy, the predominant transfer of fetal genetic material to the maternal circulation occurs during parturition. The authors also reported that irrespective of the stage of gestation or calving, as mentioned earlier, there are many occasions when fetal DNA is detected at one time point but absent in the subsequent samples. This finding indicated that the transplacental leakage of fetal genetic material to the maternal circulation might occur transiently and is not consistent throughout gestation (Turin et al., 2007).

Wang and co-workers carried out research to determine the feasibility of prenatal fetal sex determination in pregnant cows using a nested PCR methodology targeting SRY gene sequences in the male fetal genome. The blood samples were collected from 110 pregnant cows (30-242 days in gestation), and plasma was harvested for DNA extraction. The overall accuracy of correctly identifying fetal sex was 100% (43/43) for male fetuses and 91% (61/67) for female fetuses. Furthermore, the accuracy rate of correctly identifying male and female fetuses in the 30-59 days pregnant cows was 100% (3/3) and 85.7% (12/14), respectively. However, after 60 days of gestation, the accuracy rate for male and female fetus prediction was 100% (40/40) and 92.5% (49/50), respectively (G. Wang et al., 2010).

Lemos and co-workers conducted an experiment to detect the presence of fetal DNA in the peripheral blood circulation of pregnant cows. They collected blood from 84 primiparous *Bos indicus* cows in 30-270 days of gestation. The blood samples were used for DNA extraction, followed by a conventional PCR targeting TSPY (testes specific Y-encoded protein) gene amplifying a 260 bp product in the male fetal genome. The PCR identified 47/84 as the male fetuses and 37/84 as female fetuses. These PCR results showed a 100% match to the sex of the fetuses at birth. The authors, therefore, reported the presence of detectable levels of fetal DNA throughout the pregnancy in cows (Lemos et al., 2011).

Davoudi and co-workers performed a study on PCR-based prenatal fetal sex determination in pregnant cows (n=38) between 56-266 days of gestation. They used two sets of primers in this study. The first primer set targeted bAML (bovine amelogenin) gene that amplified a 341 and 467 bp product from bovine Y and X chromosomes, respectively. The second primer set targeted the TSPY gene that amplified a 260 bp product from the Y-chromosome of the male fetus. The PCR methodology used in this study predicted 24 cows to be carrying male fetuses and 14 cows to be

having female fetuses. The PCR-based fetal sex exactly matched the sex of the offspring in all 38 pregnancies. The accuracy of this methodology was hence reported to be 100%, with no false positives and negative results (Davoudi et al., 2011).

Da Cruz and co-workers performed prenatal fetal sex determination in 35 mixed-breed pregnant cows between 35-238 days of gestation using conventional PCR. They used two sets of primers targeting Y-chromosome-specific sequences in male fetal genome amplifying 210 and 196 bp products while simultaneously incorporating a primer set targeting an autosomal sequence of 280 bp from chromosome-1 of cows. Overall, the fetal sex predicted by PCR accurately matched with fetal sex at birth in 31/35 cases yielding an accuracy rate of 88.6%. There was a 63.6% concordance of fetal sex predicted by PCR to the fetal sex at birth in 35-48 days pregnant cows, whereas it was 100% on 49 days and onwards. As per the regression analysis, the PCR methodology showed an accuracy rate of 95%, 99%, and 99.9% at 43.8, 48.4, and 55 days of gestation, respectively (Da Cruz et al., 2012).

Ristanic and co-workers performed a similar study in pregnant cows to assess the accuracy of this PCR-based non-invasive genetic fetal sex determination technique. They enrolled 40 pregnant Holstein Friesian cows and divided them into two groups. The first group comprised 55-90 days pregnant cows, while the second group consisted of 240-270 days pregnant ones. The fetal sex of first and second group cows was examined using ultrasonography and at birth, respectively. Group-1 cows carried ten male and ten female fetuses; however, group-2 cows had twelve male and eight female fetuses. For conventional and qPCR, primers and PCR conditions as described by Davoudi *et al* (2011) were used. With conventional PCR, 9/10 male and 10/10 female fetuses in group-1 were correctly predicted. In group-2, none of the male fetuses could be detected by the conventional PCR. In contrast, qPCR successfully predicted 9/10 and 11/12 male fetuses in group-1 and 2, respectively. The authors thus reported that qPCR is more sensitive in amplifying the extremely low levels of fetal DNA than conventional PCR (Ristanic et al., 2018).

1.4. Objectives and hypotheses

- The overall aim of this thesis was to establish a protocol for PCR-based prenatal sex determination in cattle. The specific objectives and hypothesis of this study were:

1.4.1. Determine sample processing and DNA extraction protocol for PCR based prenatal fetal sex determination in pregnant cows

1.4.1.1. Specific objectives

- To determine the effect of stored and freshly collected plasma on cffDNA extraction and downstream PCR for fetal sexing.
- To compare different DNA extraction methods on downstream PCR for fetal sexing in cattle.

1.4.1.2. Hypothesis

- We hypothesized that plasma storage, DNA extraction methods, and target genes would differentially affect the PCR results for fetal sex determination.

1.4.2. Determination of the lower limits of cell-free fetal DNA in bovine plasma samples for PCR detection

1.4.2.1. Specific objectives

- To test the lowest limits of spiked cffDNA that can be re-extracted and amplified with PCR to identify bAML and Y-specific amplicons.
- To determine the efficacy of the PCR-based prenatal sexing in cattle at different stages of gestation.
- To compare three different commercial kits for extraction of cffDNA from spiked and neat plasma samples of pregnant cows.

1.4.2.2. Hypotheses

- cffDNA is low and variable in the plasma of pregnant cows, making it challenging to extract for conventional PCR.
- cffDNA can be successfully extracted and amplified using conventional PCR if present at amounts equal or above the minimum detection limits of a protocol.

2. DETERMINE SAMPLE PROCESSING AND DNA EXTRACTION PROTOCOL FOR PCR BASED PRENATAL FETAL SEX DETERMINATION IN PREGNANT COWS

2.1. Abstract

Objectives: The overall aim of this study was to establish a protocol for PCR-based prenatal fetal sex determination in pregnant cows. Our specific goals were to a) determine the effect of frozen and fresh plasma on cffDNA extraction and PCR for fetal sexing and b) compare different DNA extraction methods on downstream PCR for fetal sexing in cows.

Materials and methods: The current study included three trials. In the Trial 1, frozen samples from cows (n=15, three replicates, 120-150 days pregnant, each carrying a male fetus) were processed using three different DNA extraction methods followed by PCR. In the Trial 2, cfDNA from fresh plasma samples of pregnant cows (n=6, 120-150 days, each carrying a male fetus) was extracted by the phenol-chloroform method and used for PCR-based fetal sexing. In the first part of the Trial 3, fresh plasma samples of pregnant cows (150-240 days; n=5 carrying male fetii, n=3 carrying female fetii) were used for DNA extraction (three methods) and PCR. In the second part of the Trial 3, fresh plasma samples and blood (for salting-out protocol) from five pregnant cows (150-240 days pregnant; each carrying a male fetus) were processed for DNA extraction (seven different methods) followed by PCR.

Results: None of the samples yielded male fetus-specific PCR products in our Trial 1, although sequence specific to maternal cfDNA was amplified in all instances. Neither an increase in the plasma volume for DNA extraction nor template DNA volume changed the results. In the Trial 2, we observed amplification of fetal DNA sequences in 5/6 cows when 10 µl of DNA extract was used for the PCR. In the first part of the Trial 3: upon bAML PCR, correct prediction of fetal sex was observed in 4/8, 4/8, and 2/8 DNA samples obtained by Dneasy Blood & Tissue Kit, MagMAX cfDNA Isolation Kit, and Phenol-chloroform method, respectively. In the second part of the Trial 3, male fetal gene sequences were successfully amplified in 3/5 samples (DSP Virus Kit), 2/5 samples each (Blood & Tissue Kit, MagMAX Kit, Nucleomag Kit, Salting-out protocol), and 0/5 samples (Midi Kit, KAPA Kit) in PCR.

Conclusions: Fresh plasma samples but not frozen from pregnant cows could be used for cfDNA extraction and PCR for fetal sexing. Further, DNA extraction methodologies compared in the

present study showed variability in their ability to isolate fetal DNA from maternal plasma/blood of pregnant cows. Therefore, the currently tested PCR assay was observed to be unreliable for prenatal fetal sexing in pregnant cattle and was not better than the chance prediction of 50%.

2.2. Introduction

Knowledge of the sex of the fetus before birth at the time of pregnancy diagnosis has multiple benefits for cow-calf operations. Specifically, by knowing the sex ratio of the future calf-crop, producers would plan for the replacement herd (based on expected female calves) and the expected number of steers that would be available for feedlots. Furthermore, purebred producers might retain the cows carrying bull calves based on their interest in their genetics for their herds or sale. Amongst the available tools to determine the sex-ratio of calf-crop, sex-sorted semen, and sexed embryo would be best fitted for seed stock producers and least likely to be adopted by the producers using natural breeding (Holden & Butler, 2018). Ultrasonography, which is used for pregnancy diagnosis at least in some herds, is another method for accurate detection of fetal sex between 55-70 days post-conception (Curran et al., 1989; Kamimura et al., 1994) and could be used until 100 d of gestation (Heyman et al., 2002). However, in some herds, this time window would be outside the usual three to five months of gestation when cattle are often diagnosed as pregnant at the time of fall pregnancy check. Further, ultrasonographic examination for fetal sexing needs additional time and expertise, limiting its applicability. The accuracy of fetal sexing decreases beyond 100 days of gestation as the fetus grows, making the uterus drop into the abdominal cavity and beyond the reach for proper orientation of the probe to the fetus (Curran, 1992). Therefore, an alternative method that is more practical, less time-consuming, economical, and highly accurate in determining fetal sex in cattle would be desirable.

One such potential method is PCR-based detection of the cffDNA in the maternal peripheral circulation. Initially, the cffDNA was reported to be present in low proportions (3.4-6.2%) in the blood samples of pregnant women (Dennis Lo et al., 1997). Subsequently, cffDNA has been used for non-invasive prenatal genetic screening of the fetus for sex determination (Dennis Lo, Tein, et al., 1998; Sekizawa, Kondo, et al., 2001) and heritable diseases (Bianchi, 2012; Ferrari et al., 2015; Mennuti et al., 2015; Skrzypek & Hui, 2017). A limited number of studies in domesticated and wild animals have reported prenatal fetal sex determination in horses (de Leon et al., 2012; Tonekaboni et al., 2020), elephants (Vincze et al., 2019), rhinoceros (Stoops et al., 2018), and

cattle with varying degrees of accuracy (Da Cruz et al., 2012; Ristanic et al., 2018). At least in humans, isolation of cffDNA from maternal blood for downstream testing has been observed to be affected by a variety of preanalytical factors such as sample collection method, type of sample, processing as well as storage of samples, and DNA extraction methods (Meddeb et al., 2019). Only one study in cattle has compared different DNA extraction methods (Ristanic et al., 2018), while none of the studies have analyzed the effect of plasma storage on the PCR-based prenatal sex determination.

The overall aim of this study was to establish a protocol for PCR-based prenatal sex determination in cattle. Based on previous studies in humans (Meddeb et al., 2019) and cattle (Da Cruz et al., 2012; G. Wang et al., 2010), we planned to use blood plasma samples over serum samples for this study. As the cost and turnaround time for PCR results could vary with different DNA extraction methods, we decided to compare various conventional, silica-based spin column and magnetic bead-based methods to extract cfDNA from the plasma of pregnant cattle. We hypothesized that plasma storage, DNA extraction methods, and target genes would differentially affect the PCR results for fetal sex determination. Our specific objectives were to a) determine the effect of stored plasma and freshly collected plasma on cffDNA extraction and downstream PCR for fetal sexing, and b) compare different DNA extraction methods on downstream PCR for fetal sexing in cattle.

2.3. Materials and methods

2.3.1. Animals

The animal experimentation was conducted between October 2019 and October 2020 and was approved by the University of Saskatchewan Animal Care Committee (AUP#20190070). All animals (Canadian Holstein) included in the present experiment were located at Rayner Dairy Research and Teaching Facility, University of Saskatchewan. The dairy farm utilizes artificial insemination for breeding. The feeding, care, and management of the animals were done as per the routine for pregnant (mid-gestation and dry period).

2.3.2. Experimental design

A total of 34 pregnant cows (120-240 days), five heifers, and five bull calves (controls) were sampled during the experiment, which included four trials. Pregnancy diagnosis was routinely carried out within the dairy herd as part of bi-weekly herd health visits. During these visits, fetal

sexing via ultrasound was also carried out between 55-70 days of gestation and later confirmed with the determination of sex at birth.

Trial 1: Using frozen plasma for PCR-based fetal sexing

Blood samples were collected from pregnant cows (n=15, 120-150 days gestation) once weekly for 3 wks. The harvested plasma was stored at -20^{0}C until processed for cfDNA extraction and PCR. Further, blood samples from virgin heifers (n=5) and bull calves (n=5) were also collected to extract cellular DNA and stored in aliquots at -20^{0}C and used as controls in all trials (Figure 2.1).

Trial 2: Using fresh plasma for PCR-based fetal sexing

In this trial, blood samples were collected from pregnant cows (n=6, 120-150 days gestation) only once, and freshly harvested plasma was processed for cfDNA extraction and PCR (Figure 2.1).

Trial 3: Comparison of different DNA extraction methods

In the last trial under this experiment, blood samples were taken from thirteen pregnant cows (150-240 days pregnant). Depending upon the technique, the cfDNA was extracted on freshly harvested plasma or whole blood samples using three (Trial 3a, n=8) or seven different methods (Trial 3b, n=5) and then processed for PCR (Figure 2.1).

2.3.3. Collection, processing, and storage of samples

For all trials, duplicate peripheral blood samples were collected by caudal venipuncture from all animals using 6 ml K_2 EDTA tubes (VWR Cat. No. CABD367863L; BD Vacutainer) and transported on ice to the Molecular Microbiology Research Laboratory, the University of Saskatchewan within 2 hours of blood collection. Samples from pregnant cows were processed to harvest plasma for all extraction protocols except for the Salting-out method (Trial 3b), which involves the use of a whole blood sample. In general, plasma was harvested by the two-step centrifugation method at 4^{0}C. In the first step, samples were centrifuged at 1600×*g* for 10 minutes (Stovall Legend RT Centrifuge, Cat. No. 40647502; Thermo Scientific), and during the next stage, the separated plasma was re-centrifuged at 16000×*g* for 10 mins (Eppendorf Centrifuge 5415R, Cat. No. 0016957). After two-step centrifugation, the plasma samples were frozen for Trial 1 and

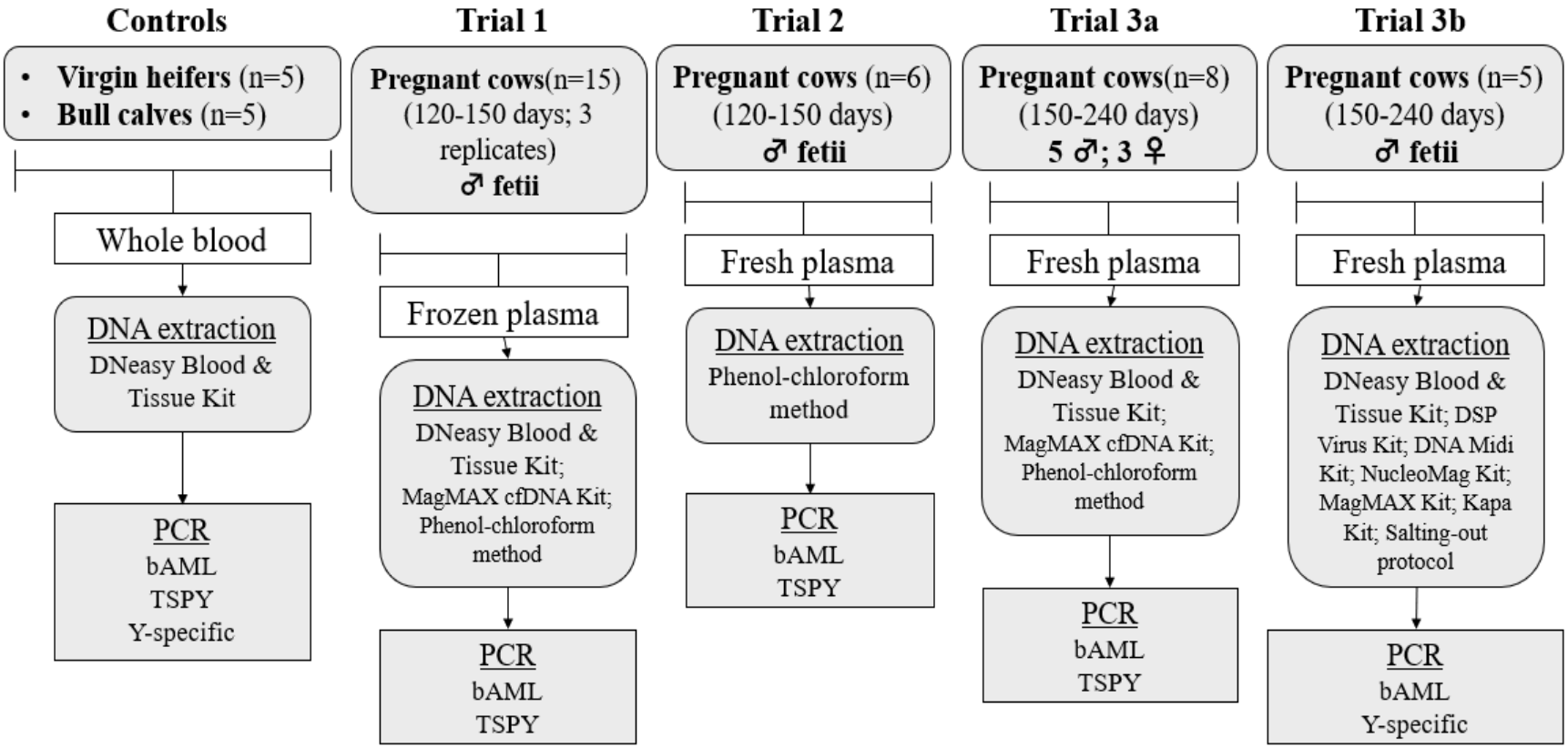


Figure 2.1*: Trials 1, 2, 3a, and 3b experimental design.* The extracted DNA from control animals was used for all trials listed above.

stored at -20^{0}C, while fresh plasma samples were used for other Trials. Samples from heifers and bull calves were used to extract DNA from blood leukocytes during the Trial 1 and then aliquoted for later use.

2.3.4. DNA extraction and quantification

The DNA extraction methods used in different trials are listed below.

Trial 1 (frozen plasma samples): During the initial phases of this experiment, DNA extraction from the frozen-thawed plasma samples of cows was performed using 100-1000 µl plasma using DNeasy Blood and Tissue Kit (Cat. No. 69506; Qiagen, Germany), 100-1000 µl plasma utilizing MagMAX cfDNA Isolation Kit (Cat. No. A29319; Applied Biosystems, USA), and 2 ml plasma using Phenol-chloroform method. Protocols provided by the manufacturer for both the commercial kits were modified to adjust for the volumes of plasma and the respective buffers. For DNeasy Blood and Tissue Kit, briefly, plasma was mixed with proteinase K and buffer AL. Following incubation at 56°C for 30 minutes, 100% ethanol was added, and samples were pipetted into the spin columns. After centrifugation for 1 minute, flow-through was discarded and two washings were performed using buffers AW1 and AW2. The DNA was eluted in 50 µl of elution buffer. For MagMAX cfDNA Isolation Kit, briefly, plasma was mixed with proteinase K and SDS. Following incubation at 60°C for 20 minutes, DNA lysis/binding solution and magnetic beads were added. After vigorous shaking for 10 minutes, one washing with the wash solution and two subsequent washings with 80% ethanol were performed. Lastly, DNA was eluted in 50 µl of elution buffer. For the phenol-chloroform-based DNA extraction, 2 ml of plasma was mixed with 50 µl of 20 mg/ml proteinase K and 100 µl of 25% SDS. The mixture was then incubated at 56°C for 3 hours following the addition of ultrapure 2 ml 25:24:1 phenol:chloroform:isoamyl alcohol (Cat. No. 77617; Sigma Aldrich, USA). The mixture was then vortexed for 30 seconds and centrifuged at 3500×*g* for 15 minutes (Stovall Legend RT Centrifuge). The supernatant was transferred to a fresh tube and an equal volume of 24:1 chloroform:isoamyl alcohol (Cat No. 25666; Sigma Aldrich, USA) was added. The mixture was again vortexed for 30 seconds and centrifuged at 14000×*g* for 10 minutes (Eppendorf Centrifuge 5415R). The supernatants were carefully transferred to clean 1.5 µl microcentrifuge tubes (Cat. No. MCT-150-L-C; AXYGEN INC., Mexico) and 1/10 volume of 5 M NaCl and two volumes of 100% ethanol were added. The mixture was incubated at -20°C for half an hour and later centrifuged at 14000×*g* for 10 minutes. The supernatant was discarded

without disturbing the pellet and the precipitated DNA was then washed twice using 70% ethanol. The DNA pellet was then air-dried and deposited with 50 µl of TE buffer. The cellular DNA of blood samples from heifers and calves were extracted using DNeasy Blood & Tissue Kit as per the manufacturer's protocol.

Trial 2 (cfDNA extraction from fresh plasma): The cfDNA was extracted using the phenol-chloroform method as described above.

Trial 3 (comparison of extraction methods). During the first part of this trial (3a), cfDNA was extracted on the same day with DNeasy Blood and Tissue Kit, MagMAX cfDNA Isolation Kit, and phenol-chloroform method using 300 µl, 500 µl, and 2000 µl, respectively, for each of the freshly harvested plasma samples.

Finally, Trial 3b involved DNA extractions using 300 µl plasma for DNeasy Blood & Tissue Kit, 500 µl plasma for DSP Virus Kit (Cat. No. 60704; Qiagen, Germany), 2 ml plasma for DNA Blood Midi Kit (Cat. No. 51183; Qiagen, Germany), 2 ml plasma for NucleoMag cfDNA Isolation Kit (Cat. No. 744550.1; Macherey-Nagel, Germany), 500 µl plasma for MagMAX cfDNA Isolation Kit, and 100 µl plasma utilizing KAPA Express Extract Kit (Cat. No. KK7100; KAPA Biosystems, South Africa) as per the manufacturer's protocols. For DSP Virus Kit, plasma was mixed with protease and lysis buffer. Following incubation at 56°C for 15 minutes, 100% ethanol was added, and the mixture was pipetted into MinElute column. Following centrifugation, two washings were performed with buffers AW1 and AW2. After this, another washing was performed with 100% ethanol and membrane of the spin column was air dried. The DNA was then eluted in 50 µl of elution buffer and immediately used as a template in the PCR. For DNA Blood Midi Kit, the extraction protocol was the same as previously described for DNeasy Blood & Tissue Kit. For NucleoMag cfDNA Isolation Kit, plasma samples were mixed with proteinase K and Buffer MCF1. Following incubation at 56°C for 30 minutes, magnetic beads and buffer MCF2 were added. After shaking the mixture for 10 minutes, one washing with buffer MCF3 and two washings with buffer MCF4 were performed. Lastly, magnetic beads were air dried, and DNA was eluted in 50 µl of elution buffer. For KAPA Express Extract Kit, plasma was mixed with KAPA Express Extract Buffer and KAPA Express Extract Enzyme. The mixture was then incubated at 75°C for 10 minutes (Lysis) followed by 95°C for 5 minutes (Enzyme activation). After this, centrifugation

was performed and 50 µl of the purified supernatant was collected, which contained the potential DNA. Additionally, whole blood samples from the same set of animals were used over plasma as per the salting-out protocol described in an earlier study (Lemos et al., 2011). Briefly, 450 µl of lysis buffer (sucrose 0.32 M, Tris–HCl 12 mM pH 7.5, MgCl2 5 mM, 1% Triton 100) was mixed with 1 ml of blood samples in an Eppendorf tube and centrifuged. Subsequently, the supernatant was discarded, and 1 ml of lysis buffer was added to the cellular debris and mixed. Again, centrifugation was performed, and this step was repeated 3-4 times until a clean pellet was obtained. The pellet was then resuspended in 80 µl of Proteinase K buffer (0.375 M NaCl; 0.12 M EDTA), and 280 µl of Milli-Q water, 10 µl of 20% SDS, and 8 µl of Proteinase K (25 mg/mL) were added to it. Samples were then incubated overnight (55°C). The following day, samples were kept at 4°C for 10 minutes, and then 120 µl of NaCl (5 M) was added to each sample, followed by centrifugation at 5000×*g* for 10 minutes (Eppendorf Centrifuge 5415R). The supernatant was transferred to clean Eppendorf tubes, 1 ml of absolute ethanol was added, and the samples were frozen at −85°C for 20 min. After removal from the freezer, samples were centrifuged again (10000×*g* for 10 minutes, Eppendorf Centrifuge 5415R), supernatants were discarded, and the pellets were washed in 70% ethanol. The pellets were allowed to air-dry, and the DNA was eluted in 50 µl of PCR grade water.

DNA quantification: The amount of cfDNA extracted from all samples obtained for all trials was assessed using a Nanodrop spectrophotometer (Cat. No. ND-1000 UV-Vis; Thermo Fisher Scientific, USA). Further, the cfDNA extracted from samples collected under Trial 3a were processed using Invitrogen Qubit dsDNA BR Assay Kit (Cat. No. Q32850; Thermo Fisher Scientific, USA) as per the manufacturer's protocol and analyzed with Qubit 3.0 Fluorometer (Cat. No. Q33216; Thermo Fisher Scientific, USA). Additionally, representative samples (positive and negative for male fetus-specific PCR amplicons) from Trial 3a were assessed for DNA size distribution using Agilent 2100 bioanalyzer (Cat. No. G2939A; Agilent Technologies, USA). For this purpose, High sensitivity DNA chips (Cat. No. 5067-4626; Agilent Technologies, USA) were used.

2.3.5. Polymerase chain reaction

Three pairs of primers from previously published studies on PCR-based bovine fetal sex determination were selected (Table 2.1). In PCR, the bovine amelogenin (bAML) primer set

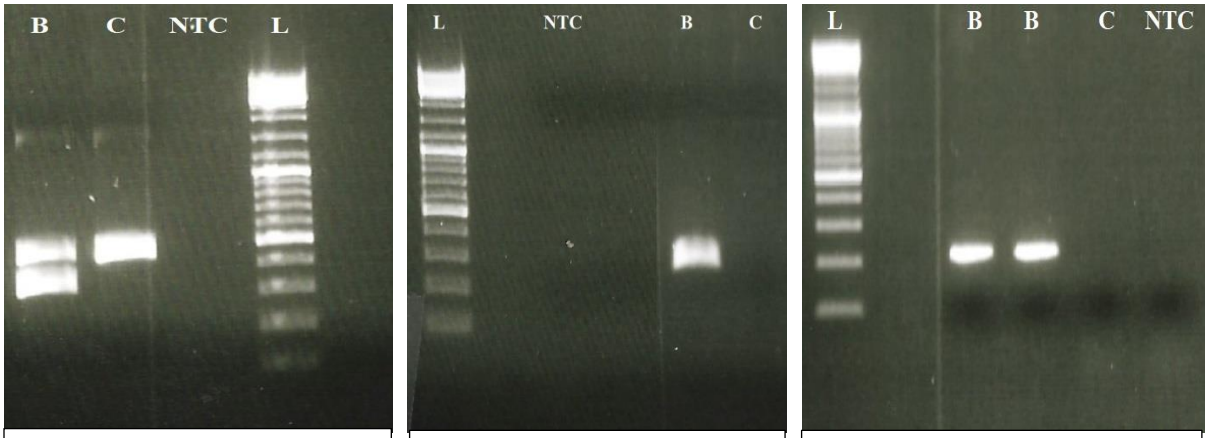
amplified a 467 bp sequence from X-chromosome and a 341 bp sequence from Y-chromosome. Thus, the amplification of both sequences in the PCR indicated a male fetus, whereas the amplification of only 467 bp sequence indicated a female fetus. The testis-specific protein Y (TSPY) primer set amplified a 260 bp sequence from Y-chromosome only. Therefore, its amplification in the PCR indicated a male fetus while its absence indicated a female fetus. Similarly, Y-specific sequence primer set amplified a 210 bp sequence from Y-chromosome only. The amplification of this sequence in the PCR indicated a male fetus, while its absence indicated a female fetus. A representative gel image following PCR with control DNA samples for each of these three genes is presented in Figure 2.2.

The PCR reactions were performed in a 25 µl volume containing 1 µl (10 µM) of each forward and reverse primer, 0.5 µl (10 µM) dNTPs, 0.75 µl (37.5 mM) $MgCl_2$, 2.5 µl 10x PCR buffer, 0.5 µl of Platinum Taq DNA polymerase. To this, either 2.5, 5, or 10 µl of DNA extracts were added as a template in each PCR reaction as indicated in the results under different trials. More specifically, 2.5, 5, and 10 µl of template DNA volumes were used in Trials 1 and 2. In Trial 3a, 2.5 and 10 µl of template DNA volumes were used. In Trial 3b, only 10 µl of template DNA volume was used. The remaining volume was compensated by adding PCR-grade water to the reaction mixture, making a final volume of 25 µl. However, 2 µl of the DNA extracts (from the blood cell fraction of control animals) were used as templates in the PCR controls, and the remaining volume (16.75 µl) was compensated by adding PCR-grade water to the reaction mixture, making a final volume of 25 µl. A non-template control (NTC) was included in every PCR batch to account for the cross-contamination of PCR tubes.

The PCR was performed using Eppendorf Master Cycler (Model: Pro 6321; Cat. No. 6321AK613475). The thermocycling protocol for Y-specific sequence PCR comprised of an initial DNA denaturation step at 94°C for 5 minutes followed by 40 cycles of DNA denaturation at 94°C for 60 seconds, primer annealing at 58°C for 30 seconds, extension at 72°C for 60 seconds, and a final extension step at 72°C for 7 minutes. On the other hand, the thermocycling protocol for bAML and TSPY PCR comprised of an initial denaturation step at 94°C for 5 minutes followed by 40 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing (at 54°C for 30 seconds for bAML; at 60°C for 30 seconds for TSPY), extension at 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes.

Table 2.1: The pairs of three different primers used for PCR.

Genes	Sequence	Primers	PCR product (bp)	Reference
Bovine amelogenin (bAML)	5′–AAATTCTCTCACAGTCCAAG–3′ 5′–CAACAGGTAATTTTCCTTTAG–3′	Forward Reverse	467 (X) 341 (Y)	(Davoudi et al., 2011; Ristanic et al., 2018)
Testis-specific protein Y (TSPY)	5′–CCCGCACCTTCCAAGTTGTG–3′ 5′–AACCTCCACCTCCTCCACGATG–3′	Forward Reverse	260 (Y)	(Davoudi et al., 2011; Ristanic et al., 2018)
Y-specific primers	5′-CCTCTTCCCGTTCAAACGCCCGGAATCATT-3′ 5′-TGCGTTGCAGGGACTGAGACCAGGTTTGGG-3′	Forward Reverse	210 (Y)	(Da Cruz et al., 2012)



2.2a (bAML) | **2.2b** (TSPY) | **2.2c** (Y-specific)

Figure 2.2: *A representative image of gel electrophoresis following bAML, TSPY, and Y-specific gene PCR. Lane 'B' represents amplification of two gene sequences in bAML PCR (341 bp from Y-chromosome and 467 bp from X-chromosome), 260 bp gene sequence in TSPY PCR, and 210 bp gene sequence in Y-specific PCR from the DNA isolated from the whole blood of a bull calf. Lane 'C' indicates amplification of a single 467 bp (X-chromosome) gene sequence in bAML PCR, no amplification in TSPY and Y-specific PCR when DNA isolated from the blood of heifer calf was used for PCR. Lane 'L' indicates a 100 bp DNA ladder while 'NTC' indicates non-template control.*

2.3.6. Gel electrophoresis

Following PCR, the amplicons were visualized on a 1% Ultrapure Agarose (Cat. No. 16500100; Invitrogen, USA) gel stained by 2 µl (20 µg) Ultrapure Ethidium Bromide (Cat. No. 15585011; Invitrogen, USA). The agarose gels containing Y-specific and TSPY PCR products were run at 110 V for 30 minutes, whereas gels containing bAML PCR products were run at 110 V for 45 minutes. Following electrophoresis, the gel images of the PCR products were captured using the ChemiDoc XRS+ system with Image lab software (Cat. No. 1708265; BIO-RAD, USA).

2.3.7. DNA sequencing of the PCR products

The PCR products that showed positive amplification of fetal sex chromosome-specific genes and PCR products of positive controls were subjected to Sanger sequencing to determine the nucleotide sequence of the amplified DNA. The 260 bp TSPY and 210 bp Y-specific PCR products were purified using QIAquick PCR Purification Kit (Cat. No. 28104; QIAGEN, Germany) as per the manufacturer's protocol for this purpose. On the other hand, bAML double bands (341 and 467 bp) were subjected to gel purification using QIAquick Gel Extraction Kit (Cat. No. 28706X4; QIAGEN, Germany) and prepared for the sequencing separately. The samples prepared for DNA sequencing were sent to Macrogen in Seoul, South Korea. The ABI file extensions received back from Macrogen were analyzed for quality using Pregap-4 and Gap-4 software (Staden package). After that, the DNA sequences were saved as FASTA files and were blasted in the NCBI database.

2.3.8. Data handling and analyses

The data generated from DNA quantification and PCR has been presented in a descriptive tabulated manner. No statistical comparisons were performed for the endpoints described in this chapter.

2.4. Results

2.4.1. cfDNA and cffDNA in frozen plasma (Trial 1)

None of the frozen plasma samples from three replicates processed using three different DNA extraction methods yielded a positive result for either bAML or TSPY amplicons expected with the presence of cffDNA from a male fetus. Even after increasing the amount of plasma sample volumes (100 to1000 µl) or amount of extracted DNA used as PCR template (2.5, 5, and 10 µl),

results did not change (data not presented). All samples from all replicates using Blood and Tissue kit and MagMax cfDNA kits showed successful amplification of 467 bp bAML amplicons, indicating cfDNA of maternal origin. However, products representing maternal cfDNA could not be amplified following PCR in all samples processed with the phenol-chloroform method.

2.4.2. cfDNA and cffDNA in fresh plasma (Trial 2)

When DNA extracted from freshly processed plasma samples were used at different volumes (2.5, 5, and 10 µl) as PCR templates in three separate reactions targeting both bAML and TSPY genes, none of the samples showed amplicons for TSPY gene. However, we observed 341 bp PCR products for the bAML gene sequence (consistent with cffDNA from a male fetus) in 4/6, 3/6, and 5/6 samples when 2.5, 5.0, and 10 µl of DNA extracts were used as PCR templates, respectively (Figure 2.3). Moreover, 3/6, 2/6, and 5/6 samples showed 467 bp amplicons consistent with maternal cfDNA when 2.5, 5.0, and 10 µl of DNA extracts were used as PCR templates, respectively (Figure 2.3).

2.4.3. Comparison of cfDNA extraction methods for fetal sex determination (Trials 3a and 3b)

The amount of cfDNA extracted from different samples using different methods was observed to be either very low or below the detection limits of the Qubit fluorometer (Table 2.2).

Table 2.2: Quantity of cfDNA detected in samples processed with different DNA extraction methods.

Cow ID	dsDNA concentration (ng/µl)		
	DNeasy Blood & Tissue Kit	MagMAX cfDNA Isolation Kit	Phenol-chloroform method
1	Too low	Too low	0.285
2	Too low	Too low	0.260
3	Too low	Too low	0.157
4	Too low	Too low	0.257
5	Too low	Too low	0.067
6	Too low	Too low	0.395
7	Too low	Too low	0.257
8	Too low	Too low	0.466

Qubit Fluorometer was used for DNA quantification.

Following PCR using cfDNA extracted (with DNeasy Blood & Tissue Kit, phenol-chloroform method) using fresh plasma, we did not observe amplification for the TSPY gene with 2.5 µl and

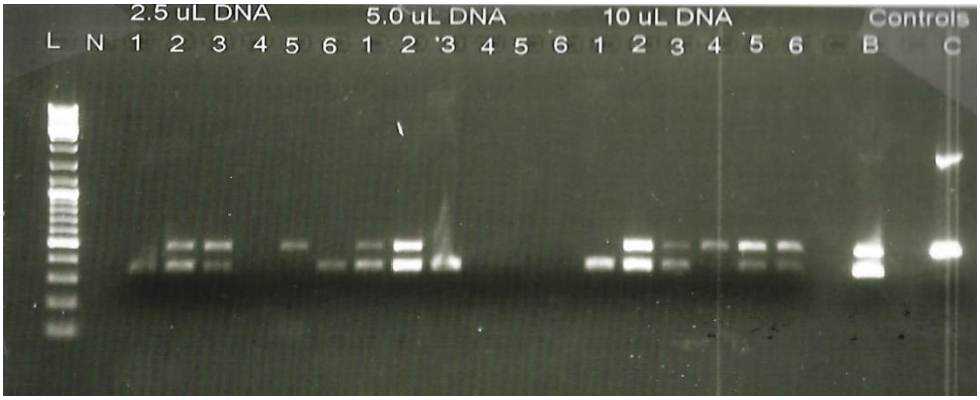


Figure 2.3: *bAML PCR results following phenol-chloroform based DNA extraction from freshly harvested plasma samples of six pregnant cows. The cows were 120-150 days pregnant and carrying male fetii. DNA extracted from 2 ml of plasma was eluted in 50 µl of TE buffer and PCR was carried out in triplicate using 2.5, 5, and 10 µl of template DNA. 'L': 100 bp DNA ladder; 'N': Non-template control; '1-6': presence/absence of 341 bp and/or 467 bp bAML gene sequences in the DNA samples from pregnant cows; 'B' and 'C' indicate bull and heifer PCR controls, respectively.*

10 µl of DNA templates in the PCR mix. However, 1/8 and 2/8 samples yielded positive TSPY amplification when 2.5 µl and 10 µl, respectively, of the DNA extracted by MagMAX cfDNA Isolation Kit were used as PCR templates (Table 2.3; Figure 2.4c). Further, one out of two samples that predicted a male fetus by TSPY PCR was identified as a false positive based on fetal sex determination by ultrasonography and fetal sex at birth (Table 2.3). Variations were also observed for bAML PCR between three extraction methods depending on the amount of PCR template used. None of the samples extracted by DNeasy Blood & Tissue and MagMAX cfDNA kits yielded 341 bp bAML PCR product when 2.5µl template was used (Figures 2.4a and 2.4b), while 2/8 samples processed with phenol-chloroform method showed the presence of male fetal-specific 341 bp amplicon. On the other hand, 3/8 (Figure 2.4a), 3/8 (Figure 2.4b), and 1/8 samples resulted in a bAML PCR product (341 bp), indicating the presence of a male fetus with 10 µl of template obtained by DNeasy Blood & Tissue kit, MagMAX cfDNA kit, and phenol-chloroform method, respectively. Additionally, one positive 341 bp amplification in each of the PCR batches from the DNA extracts of three DNA extraction methods was found to be false positives as female calves were born to those dams (Table 2.3).

Further, the Agilent High Sensitivity DNA assay for size distribution analyses on representative DNA samples obtained from all three DNA extraction methods (that showed successful amplification in bAML and/or TSPY gene PCR) was unsuccessful (Figure 2.5). These observations again indicated that the cfDNA concentration in the plasma DNA extracts might be too low and could be beyond the detection limits of these techniques despite the successful PCR amplification. In contrast, only one DNA extract from the phenol-chloroform method (PC-8 in Figure 2.5) showed a little 980 bp peak with only 3.8 pg/µl DNA concentration; however, the DNA template from this extract failed to amplify in the PCR (phenol-chloroform extract from cow ID-8 in Table 2.3).

Table 2.3: PCR (bAML) results for fresh plasma samples from cows (n=8, 150-240 days pregnant) following DNA extraction with three different methods.

Cow ID		DNeasy Blood & Tissue Kit	MagMAX cfDNA Isolation Kit	Phenol-chloroform method	Fetal sex at birth
1	341 bp	+	-	-	F
	467 bp	-	+	-	
	Predicted Sex	M	F	-	
2	341 bp	+	+	+	M
	467 bp	+	+	-	
	Predicted Sex	M	M	M	
3#	341 bp	-	+	+	F
	467 bp	+	+	+	
	Predicted Sex	F	M	M	
4	341 bp	+	-	-	M
	467 bp	+	+	-	
	Predicted Sex	M	F	-	
5	341 bp	-	-	-	M
	467 bp	+	+	+	
	Predicted Sex	F	F	F	
6	341 bp	-	+	-	M
	467 bp	+	+	+	
	Predicted Sex	F	M	F	
7	341 bp	-	-	-	F
	467 bp	+	+	+	
	Predicted Sex	F	F	F	
8#	341 bp	-	-	-	M
	467 bp	-	+	-	
	Predicted Sex	-	F	-	

'#' indicates successful amplification of 260 bp TSPY amplicon in the respective DNA extract of MagMAX cfDNA Isolation Kit.

'F' and 'M' indicate female and male fetal sex, respectively.

'+' and '–' indicate the presence and absence, respectively, of gene amplicon in PCR.

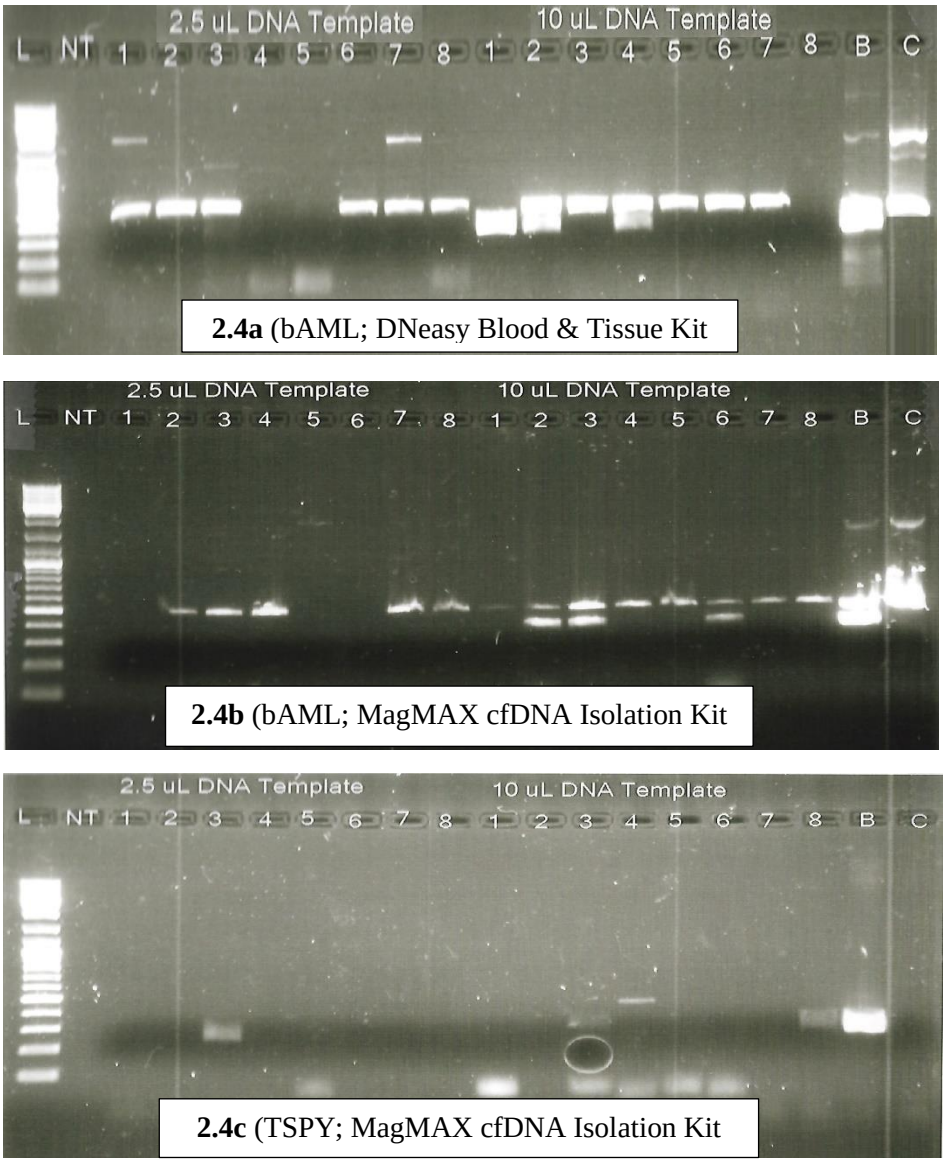


Figure 2.4: *bAML (DNeasy Blood & Tissue Kit; MagMAX cfDNA Isolation Kit) and TSPY (MagMAX cfDNA Isolation Kit) PCR results following DNA extraction from freshly harvested plasma samples of eight pregnant cows. The cows were more 150-240 days pregnant (5 carrying male fetii, 3 carrying female fetii as indicated in Table 2.3). Following extraction, DNA was eluted in 50 µl of elution buffer and both bAML and TSPY PCR were carried out in duplicate using 2.5 and 10 µl of template DNA volume. 'L': 100 bp DNA ladder; 'NT': Non-template control; '1-8': presence/absence of 341 bp and/or 467 bp bAML as well as 260 bp TSPY gene sequences in the DNA samples from pregnant cows; 'B' and 'C' indicate bull and heifer PCR controls, respectively.*

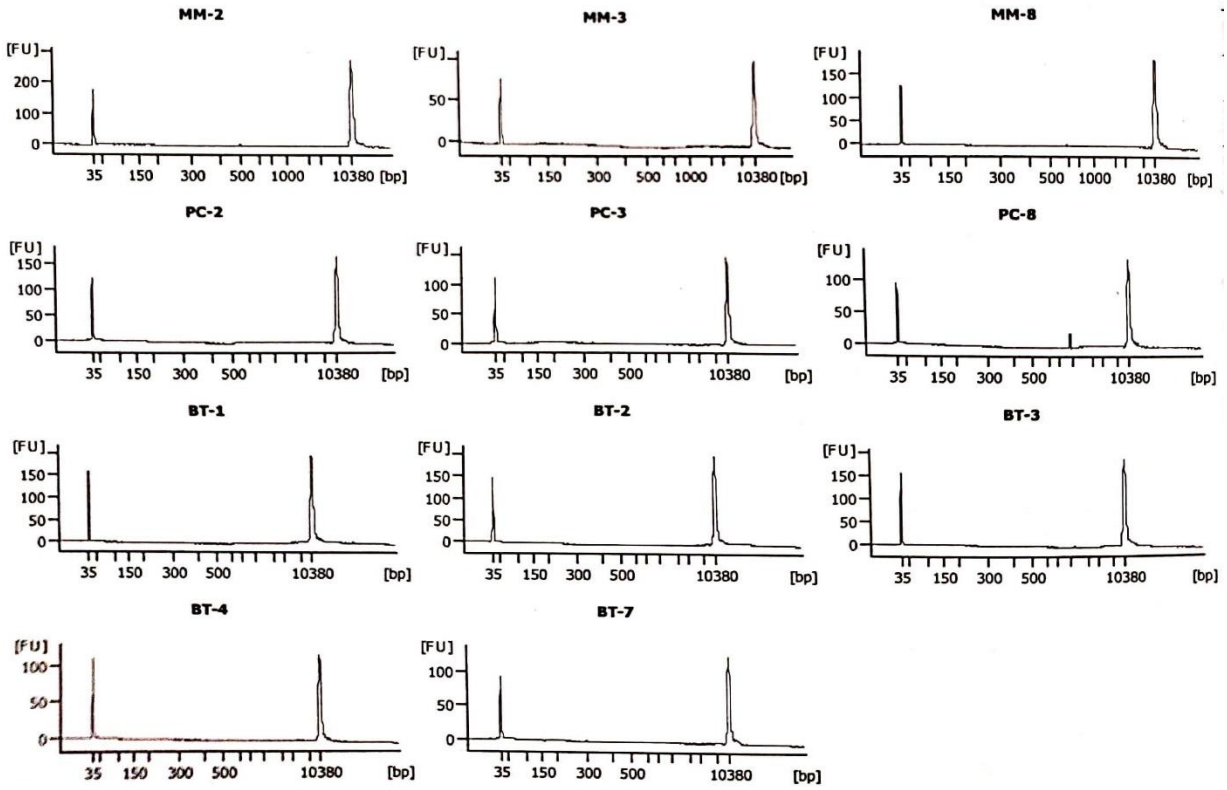


Figure 2.5: *Agilent High Sensitivity DNA assay depicting the size range of DNA in the plasma DNA extracts of three DNA extraction methods. The X-axis indicates the length of DNA fragments in base pairs (bp) with a 35 bp peak indicating 'low DNA marker' and 10380 bp peak indicating 'high DNA marker'. The Y-axis indicates the fluorescence units (FU). MM: MagMAX cfDNA Isolation Kit; PC: Phenol-chloroform method; BT: DNeasy Blood & Tissue Kit.*

The results of PCR (bAML and Y-specific) for 10 µl of cfDNA template obtained using seven different methods are shown in Table 2.4. Among the spin-column-based DNA extraction techniques, the PCR on DNA isolated from fresh maternal plasma using the DNeasy Blood & Tissue and QIAamp DSP Virus kits correctly predicted the presence of male fetii in 2/5 and 3/5 cows (Figures 2.6a and 2.6b), respectively. On the other hand, PCR on the cfDNA obtained from QIAamp DNA Midi Kit (Figures 2.6a and 2.6b) and single tube extraction kit (KAPA express extract kit) failed to predict the presence of male fetii in all five cows. Furthermore, PCR on the DNA isolated using magnetic beads-based techniques (NucleoMag cfDNA Isolation and MagMAX cfDNA Isolation kits) correctly predicted the presence of male fetii in 2/5 cows each. The PCR on DNA obtained from the Salting out protocol correctly predicted the presence of male fetii in 2/5 cows. However, the PCR lanes showed smearing on the agarose gel (data not shown).

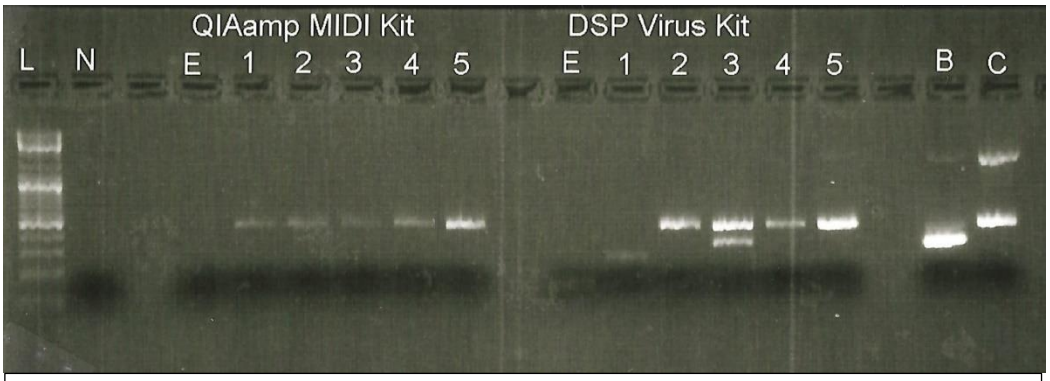
2.4.4. DNA sequencing results

Sequencing results of the PCR products of 341 bp bAML amplicons from both bull controls and test samples showed that the amplified products were part of the Amelogenin-Y gene of *Bos taurus,* showing 94-100% similarity to the bovine bAML gene sequence. Similarly, 467 bp bAML amplicons were found to be part of the Amelogenin-X gene of *Bos taurus* showing 96-100% similarity to the actual gene sequence. Furthermore, sequencing results of 260 bp TSPY amplicons from both bull controls and test samples showed that the products amplified in the PCR were part of the Testes specific protein-Y gene of *Bos taurus,* showing 93-100% similarity to the true gene sequence. Likewise, 210 bp Y-specific sequence PCR products from both bull controls and test samples were found to be the part of *Bos taurus* Y-chromosome showing 90-100% similarity to the actual gene sequence.

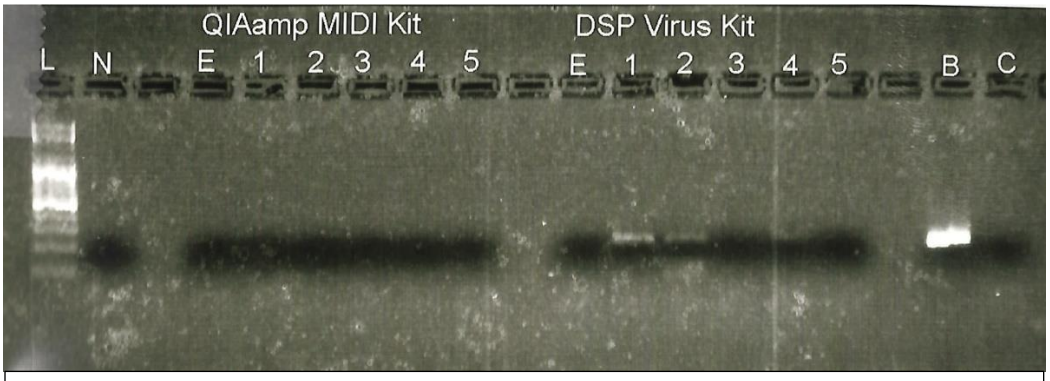
Table 2.4: PCR results of the cfDNA isolated by seven different extraction methods.

Cow ID	Gene amplicons	Spin-column based kits			Magnetic beads-based kits		Single tube extraction kit	Manual in-house protocol
		Blood & Tissue Kit	DSP Virus Kit	DNA Midi Kit	NucleoMag Kit	MagMAX Kit	Kapa Express Extract Kit	Salting-out protocol
1	341 bp bAML	-	-	-	-	-	-	-
	467 bp bAML	+	-	+	+	+	+	+
	210 bp Y-specific	-	+	-	+	-	-	+
2	341 bp bAML	+	-	-	-	+	-	-
	467 bp bAML	+	+	+	+	+	+	+
	210 bp Y-specific	-	+	-	+	-	-	-
3	341 bp bAML	-	+	-	-	+	-	-
	467 bp bAML	+	+	+	+	+	+	+
	210 bp Y-specific	-	-	-	-	-	-	-
4	341 bp bAML	-	-	-	-	-	-	-
	467 bp bAML	+	+	+	+	+	+	+
	210 bp Y-specific	+	-	-	-	-	-	+
5	341 bp bAML	-	-	-	-	-	-	-
	467 bp bAML	+	+	+	+	+	-	+
	210 bp Y-specific	-	-	-	-	-	-	-
Correct prediction of male fetii		2	3	0	2	2	0	2

'+' and '-' indicate the presence and absence, respectively of gene amplicon in the PCR.



2.6a (bAML; QIAamp DNA Midi Kit, QIAamp DSP Virus Kit



2.6b (Y-specific; QIAamp DNA Midi Kit, QIAamp DSP Virus Kit

Figure 2.6: *bAML and Y-specific PCR results following DNA extraction from freshly harvested plasma samples of five pregnant cows utilizing QIAamp DNA Midi Kit and QIAamp DSP Virus Kit. The cows were 150-240 days pregnant and carrying male fetii. Following extraction, DNA was eluted in 50 µl of elution buffer and both bAML and Y-specific PCR were carried out separately using 10 µl of template DNA. 'L': 100 bp DNA ladder; 'N': Non-template control; 'E': Extraction control; '1-5': presence/absence of 341 bp and/or 467 bp bAML as well as 210 bp Y-specific gene sequences in the DNA samples from pregnant cows; 'B' and 'C' indicate bull and heifer PCR controls, respectively).*

2.5. Discussion

The present study provided initial information on developing a PCR test targeting the sex-chromosome-specific genes for use in prenatal fetal sex determination in pregnant cows. We determined that the fresh plasma would be better than frozen plasma samples to extract cfDNA and carry out PCR for fetal sexing. Further, we identified Y-specific and bAML genes to be better PCR targets for fetal sex determination. Additionally, we observed variability in the PCR results between animals and also due to different DNA extraction methods.

Previous studies in cattle have either utilized the frozen (Davoudi et al., 2011; G. Wang et al., 2010) or fresh plasma (Da Cruz et al., 2012) and reportedly achieved successful amplification of PCR products specific to the presence of male fetii in the pregnant cows. Interestingly, none of the frozen samples (of three replicates) processed for DNA extraction with different methods in the current study yielded PCR products specific to the male fetus even though the pregnant cows were carrying a male fetus. Earlier studies had shown that freeze-thaw cycles before DNA extraction could cause DNA fragmentation and compromise the integrity of cfDNA (K. A. Chan et al., 2005; El Messaoudi et al., 2013). This might have been the case in the present study with the frozen samples because the cfDNA has been observed to undergo rapid degradation (Zandvakili & Lazaridis, 2019). On the other hand, the lack of amplicons expected for maternal cfDNA for frozen samples processed with the phenol-chloroform method could be attributed to increased steps and carryover of PCR inhibitors such as large amounts of salts, proteins, and phenol in the isolated DNA samples (Lemarchand et al., 2005; Nolan et al., 2006). On the contrary, PCR on some of the fresh plasma samples did show expected amplicons consistent with the presence of a male fetus when different extraction methods were used. Overall, our observations using fresh plasma from the pregnant cattle for PCR to ascertain the fetal sex were different from previous reports that showed higher accuracy, sensitivity, and specificity for cattle >60 days pregnant (Da Cruz et al., 2012). Since the cfDNA in human circulation is highly degraded due to nuclease activity (Sanchez et al., 2018), AACC recommends that plasma harvested from blood must immediately be cooled to refrigeration temperature, and if cfDNA extraction has to be further delayed, it must be stored between -20 to -80°C to minimize ex-vivo nuclease activity (Meddeb et al., 2019). In our case, even when fresh plasma samples were used for DNA extraction with different methods, the accuracy of fetal sexing was lesser. This might be because of the variability of cfDNA concentrations between subjects as reported in humans (Wright & Burton, 2009), although no such

study has been carried out in cattle. Another explanation could be the variable efficacies of different DNA extraction methods to isolate cffDNA from maternal plasma or blood as reported in cattle (Davoudi et al., 2011; Ristanic et al., 2018). To date, no study has compared the fresh and frozen plasma for DNA extraction and PCR for fetal sexing in cows. We used both fresh and frozen plasma samples from pregnant cows in different experimental trials to determine the appropriate sample type for fetal DNA extraction. After ascertaining that fresh plasma was better than frozen, we performed DNA extraction utilizing fresh plasma samples from pregnant cows (using several DNA extraction methods) following PCR targeting bAML and TSPY/Y-specific genes.

In our trial comparing three different DNA extraction methods with fresh plasma samples from pregnant cows, we found that only a couple of DNA extracts obtained by the MagMAX cfDNA Isolation kit yielded amplicons specific to TSPY in the PCR while all the other samples came out TSPY negative. Although male fetal bAML amplicons in the PCR were detected in several DNA extracts obtained by all three methods, lack of amplification in TSPY PCR could be because of the lower sensitivity of these primers compared to bAML primers as different primers have been found to show variable sensitivities in detecting a particular target (Marcon et al., 2011). Another explanation could be the variability of various DNA extraction methods in extracting the fetal DNA fragments from the plasma of pregnant cows (Davoudi et al., 2011; Ristanic et al., 2018). Our findings are contrary to earlier studies that have reported consistent amplification of male fetal DNA in the PCR targeting the TSPY gene using the same set of primers (Davoudi et al., 2011; Lemos et al., 2011).

Within the limitations of a smaller number of samples included in our trials comparing three and seven different DNA extraction methods periodically, we observed wide variations in the PCR results for bAML and TSPY/Y-specific genes, which are similar to some reports in cattle (Davoudi et al., 2012; Ristanic et al., 2018). Since the proportion of cffDNA is extremely low in maternal circulation (3.4-6.2%) as reported in women (Dennis Lo, Tein, et al., 1998), larger yields of maternal cfDNA inhibit the retention of shorter fetal DNA sequences during extraction (Suzuki et al., 2008). Furthermore, it has been reported that the proportion of cfDNA is the only factor that determines the success of cfDNA based diagnostic assays (Canick et al., 2013). Therefore the extremely low fetal fraction in maternal blood of pregnant women is the chief culprit of negative assay results (Lench et al., 2013). This might be true in our case because the DNA extracts had extremely low DNA concentrations that were beyond the Qubit fluorometer's detection limits. The

failure of amplification of male fetus-specific sequences in bAML and TSPY/Y-specific PCR might be attributed to the fact that the quality and quantity of DNA templates can significantly impact the accuracy, sensitivity, and reliability of the PCR-based methods (Nolan et al., 2006). Moreover, we observed a variation in the comparative efficacy of different extraction methodologies in isolating cffDNA from the maternal plasma, which is congruent to other studies carried out in humans (Akbariqomi et al., 2019) and cows (Davoudi et al., 2011; Ristanic et al., 2018). Differences in methodologies and underlying extraction principles for each kit might determine the extent of retention of highly fragmented fetal DNA sequences (Cicchillitti et al., 2017; Fleischhacker & Schmidt, 2007). On the other hand, some studies have indicated consistent and highly accurate results of PCR-based prenatal sexing of the fetus in cattle (Lemos et al., 2011; G. Wang et al., 2010).

Although we had a limited sample size, overall, different kits yielded wide variations in PCR results. Contrary to the previous report on the single tube KAPA Express Extract Kit to be better than both spin-column and manual in-house DNA extraction methods (Ristanic et al., 2018), we did not amplify any male fetus-specific sequence in the samples from all cows. Similarly, we had lower success (2/5 samples) with the salting-out protocol than a previous study that used the same extraction method and reported a 100% accuracy for TSPY PCR in pregnant cattle (Lemos et al., 2011). The use of whole blood might have released maternal DNA that caused the background smearing in the gel images in the present study.

2.6. Conclusions

In conclusion, the freshly harvested but not frozen-thawed plasma from pregnant cattle could be used for PCR-based prenatal fetal sexing. The fresh plasma samples from pregnant cows were found to be better than the frozen-thawed plasma samples in terms of their relative amplification of fetal DNA in the PCR. Although the fetal gene sequences were successfully amplified in some samples when DNA extracts from three different DNA extraction methodologies were subjected to PCR, the size distribution of these extracted cfDNA samples could not be ascertained using Agilent High Sensitivity Bioanalyzer owing to the extremely low yields of cfDNA as reported by Qubit Fluorometer. Furthermore, when compared, seven different DNA extraction methods were found to yield variable results in their ability to isolate fetal DNA from the plasma of pregnant

cows. Therefore, the current PCR methodology for fetal sexing in pregnant cattle is unreliable for field applications as the obtained results were no better than chance predictions.

2.7. Transition

The relative variation in the extraction efficiency of different DNA extraction methodologies could be because of the extremely low and variable concentrations of fetal DNA in the plasma of pregnant cows. So, chapter 3 was focused on a series of spiking experiments aimed to determine the lower limits of cffDNA that could be extracted back from plasma for successful PCR targeting sex-chromosome-specific genes.

3. DETERMINATION OF THE LOWER LIMITS OF CELL-FREE FETAL DNA IN BOVINE PLASMA SAMPLES FOR PCR DETECTION

3.1. Abstract

Objectives: The specific objectives of this study were to a) determine if spiked fetal DNA could be recovered from maternal plasma, b) ascertain the minimum concentrations of cffDNA that can be extracted from maternal plasma and amplified with PCR to identify bAML and Y-specific amplicons, c) compare the PCR results of samples taken from cows at different stages of gestation and d) compare three different commercial kits for extraction of cffDNA from spiked and neat plasma samples of pregnant cows.

Materials and methods: In the Trial 4, serum and cellular DNA from five aborted male fetii were spiked in the plasma of their respective dams at different proportions, followed by re-extraction and PCR. In the Trial 5, male fetal cellular DNA at different dilutions was spiked in the plasma of unrelated pregnant cows (n=10, 120-150 days pregnant, each carrying a male fetus) and non-pregnant heifers (n=10) followed by re-extraction and PCR. We used spiked (with 35 pg/ml male fetal DNA) and neat plasma samples from pregnant cows carrying male fetii (n=9, 60-150 days pregnant versus n=10, 151-240 days pregnant) in the Trial 6 for DNA extraction and PCR. In the Trial 7, spiked (with 35 pg/ml male fetal DNA) and neat plasma samples from pregnant cows (n=5, 181-240 days, carrying a male fetus) were used for DNA extraction using three different kits and followed by PCR. Fisher's exact test was used to compare the proportion of samples from two gestational groups with the correct prediction of fetal sex using PCR.

Results: In the Trial 4, all maternal plasma samples spiked with either the fetal serum or fetal cellular DNA at all dilution rates (0.5-100%) showed amplicons following Y-specific PCR. In the Trial 5, male fetus-specific amplicons were detected in the maternal plasma spiked with >31.25 pg/ml and >2ng/ml male fetal DNA for Y-specific PCR and bAML PCR, respectively. In the Trial 6, the proportion of cows with a positive PCR in the 60-150 days gestation group were lower ($P<0.05$) than the 151-240 days group (3/9 versus 9/10, respectively). In the Trial 7, spiked fetal DNA was recovered in all the samples processed with three kits; however, only 3/5 (Blood & Tissue Kit), 1/5 (DSP Virus Kit), and 0/5 (NucleoMag Kit) neat plasma samples yielded male fetal sequences in the Y-specific PCR.

Conclusions: Y-specific primers were more sensitive than bAML primers in amplifying lower limits of cffDNA in maternal plasma (i.e., 31.25 pg/ml). In addition, the sensitivity of fetal sexing using Y-specific PCR was higher during the second-half of the gestation but the finding needs to be further tested on a large number of samples.

3.2. Introduction

Following the discovery of cffDNA in the maternal blood circulation of pregnant women (Dennis Lo et al., 1997), research work over the next three decades determined that individual variations (Wright & Burton, 2009), stage of gestation (Dar et al., 2016), and laboratory factors (El Messaoudi et al., 2013; Meddeb et al., 2019) could affect the extraction of cffDNA. These efforts lead to the development of non-invasive prenatal testing of the unborn baby for sex-chromosome-related defects (Yao et al., 2014) and genetic disorders (Drury et al., 2016) besides fetal sexing (Kazachkova et al., 2019). Despite the presence of additional tissue layers of the interhemal membrane in ruminants compared to humans, the synepitheliochorial placenta in cattle might allow the passage of minute quantities of cffDNA in the maternal circulation of pregnant cows (P. Wooding & Burton, 2008). As a result, various studies have periodically focused on bovine prenatal fetal sex determination utilizing circulating cffDNA in maternal peripheral blood and have reported varying degrees of accuracy (Da Cruz et al., 2012; Davoudi et al., 2011; Kadokawa et al., 1996; Lemos et al., 2011; Ristanic et al., 2018; Turin et al., 2007; G. Wang et al., 2010). While the initial study demonstrated the absence of bovine fetal cells and cffDNA in the blood circulation of pregnant cows (Kadokawa et al., 1996), subsequent studies showed that fetal sex chromosome-specific genes could be targeted using PCR to determine fetal sex in pregnant cows (Da Cruz et al., 2012; Davoudi et al., 2011; Lemos et al., 2011; Ristanic et al., 2018; G. Wang et al., 2010). Our first experiment exhibited limited success and wide variability in prenatal determination of fetal sex using PCR. In pregnant mares, which have epitheliochorial placenta, the accuracy of PCR-based fetal sex determination was observed to be 48.7% and 68.7% in first and second-trimester pregnant mares, respectively (Tonekaboni et al., 2020). It has been proposed that fetal genetic material might transfer across the placenta in pregnant cattle, which could occur transiently but predominantly during parturition (Turin et al., 2007). In such a situation, the PCR results for fetal sexing would vary between cows at a similar stage of gestation and repeat samples from the same cows would yield different outcomes.

Variability in the PCR-based fetal sex determination assays in the case of pregnant cows and other domestic animals might also occur due to lower amounts of cffDNA in the maternal plasma. Studies in humans have indicated that the absence or meager amounts of cffDNA is likely the leading cause of negative assay results (Lench et al., 2013). Furthermore, these low quantities of cffDNA molecules are overshadowed by the presence of a relatively large number of maternal cfDNA molecules (Wright & Burton, 2009). Therefore, a fetal fraction of at least 4% is recommended for adequate assay results in the case of humans (Grace et al., 2016), but no such information for cattle exists in the literature.

We hypothesized that cffDNA might be low and variable in the plasma of pregnant cows, making it challenging to extract for conventional PCR. Therefore, our main objective was to determine minimum concentrations of cffDNA that can be re-extracted from maternal plasma to use for PCR. Specifically, we wanted to test the lowest limits of spiked cffDNA that can be re-extracted and amplified with PCR to identify bAML and Y-specific amplicons. Our secondary objectives were to a) determine the efficacy of the PCR-based prenatal sexing in cattle at different stages of gestation and b) compare three different commercial kits for extraction of cffDNA from spiked and neat plasma samples of pregnant cows.

3.3. Materials and methods

3.3.1. Animals and their management

In the present study, 15 non-pregnant dairy heifers (Canadian Holstein), 34 pregnant dairy cows (Canadian Holstein), and 20 pregnant beef cows (Angus) were included. Dairy cows were located at the Rayner Dairy Research and Teaching Facility and managed as mentioned in chapter 2. Beef cows were from the Livestock Centre for Forage Research and Excellence, University of Saskatchewan, and were 60-150 days pregnant following natural breeding with a bull. The beef cows were being aborted as part of a parallel metabolic study. Furthermore, five each of the virgin heifers and bull calves (Rayner Dairy Research and Teaching Facility, University of Saskatchewan) were also included in the study, and the blood samples collected from them were used for DNA extraction that served as controls in all the PCR batches. The study was approved by the University of Saskatchewan under the AUP# 20190070.

3.3.2. Sample collection from aborting cows and their aborted fetii

The abortions were induced in pregnant beef cows using 500 µg Cloprostenol sodium (Estrumate®, MSD) and 25 mg Dexamethasone sodium phosphate (Dexamethasone 5®, Vetoquinol). Cows were then observed at eight hours intervals for the expulsion of an aborted fetus. Based on vaginal discharge, rectal palpation, and stage of gestation at the induction of abortion, 17/20 aborted fetii were identified with the corresponding mothers. The aborted fetii were placed in a portable cooler and transported to the Westgen Research Suite, University of Saskatchewan. The sex of the aborted fetus was confirmed based on the examination of the external genitalia. Out of 17 aborted fetii, nine were identified to be male, and blood was successfully collected by cardiac puncture from 5/9 male fetii using 10 ml serum separator tubes (VWR Cat. No. CABD366430; BD Vacutainer). Within 24 h of abortion, duplicate blood samples were collected from the respective mothers of aborted fetii by coccygeal venipuncture in 6 ml K_2 EDTA tubes (VWR Cat. No. CABD367863L; BD Vacutainer). All collected samples from the cows and aborted fetii were immediately transported on ice to the Molecular Microbiology Research Laboratory for further processing.

3.3.3. Collection of blood samples from non-pregnant heifers and unrelated pregnant cows

Peripheral blood samples from the caudal vein were collected from 15 non-pregnant Holstein Friesian heifers and 34 pregnant Holstein Friesian cows (carrying male fetii, as described earlier) grouped in different gestational stages at varying points in time. The blood samples were collected in duplicate from each heifer/cow in 6 ml K_2 EDTA tubes (BD Vacutainer). The collected blood samples were placed in the icebox and carefully transported to the Molecular Microbiology Research Laboratory, the University of Saskatchewan, within two hours of blood collection. The blood samples were immediately processed for harvesting plasma.

3.3.4. Processing of blood samples

All the blood samples collected from aborted fetii and their dams, non-pregnant heifers, and pregnant dairy cows were immediately processed for separation of serum/plasma as explained previously (subsection 2.3.3). Fresh plasma, serum, and fetal blood cell fractions were immediately used for DNA extraction.

3.3.5. Experimental design

The current experiment included the following series of trials to fulfill the study objectives.

Trial 4: Spiking of maternal plasma from aborting cows with fetal serum and DNA

This experiment was aimed to determine the possibility of re-extraction of fetal serum/DNA spiked into the plasma of their respective mother at different proportions of v/v dilutions. For this, freshly harvested fetal serum and DNA extracted from fetal blood cell fraction from five aborted male fetii were spiked into the plasma of their respective dams in two separate experiments, as described in Table 3.1 and Figure 3.1. The final 300 µl volume of neat or spiked maternal plasma was used for DNA extraction and downstream PCR targeting Y-specific gene.

Table 3.1: Different concentrations (v/v) of fetal serum/fetal cellular DNA spiked into maternal plasma.

Maternal plasma (µl)	Fetal serum/Fetal cellular DNA (µl)	Total volume (µl)	% of fetal serum/fetal cellular DNA in maternal plasma (v/v)
300	0	300	0
298.5	1.5	300	0.5
285	15	300	5
262.5	37.5	300	12.5
225	75	300	25
150	150	300	50
0	300	300	100

Trial 5: Spiking of plasma from non-pregnant heifers and pregnant dairy cows with fetal DNA

Trial 5a: To determine the lower range of cffDNA concentrations needed for successful re-extraction and PCR, we spiked male fetal DNA in the plasma of randomly assigned non-pregnant heifers (n=5) and pregnant unrelated dairy cows (n=5, 120-150 days pregnant; carrying male fetii) as mentioned in Table 3.2. Based on the known amounts of fetal DNA (quantified by Qubit) at the initial dilution step, the estimated amount of cffDNA at subsequent dilutions is shown in Table 3.2. The final 300 µl volume of neat or spiked maternal plasma was used for DNA extraction and downstream PCR targeting bAML and Y-specific genes.

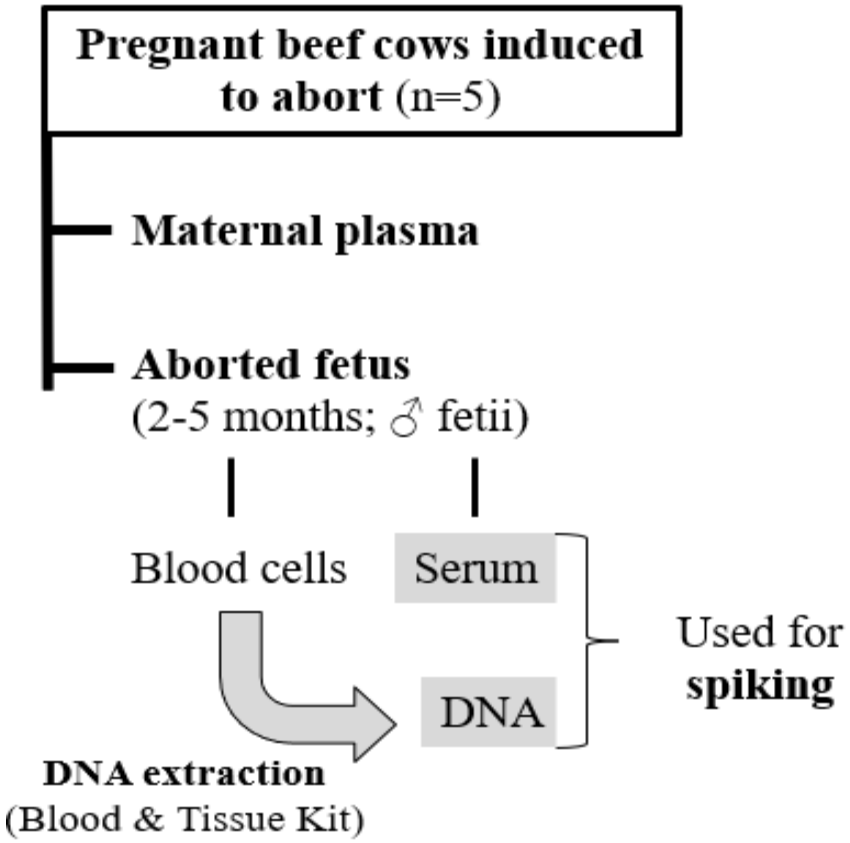


Figure 3.1: *Trial 4 experimental design.*

Table 3.2: Serial dilution rate of fetal DNA in the plasma of unrelated non-pregnant heifers (H1-H5) and pregnant cows (C1-C5: 120-150 days gestation, carrying male fetus).

v/v (%) dilution	Serial dilution	Volume of fetal DNA used for spiking of plasma	Volume of maternal plasma	Volume of spiked plasma used for DNA extraction	Known (5% v/v) and expected amounts (subsequent serial dilutions) of fetal DNA in 300 µl heifer/cow plasma (n=5 each) used for re-extraction of DNA				
					H1/C1	H2/C2	H3/C3	H4/C4	H5/C5
5	N/A	20 µl (neat)	380 µl	300	556.5 ng	2505 ng	304.5 ng	250.5 ng	525 ng
2.5×10^{-1}	$2x10^{-1}$	20 µl (5%)	380 µl	300	27.82 ng	125.2 ng	15.22 ng	12.52 ng	26.25 ng
1.25×10^{-2}	$2x10^{-2}$	20 µl (20^{-1})	380 µl	300	1.39 ng	6.26 ng	761 pg	626 pg	1.31 ng
6.25×10^{-4}	$2x10^{-3}$	20 µl (20^{-2})	380 µl	300	69.5 pg	313 pg	38.05 pg	31.3 pg	65.5 pg
3.12×10^{-5}	$2x10^{-4}$	20 µl (20^{-3})	380 µl	300	3.47 pg	15.65 pg	1.9 pg	1.56 pg	3.27 pg
1.56×10^{-6}	$2x10^{-5}$	20 µl (20^{-4})	380 µl	300	173.5 fg	782.5 fg	95 fg	78 fg	163.5 fg
7.80×10^{-8}	$2x10^{-6}$	20 µl (20^{-5})	380 µl	300	8.67 fg	39.12 fg	4.75 fg	3.9 fg	8.17 fg
3.91×10^{-9}	$2x10^{-7}$	20 µl (20^{-6})	380 µl	300	433.5 ag	1.96 fg	237.5 ag	195 ag	408.5 ag

ng : Nano grams ; **pg** : Pico grams ; **fg** : Femto grams ; **ag** : Atto grams

Trial 5b: To further determine the lowest concentration of fetal DNA needed for a successful re-extraction, a known amount of fetal DNA (2 ng) was spiked in 1000 µl plasma from a separate set of unrelated five non-pregnant heifers and five pregnant cows (120-150 days pregnant; carrying male fetii). Further dilutions were made for each set of spiked plasma so that progressive dilutions had a gradually halving fetal DNA concentration/ml of maternal plasma (Figure 3.2). The DNA re-extraction was performed from all the dilutions of each replicate and PCR was performed to amplify both Y-specific and bAML gene sequences. In addition, 300 µl of non-spiked (neat) plasma samples of each heifer/cow were also simultaneously used for DNA extraction and PCR (Figure 3.2).

Trial 6: Assessing the effect of gestation stages at sampling on prenatal fetal sexing using PCR

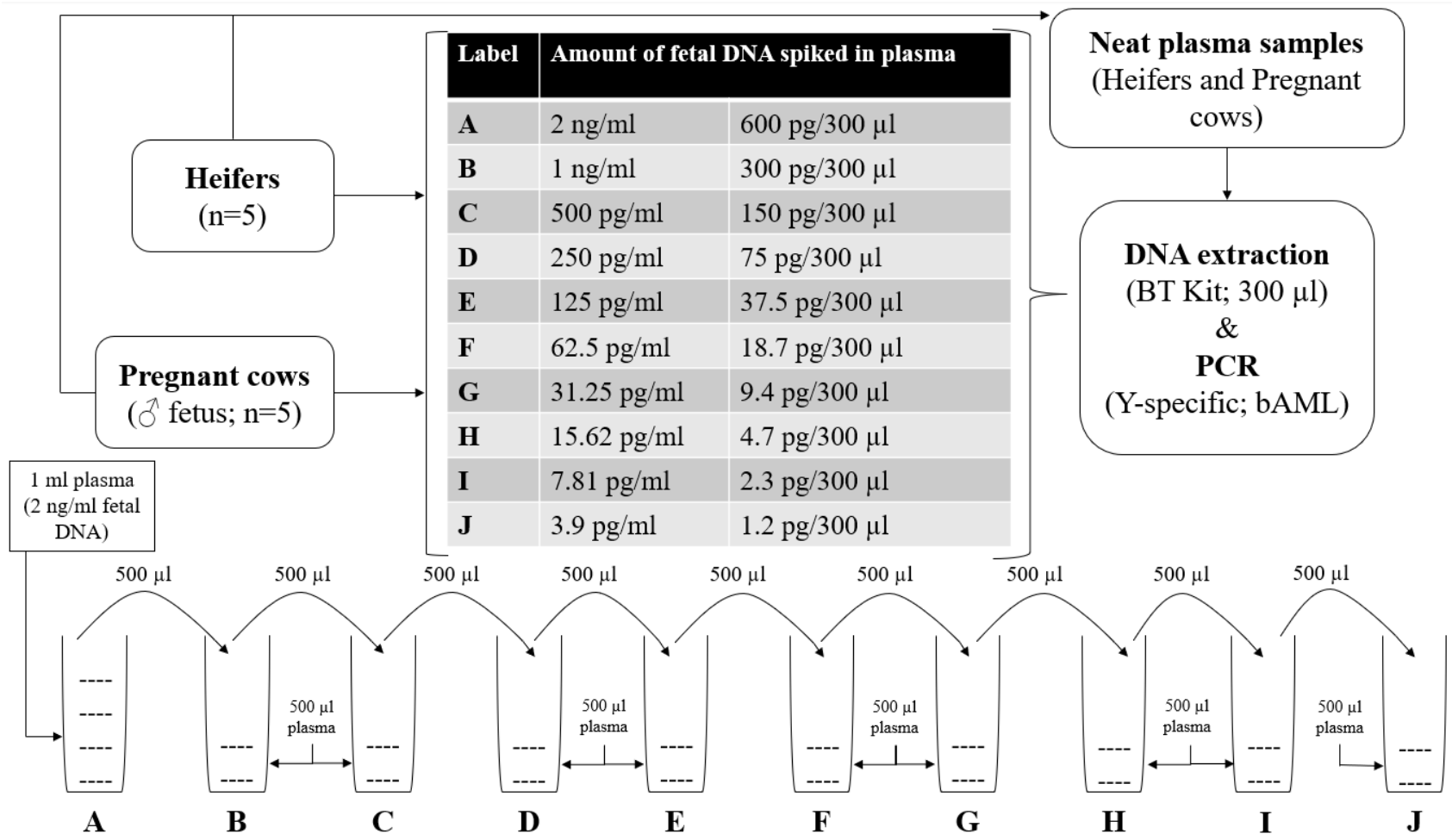
Using the minimum re-extractable concentration of fetal DNA identified in Trial 5b as our positive control, we compared the two gestational stages of pregnant cows for prenatal fetal sexing using PCR. For this trial, male fetal DNA was spiked at the lowest re-extractable concentrations in the plasma from a separate set of non-pregnant heifers (n=5), pregnant cows carrying male fetii (n=9 cows 60-150 days pregnant; n=10 cows 151-240 days pregnant carrying male fetus). The DNA extraction and PCR for Y-specific sequence were carried out on spiked and neat plasma samples of nineteen pregnant cows and five non-pregnant heifers (Figure 3.3).

Trial 7: Assessing the effect of the DNA extraction method on prenatal fetal sexing using PCR

In this trial, a separate set of pregnant cows (n=5, 181-240 days gestation carrying male fetus) were selected for blood collection. The neat plasma samples and plasma samples spiked with the lowest re-extractable concentration of male fetal DNA were used for DNA extraction using three different kits and downstream PCR for Y-specific sequence (Figure 3.4).

3.3.6. DNA extraction and quantification

The fetal blood cell fraction (10 µl) and plasma samples (300 µl, non-spiked and spiked) from Trials 4, 5, and 6 were processed for DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen). Whereas, for trial 7, DNeasy Blood & Tissue (Qiagen), QIAamp DSP Virus (Qiagen), and NucleoMag cfDNA Isolation (Macherey-Nagel) kits were used for DNA extraction from each set of samples. DNA extractions were performed as per the previously mentioned protocols for



Label	Amount of fetal DNA spiked in plasma	
A	2 ng/ml	600 pg/300 µl
B	1 ng/ml	300 pg/300 µl
C	500 pg/ml	150 pg/300 µl
D	250 pg/ml	75 pg/300 µl
E	125 pg/ml	37.5 pg/300 µl
F	62.5 pg/ml	18.7 pg/300 µl
G	31.25 pg/ml	9.4 pg/300 µl
H	15.62 pg/ml	4.7 pg/300 µl
I	7.81 pg/ml	2.3 pg/300 µl
J	3.9 pg/ml	1.2 pg/300 µl

Figure 3.2: *Trial 5b experimental design.*

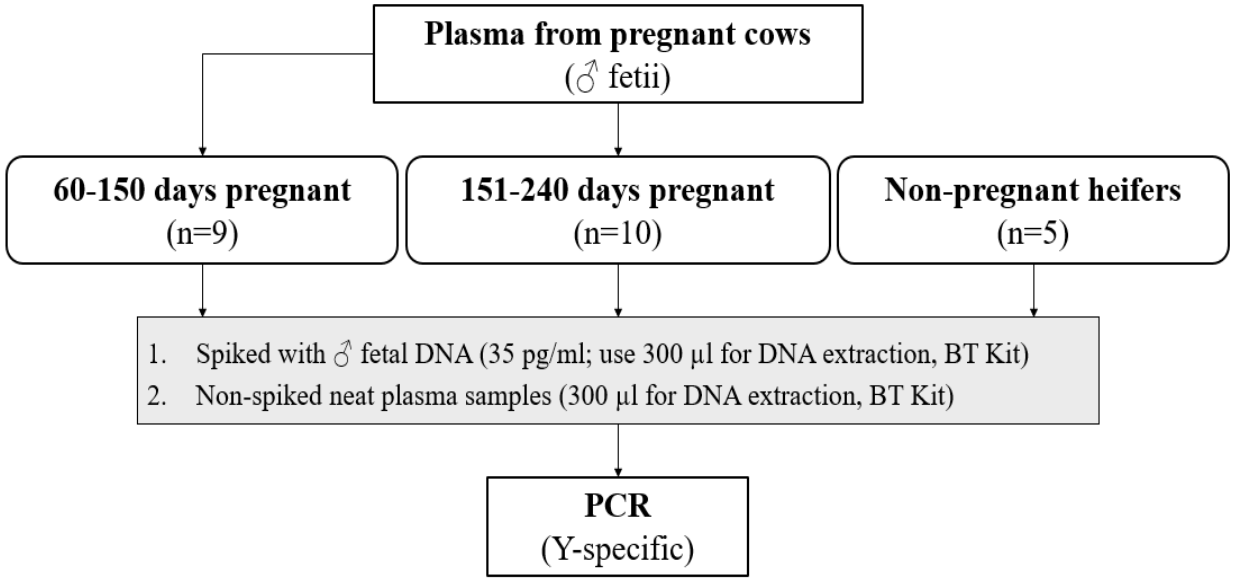


Figure 3.3: *Trial 6 experimental design.*

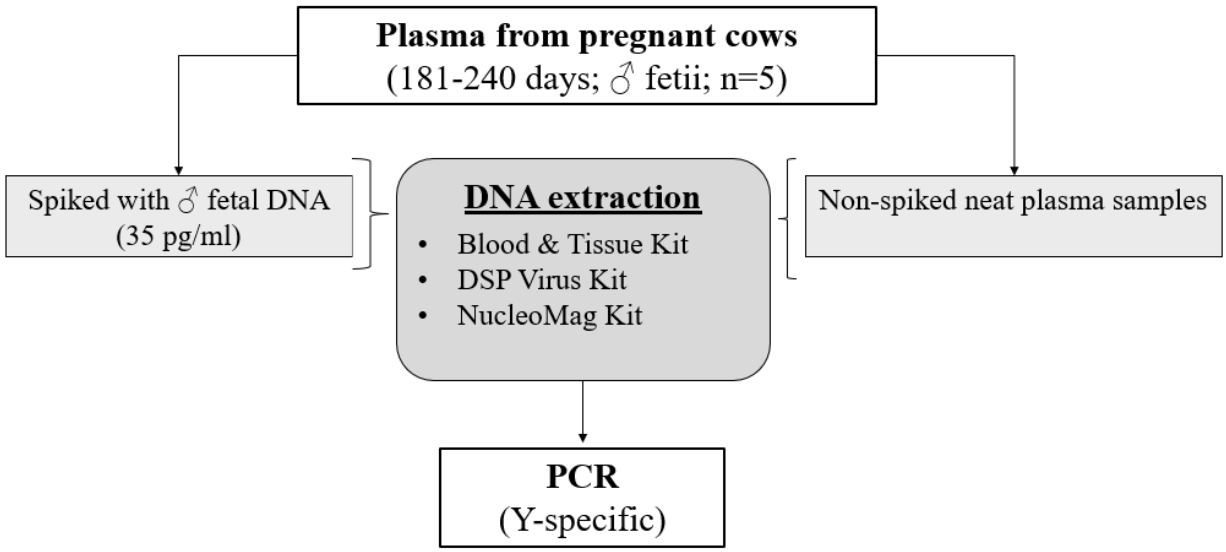


Figure 3.4: *Trial 7 experimental design.*

these kits in subsection 2.3.4. The extracted DNA from fetal blood cell fraction and blood cells of positive control animals was eluted in 200 µl of elution buffer and stored at -20°C until further use. The DNA extracted from spiked and neat plasma samples of heifers/pregnant cows was eluted in 50 µl (Trials 4, 5b, 6, and 7) and 80 µl (Trial 5a) elution buffer and was immediately used for PCR.

DNA quantification: The DNA extracted from the blood cell fraction of aborted male fetii was quantified using Qubit 3.0 Fluorometer (Thermo Fisher Scientific), as explained previously under subsection 2.3.4.

3.3.7. PCR and gel electrophoresis

Based on previous studies and the observations made in our research (chapter 2), primer pairs targeting Y-specific sequences and bAML gene (Table 2.1) were used. For all PCR reactions, 10 µl of DNA extract (non-spiked and spiked samples) was used, while other contents of the PCR mixture were similar to our previous study (subsection 2.3.5). However, 2 µl of the DNA extracts (from the blood cell fraction of control animals) were used as templates in the PCR controls. The remainder of the volume (16.75 µl) was compensated by adding PCR-grade water to the reaction mixture, making a final volume of 25 µl. A non-template control was included in every PCR batch to account for the cross-contamination of PCR tubes. The thermocycling protocols for Y-specific and bAML PCR were used as described earlier (subsection 2.3.5). For gel electrophoresis, the protocol for separating amplicons based on size was carried out as explained in subsection 2.3.6.

3.3.8. Statistical analysis

The proportions of correct prediction of fetal sex between two groups of pregnant cows using PCR (Trial 6) were compared by Fisher's exact test using SPSS 28.0 (SPSS for windows by International Business Machines). P-value <0.05 was considered statistically significant.

3.4. Results

3.4.1. Re-extraction of fetal serum and DNA spiked into plasma samples of their respective dams (Trial 4)

We observed that spiking of mother's plasma with fetal serum samples (Table 3.3; Figure 3.5a) or fetal DNA from cell fractions (Table 3.4; Figure 3.5b) of a male fetus at all dilution rates resulted

in amplification of Y-specific amplicon. No PCR product was observed in the neat plasma of cows induced to abort and non-template control, whereas appropriate amplification in positive controls indicated successful PCR.

Table 3.3: PCR results of male fetal serum spiked at different concentrations (v/v) in the maternal plasma.

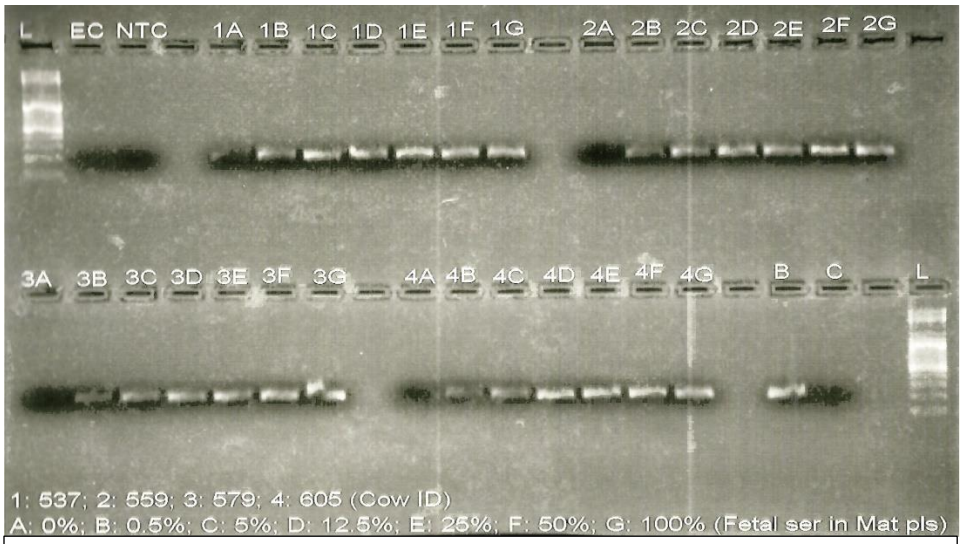
% of fetal serum in maternal plasma (v/v)	**Y-specific PCR result** (n=5)				
	1	**2**	**3**	**4**	**5**
0	-	-	-	-	-
0.5	+	+	+	+	+
5	+	+	+	+	+
12.5	+	+	+	+	+
25	+	+	+	+	+
50	+	+	+	+	+
100	+	+	+	+	+

'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.

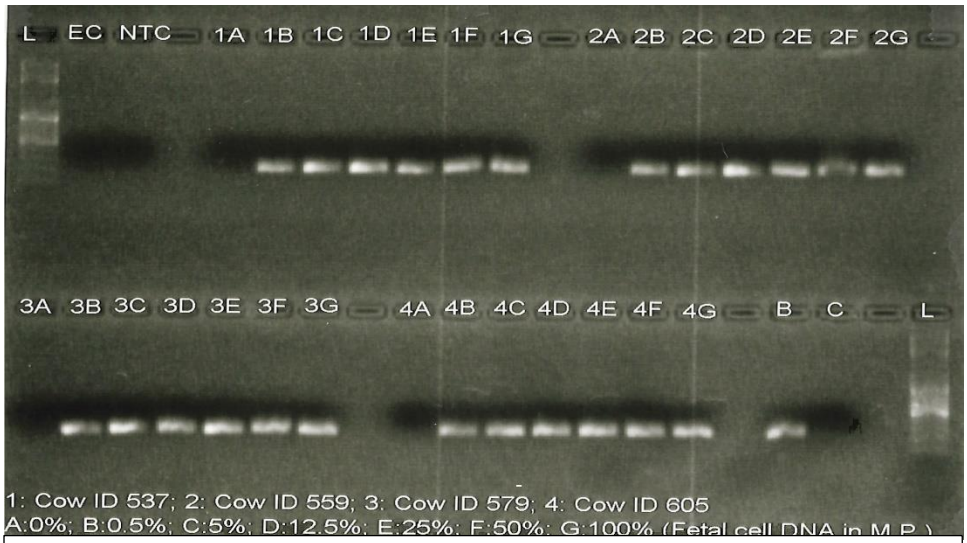
Table 3.4: PCR results of male fetal cellular DNA spiked at different concentrations (v/v) in the maternal plasma.

% of fetal DNA in maternal plasma (v/v)	**Expected fetal DNA concentration (ng/µl) spiked in maternal plasma** (n=5)					**Y-specific PCR result**
	1	**2**	**3**	**4**	**5**	
0	0	0	0	0	0	-
0.5	24.9	34.8	7.9	2.2	5	+
5	249	348	79	22	50	+
12.5	622.5	870	197.6	55.8	124.9	+
25	1245	1740	395.2	111.7	249.7	+
50	2490	3480	790.5	223.5	499.5	+
100	4980	6960	1581	447	999	+

'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.



3.5a (Maternal plasma spiked with male fetal serum)

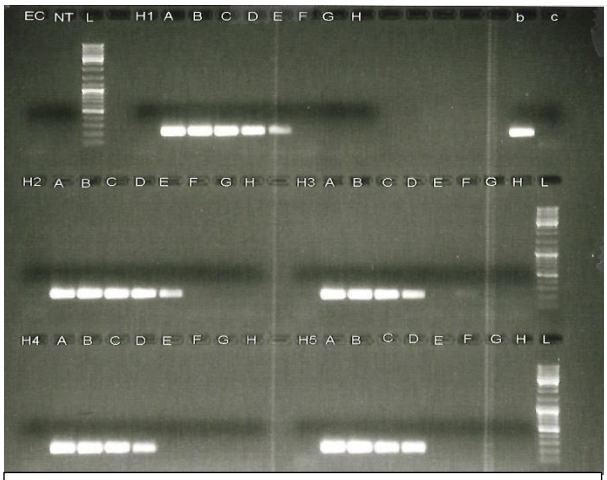


3.5b (Maternal plasma spiked with male fetal cellular DNA)

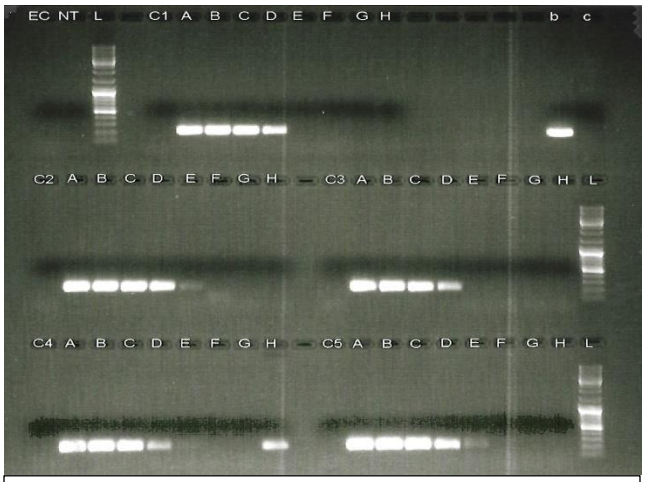
Figure 3.5: *Y-specific PCR results following DNA extraction from the plasma samples (spiked with the fetal serum/fetal cellular DNA from their respective aborted male fetii) of aborted beef cows. Spiking of fetal serum/fetal cellular DNA in the maternal plasma was performed at different levels of v/v dilutions viz. 0% (A), 0.5% (B), 5% (C), 12.5% (D), 25% (E), 50% (F), and 100% (G); 'L': 100 bp DNA ladder; 'EC': Extraction control; 'NTC': Non-template control; '1-4': aborting cows from whom the plasma samples were obtained for spiking with fetal serum/cellular DNA followed by DNA extraction and PCR. 'B' and 'C': Bull and heifer PCR controls with presence and absence of 210 bp Y-specific gene sequence, respectively.*

3.4.2. Minimum range of spiked fetal DNA concentration needed for successful re-extraction (Trial 5a)

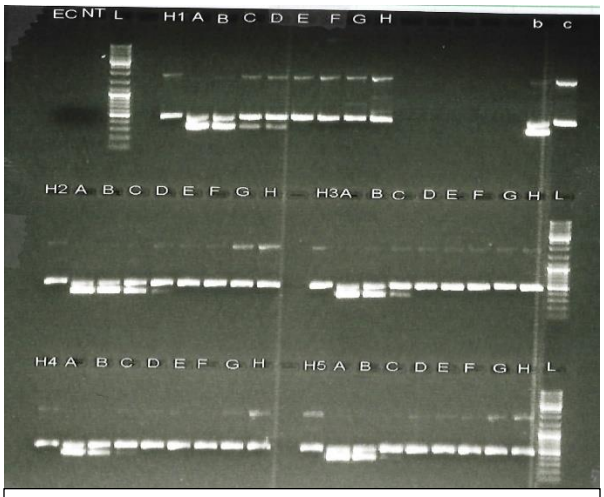
The PCR results for Y-specific (Figures 3.6a and 3.6b) and bAML (Figures 3.6c and 3.6d) genes in plasma samples of non-pregnant heifers and pregnant cows spiked with different concentrations of male fetal DNA are shown in Tables 3.5 and 3.6, respectively. The lower range of cffDNA re-extracted and detectable by Y-specific and bAML gene PCR was 16-69 pg/300 µl (approximately 53-230 pg/ml) and 0.63-1.3 ng/300 µl of maternal plasma (approximately 2.1-4.3 ng/ml), respectively. We did not observe Y-specific 210 bp and male fetus-specific bAML (341 bp) amplicons in the non-spiked neat plasma samples from non-pregnant heifers and pregnant cows. No bands in the negative controls confirmed a contamination-free DNA extraction and PCR process, while appropriate amplification in positive controls indicated the successful PCR.



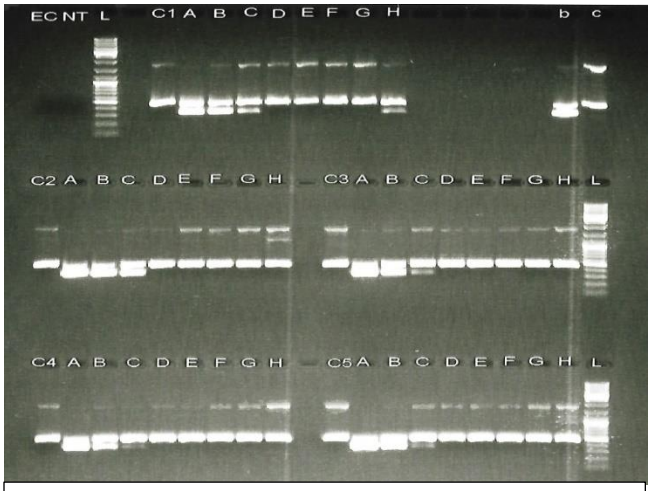
3.6a (Y-specific; Heifer plasma samples spiked with fetal cellular DNA)



3.6b (Y-specific; Pregnant cows' plasma samples spiked with fetal cellular DNA)



3.6c (bAML; Heifer plasma samples spiked with fetal cellular DNA)



3.6d (bAML; Pregnant cows' plasma samples spiked with fetal cellular DNA)

Figure 3.6: *Y-specific and bAML PCR results when known amount of cellular DNA from aborted male fetii was spiked in plasma of unrelated heifers and pregnant cows (120-150 days pregnant; carrying male fetii). From this initial dilution step, subsequent serial dilutions were made in a 1:20 manner and DNA re-extraction was performed from all the diluted plasma samples followed by PCR. 'EC': Extraction control; 'NT': Non-template control; 'L': 100 bp DNA ladder; 'H1-H5'and 'C1-C5': neat plasma from heifers and pregnant cows, respectively used for DNA extraction and PCR; 'A': DNA re-extraction and PCR from plasma samples of each heifer/cow spiked with known amount of male fetal cellular DNA; 'B-H': subsequent serially diluted plasma samples of the respective heifers/cows with an expected known amount of male fetal cellular DNA (A: No serial dilution; B: 2×10^{-1}, C: 2×10^{-2}, D: 2×10^{-3}, E: 2×10^{-4}, F: 2×10^{-5}, G: 2×10^{-6}, H: 2×10^{-7}); 'b' and 'c': Bull and heifer PCR controls, respectively.*

Table 3.5: PCR results of plasma samples spiked with serial dilutions of fetal DNA from the aborted male fetii and tested for Y-specific gene. Non-pregnant heifers (H1-H5) and pregnant cows (C1-C5: 120-150 days gestation, carrying male fetus).

Dilution rate	Male fetus specific amplicon in Y-specific primers PCR									
	H1	C1	H2	C2	H3	C3	H4	C4	H5	C5
Undiluted	+	+	+	+	+	+	+	+	+	+
$2x10^{-1}$	+	+	+	+	+	+	+	+	+	+
$2x10^{-2}$	+	+	+	+	+	+	+	+	+	+
$2x10^{-3}$	+	+	+	+	+	+	+	+	+	+
$2x10^{-4}$	+	-	+	+	-	-	-	-	-	+
$2x10^{-5}$	-	-	-	-	-	-	-	-	-	-
$2x10^{-6}$	-	-	-	-	-	-	-	-	-	-
$2x10^{-7}$	-	-	-	-	-	-	-	+	-	-
Non-spiked neat heifer/cow samples	-	-	-	-	-	-	-	-	-	-

'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.

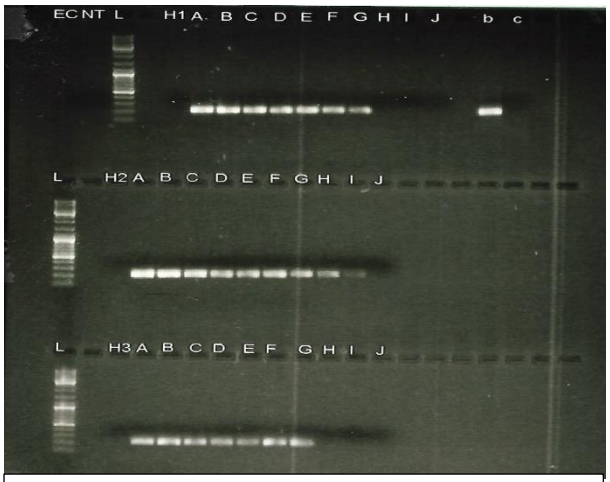
Table 3.6: PCR results of plasma samples spiked with serial dilutions of fetal DNA from the aborted male fetii and tested for bAML gene. Non-pregnant heifers (H1-H5) and pregnant cows (C1-C5: 120-150 days gestation, carrying male fetus).

Dilution rate	Male fetus specific amplicon in bAML PCR									
	H1	C1	H2	C2	H3	C3	H4	C4	H5	C5
Undiluted	+	+	+	+	+	+	+	+	+	+
$2x10^{-1}$	+	+	+	+	+	+	+	+	+	+
$2x10^{-2}$	+	+	+	+	+	+	+	+	+	+
$2x10^{-3}$	+	-	+	-	-	-	-	-	-	-
$2x10^{-4}$	-	-	-	-	-	-	-	-	-	-
$2x10^{-5}$	-	-	-	-	-	-	-	-	-	-
$2x10^{-6}$	-	-	-	-	-	-	-	-	-	-
$2x10^{-7}$	-	-	-	-	-	-	-	-	-	-
Non-spiked neat heifer/cow samples	-	-	-	-	-	-	-	-	-	-

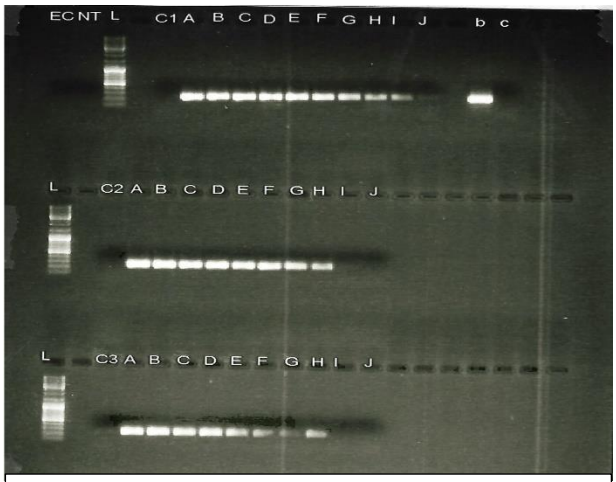
'+' and '–' indicate the presence and absence, respectively, of gene amplicon (341 bp) in PCR.

3.4.3. Lowest concentration of spiked male fetal DNA for a successful PCR (Trial 5b)

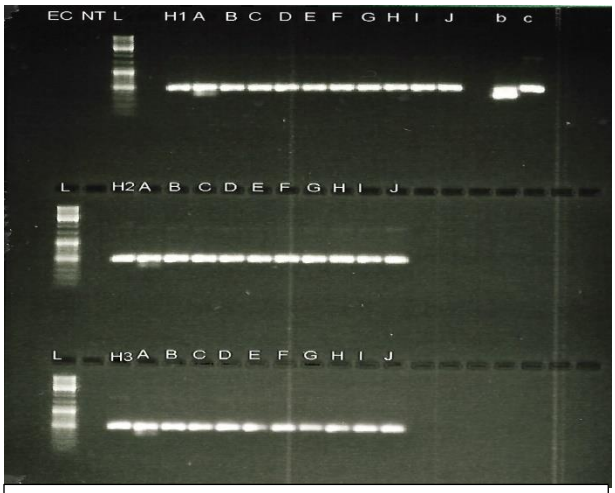
The Y-specific PCR (Figures 3.7a and 3.7b) results showed that male fetus-specific amplicons could be detected in maternal plasma at spiking concentrations $\geq$ 9.4 pg/300 µl ($\geq$31.25 pg/ml, Table 3.7). When this concentration was converted to the DNA copy number, 10 copies of the whole fetal genome/ml of maternal plasma are needed for a successful PCR. Further, the PCR designed to target the bAML sequence (Figures 3.7c and 3.7d) appeared to be less sensitive as even at 600 pg/300 µl (2 ng/ml) concentration of male fetal DNA in maternal plasma, 341 bp bAML sequence (specific to the male fetus) was observed in four out of five non-pregnant heifer/pregnant cows (Table 3.8). When this concentration was converted to the DNA copy number, the figure came out to be 648 copies of the whole fetal genome/ml of maternal plasma. We did not observe male fetus-specific amplicons for Y-specific and bAML genes in the non-template control and the neat plasma samples of any of the pregnant cows carrying a male fetus. Appropriate working of the positive controls implied successful PCR.



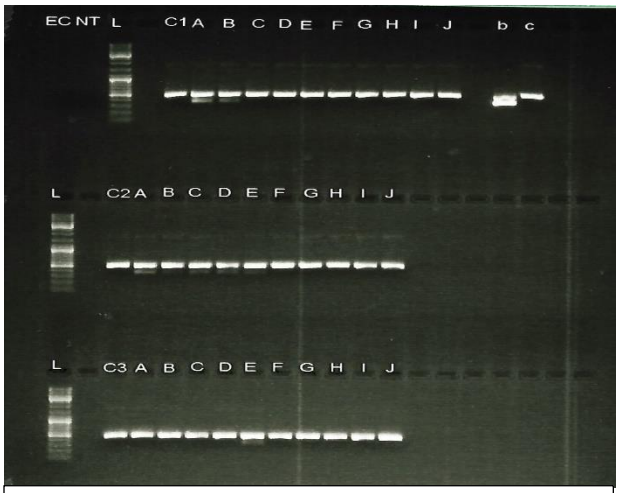
3.7a (Y-specific; Heifer plasma samples spiked with fetal cellular DNA)



3.7b (Y-specific; Pregnant cows' plasma samples spiked with fetal cellular DNA)



3.7c (bAML; Heifer plasma samples spiked with fetal cellular DNA)



3.7d (bAML; Pregnant cows' plasma samples spiked with fetal cellular DNA)

Figure 3.7: *A representative gel image of Y-specific and bAML PCR results when known amount of cellular DNA (2 ng) from aborted male fetii was spiked in 1000 µl plasma of unrelated heifers and pregnant cows (120-150 days pregnant; carrying male fetii). From this initial dilution, further dilutions were made in a way to have half the amount of fetal DNA than the previous one. 'EC': Extraction control; 'NT': Non-template control; 'L': 100 bp DNA ladder; 'H1-H3' and 'C1-C3': neat plasma from heifers and pregnant cows, respectively used for DNA extraction and PCR; 'A': DNA re-extraction and PCR from plasma samples of each heifer/cow spiked with known amount of male fetal cellular DNA; 'B-J': subsequently diluted plasma samples of the respective heifers/cows with an expected known amount of male fetal cellular DNA/ml of maternal plasma (A: 2 ng, B: 1 ng, C: 500 pg, D: 250 pg, E: 125 pg, F: 62.5 pg, G: 31.25 pg, H: 15.62 pg, I: 7.81 pg, J: 3.9 pg) ; 'b' and 'c': Bull and heifer PCR controls, respectively.*

Table 3.7: Results of Y-specific PCR conducted on plasma samples spiked with male cffDNA. Plasma of unrelated non-pregnant heifers (H1-H5) and pregnant cows (C1-C5: 120-150 days gestation, male fetus) were used.

Amount of male fetal DNA expected in the spiked plasma of heifers/cows		**Y-specific PCR**									
Fetal DNA/ml of plasma	**Fetal DNA/300 µl of plasma (used for re-extraction)**	**H1**	**H2**	**H3**	**H4**	**H5**	**C1**	**C2**	**C3**	**C4**	**C5**
2 ng	600 pg	+	+	+	+	+	+	+	+	+	+
1 ng	300 pg	+	+	+	+	+	+	+	+	+	+
500 pg	150 pg	+	+	+	+	+	+	+	+	+	+
250 pg	75 pg	+	+	+	+	+	+	+	+	+	+
125 pg	37.5 pg	+	+	+	+	+	+	+	+	+	+
62.5 pg	18.7 pg	+	+	+	+	+	+	+	+	+	+
31.25 pg	9.4 pg	+	+	+	+	+	+	+	+	+	+
15.62 pg	4.7 pg	-	+	-	+	-	+	+	+	-	-
7.81 pg	2.3 pg	-	+	-	-	-	+	-	-	-	-
3.9 pg	1.2 pg	-	-	-	-	-	-	-	-	-	-
Non-spiked neat heifer/cow plasma (300 µl) used for DNA extraction		-	-	-	-	-	-	-	-	-	-

'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.

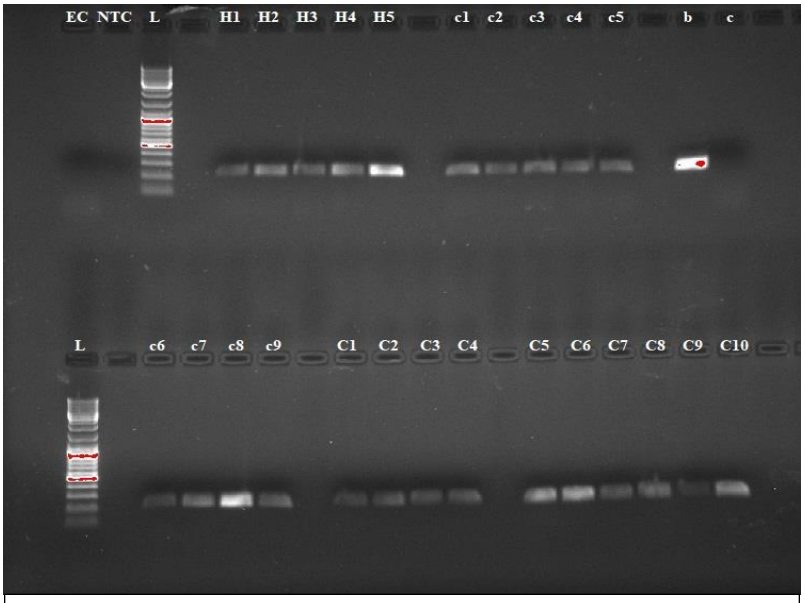
Table 3.8: Results of bAML PCR conducted on plasma samples spiked with male cffDNA. Plasma of unrelated non-pregnant heifers (H1-H5) and pregnant cows (C1-C5: 120-150 days gestation, male fetus) were used.

Amount of male fetal DNA expected in the spiked plasma of heifers/cows		**bAML PCR**									
Fetal DNA/ml of plasma	**Fetal DNA/300 µl of plasma (used for re-extraction)**	**H1**	**H2**	**H3**	**H4**	**H5**	**C1**	**C2**	**C3**	**C4**	**C5**
2 ng	600 pg	+	+	+	+	-	+	+	-	+	+
1 ng	300 pg	-	-	-	-	-	+	-	-	+	-
500 pg	150 pg	-	-	-	-	-	-	+	-	-	-
250 pg	75 pg	-	-	-	-	-	-	+	-	-	-
125 pg	37.5 pg	-	-	-	-	-	-	+	+	-	-
62.5 pg	18.7 pg	-	-	-	-	-	-	-	-	-	-
31.25 pg	9.4 pg	-	-	-	-	-	-	-	-	-	-
15.62 pg	4.7 pg	-	-	-	-	-	-	-	-	-	-
7.81 pg	2.3 pg	-	-	-	-	-	-	-	-	-	-
3.9 pg	1.2 pg	-	-	-	-	-	-	-	-	-	-
Non-spiked neat heifer/cow plasma (300 µl) used for DNA extraction		-	-	-	-	-	-	-	-	-	-

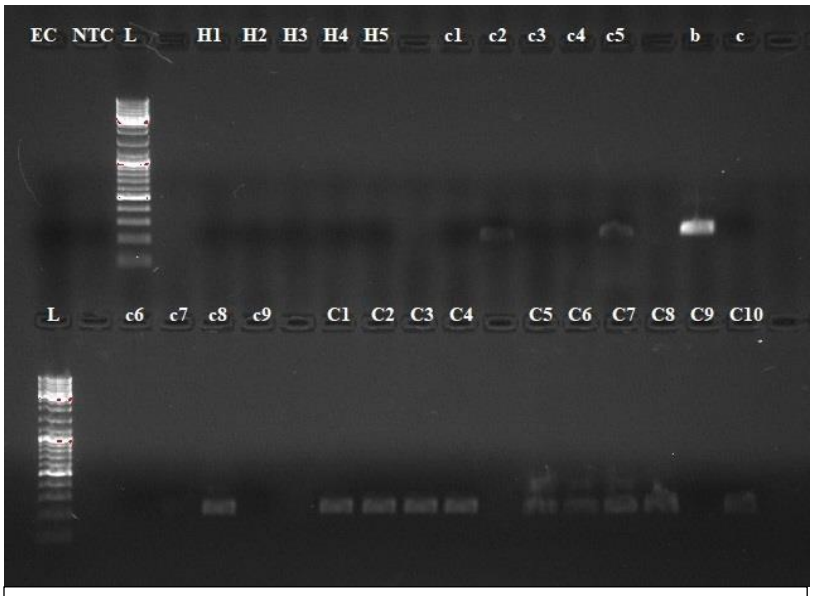
'+' and '–' indicate the presence and absence, respectively, of gene amplicon (341 bp) in PCR.

3.4.4. Effect of gestation stage at sampling on prenatal fetal sexing using PCR (Trial 6)

Considering our observation that Y-specific PCR would detect lower concentrations of cffDNA than bAML PCR, we decided to use the former to compare two groups of cows at different stages of gestation for prenatal fetal sexing. The result of Y-specific PCR on spiked (Figure 3.8a) plasma samples (positive control) and neat (Figure 3.8b) plasma samples from cows 60-150 days pregnant (n=9) versus cows 151-240 d pregnant (n=10) are described in Table 3.9. The proportion of cows with a PCR positive sample were lower in the 60-150 d gestation group (CI: 7.5-70.1) than the 151-240 d group (CI: 55.5-99.75), 3/9 versus 9/10 ($P<0.05$). All spiked samples of pregnant cows and non-pregnant heifers showed the expected 210 bp Y-specific amplicons, while the neat samples from non-pregnant heifers and non-template control did not yield any PCR product. Appropriate working of the positive controls implied successful PCR.



3.8a (Heifers and pregnant cows' plasma samples spiked with lower limits of male fetal DNA)



3.8b (Neat plasma samples of heifers and pregnant cows)

Figure 3.8: *Y-specific PCR results when spiked (with 35 pg male fetal DNA/ml) and neat plasma samples from non-pregnant heifers and pregnant cows carrying male fetii grouped in two gestational stages (60-150 days pregnant versus 151-240 days pregnant) were subjected to DNA extraction. 'EC': Extraction control; 'NTC': Non-template control; 'L': 100 bp DNA ladder; 'H1-H5': Heifer DNA samples; 'c1-c9': DNA samples from 60-150 days pregnant cows; 'C1-C10': DNA samples from 151-240 days pregnant cows; 'b' and 'c': Bull and heifer PCR controls, respectively.*

Table 3.9: Results of Y-specific PCR. Extracted DNA from neat and spiked (with 35 pg/ml male cffDNA) plasma samples from non-pregnant heifers (n=5) and pregnant cows (n=19) were used.

Non-pregnant heifers			**60-150 days pregnant cows**			**151-240 days pregnant cows**		
Sr. No.	**Spiked plasma**	**Neat plasma**	**Sr. No.**	**Spiked plasma**	**Neat plasma**	**Sr. No.**	**Spiked plasma**	**Neat plasma**
1	+	-	**1**	+	-	**1**	+	+
2	+	-	**2**	+	+	**2**	+	+
3	+	-	**3**	+	-	**3**	+	+
4	+	-	**4**	+	-	**4**	+	+
5	+	-	**5**	+	+	**5**	+	+
			6	+	-	**6**	+	+
			7	+	-	**7**	+	+
			8	+	+	**8**	+	+
			9	+	-	**9**	+	-
						10	+	+

'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.

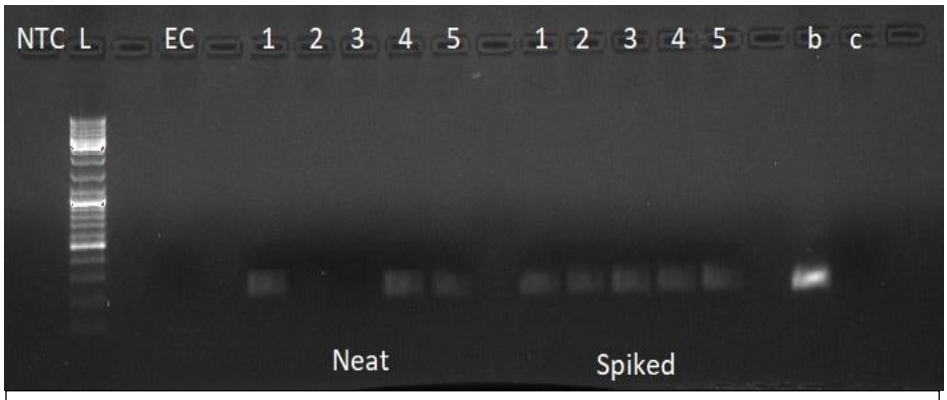
3.4.5. Comparison of DNA extraction methods for Y-specific PCR (Trial 7)

The results of Y-specific PCR conducted on DNA extracted from neat and spiked plasma samples using three commercial kits are shown in Table 3.10. The Y-chromosome-specific sequence was successfully amplified in all the DNA extracts obtained from the spiked plasma samples using all three kits compared (Figures 3.9a, 3.9b, and 3.9c). However, the Y-specific sequences were amplified in 3/5, 1/5, and 0/5 DNA extracts obtained from neat plasma samples using DNeasy Blood and Tissue Kit (Figure 3.9a), Qiagen DSP Virus Kit (Figure 3.9b), and NucleoMag cfDNA Isolation Kit (Figure 3.9c), respectively (Table 3.10). A clean non-template control and appropriate amplification in positive controls indicated successful PCR.

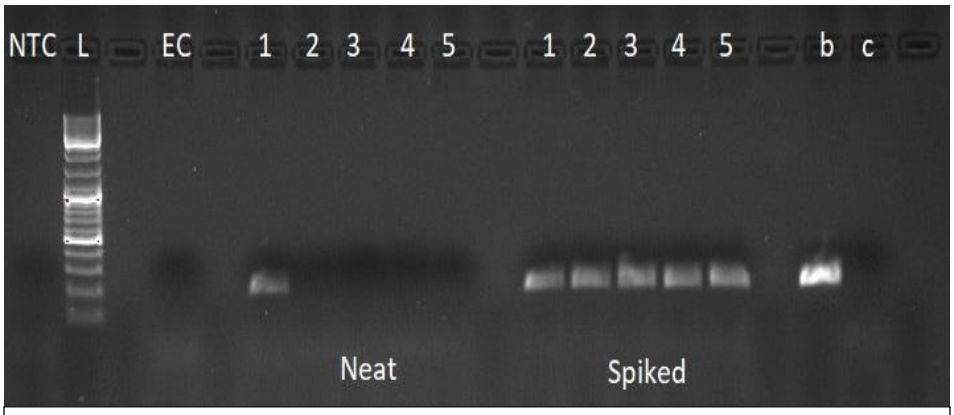
Table 3.10: Results of Y-specific PCR comparing different extraction methods. Neat and spiked (with 35 pg/ml male cffDNA) plasma samples from pregnant cows (181-240 days gestation, carrying male fetus) were extracted using three commercial kits.

Kits used for DNA extraction	**Spiking status**	**Y-specific PCR output** (n=5 replicates each)				
		1	**2**	**3**	**4**	**5**
DNeasy Blood & Tissue Kit	Neat	+	-	-	+	+
	Spiked	+	+	+	+	+
DSP Virus Kit	Neat	+	-	-	-	-
	Spiked	+	+	+	+	+
NucleoMag cfDNA Isolation Kit	Neat	-	-	-	-	-
	Spiked	+	+	+	+	+

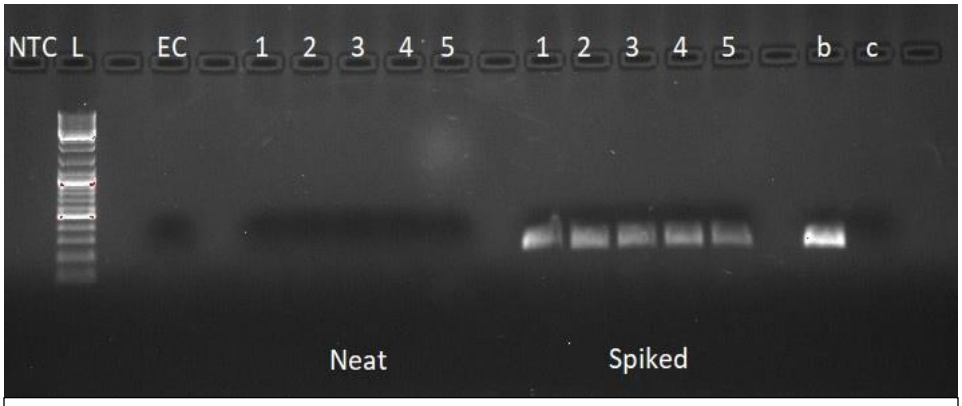
'+' and '–' indicate the presence and absence, respectively, of gene amplicon (210 bp) in PCR.



3.9a (DNeasy Blood & Tissue Kit)



3.9b (Qiagen DSP Virus Kit)



3.9c (NucleoMag cfDNA Isolation Kit)

Figure 3.9: *Y-specific PCR results following DNA extraction using neat and spiked (with 35 pg male fetal DNA/ml) plasma samples from pregnant cows carrying male fetii (181-240 days pregnant). DNeasy Blood & Tissue Kit, Qiagen DSP Virus Kit, and NucleoMag cfDNA Isolation Kit were used on same set of plasma samples. 'NTC': Non-template control; 'L': 100 bp DNA ladder; 'EC': Extraction control; '1-5': DNA samples obtained from neat and spiked plasma samples of pregnant cows; 'b' and 'c': Bull and heifer PCR controls, respectively.*

3.5. Discussion

To the best of our knowledge, the present study is novel in identifying the lower limits of cffDNA in the plasma of pregnant cattle for a PCR-based prenatal fetal sexing. We also determined that the a) Y-specific PCR would amplify spiked cffDNA at lower concentrations than the bAML PCR, and b) the sensitivity of prenatal fetal sex determination using PCR was superior in cows sampled in the second half of gestation than during the first half of gestation. Lastly, the lowest concentrations of spiked cffDNA could be successfully re-extracted with all extraction methods and amplified using Y-specific PCR; however, the results of PCR were variable when neat plasma was used.

Findings of our initial spiking experiment showed that cffDNA could be successfully extracted and amplified with the Y-specific gene PCR when the fetal serum fraction in the maternal plasma was as low as 0.5% (v/v). Results were similar when extracted fetal cellular DNA was added at the same (0.5% v/v) dilution rates. A couple of previous studies have indicated that PCR could successfully amplify Y-specific PCR products if the proportion of male to female DNA was 1% but not at 0.1% proportion (Cruz et al., 2011; Da Cruz et al., 2012). Interestingly, we could not amplify PCR products in the non-spiked samples of cows that aborted a male fetus. At least in women, the mean half-life of fetal DNA in maternal circulation was reported to be 16.3 minutes, indicating a rapid clearance of cffDNA following delivery (Dennis Lo et al., 1999). Since we collected the plasma samples from aborting cows within 24 h of abortion, it is possible that the cffDNA either was below the detection limits or was cleared out from the maternal peripheral circulation.

Our subsequent spiking trials provided more insights into the lowest amount of cffDNA that could be successfully re-extracted and used for PCR (both bAML and Y-specific). We observed that Y-specific PCR successfully amplified PCR products if the fetal DNA concentration was between 53-230 pg/ml of maternal plasma. When the same primers were used in a previous report, the lowest concentration of DNA needed for a successful Y-specific PCR was indicated to be 0.4 pg (Cruz et al., 2011; Da Cruz et al., 2012). In the current study, with the same elution volume as for Y-specific PCR, the successful amplification of male fetus-specific bAML gene sequences was observed when male fetal DNA was present in the range of approximately 2.1-4.3 ng/ml. Selected Y-specific primers might likely have better efficiency than bAML primers. In a follow-up spiking

experiment, we determined the threshold limits for consistent amplification of male fetal Y-specific and bAML gene sequences with PCR as >31.25 pg/ml and 2ng/ml of maternal plasma, respectively, when DNA elution volume was decreased. Considering the size of the whole bovine fetal genome to be 2,857,605,192 base pairs (Zimin et al., 2009), a minimum of at least ten copies of the entire fetal genome must be present in one milliliter of maternal plasma for successful extraction of fetal DNA for Y-specific 210 bp primer PCR used in this study. Our observation corresponds with an older study in humans that has suggested approximately ten copies of fetal DNA circulating per milliliter of maternal plasma for prenatal fetal sex determination (Dennis Lo et al., 1989). However, about 648 copies of bovine fetal DNA must be present per milliliter of maternal plasma for successful extraction and amplification using bAML PCR.

Our subsequent trial indicated that the sensitivity of the Y-specific PCR for fetal sexing was higher for cows between 151-240 days pregnant (CI: 55.5-99.75) compared to 60-150 days pregnant (CI: 7.5-70.1). Although a limited number of animals were included in our trial, this finding suggested that cffDNA fraction may be higher during the second-half of gestation, similar to the observations made in pregnant women (Dar et al., 2016; Dennis Lo, Tein, et al., 1998). Collectively, these findings support the notion that the concentration of fetal DNA might gradually rise with advancement in gestation but needs to be tested in a larger number of animals. A similar trend of correctly predicting bovine fetal sex using PCR with advancement in gestation was also reported in an earlier study (Da Cruz et al., 2012), however, the authors observed that fetal sex could be correctly predicted with 99.9% accuracy 55 days post-conception. In our case, the sensitivity of correctly predicting male fetal sex in cows lesser than five months pregnant was just 33%, which is in concordance with an earlier study where authors had reported a 25% sensitivity of successfully amplifying fetal Y-chromosome specific sequences in pregnant cows around their mid-gestation (i.e., 150 days pregnant) (Turin et al., 2007). The sensitivity of correctly identifying fetal sex using Y-specific PCR in cows greater than 150 days pregnant was 90% in our case which is in agreement with an earlier finding by Davoudi and co-workers who have reported that the ease of identifying bovine fetal DNA using PCR rises as the pregnancy advances (Davoudi et al., 2011). However, our observations need to be further tested on a larger dataset to obtain a statistically meaningful comparison with regards to the sensitivity, specificity, and accuracy of the PCR assay for fetal sexing in pregnant cows. In another study, the accuracy of PCR-based bovine fetal sexing was found to be 60% and 92% for cows 30-60 and 121-240 days pregnant, respectively (Xi, 2006).

A study conducted on pregnant mares also reported a gradual increase in the accuracy of PCR-based fetal sexing from the first to the third trimester of pregnancy (48.75%, 68.75, and 75%, respectively) (Tonekaboni et al., 2020). In the same trial, the spiked cffDNA (@ 35 pg/ml of maternal plasma) was successfully re-extracted and detected using Y-specific PCR from both gestational groups of cows. It is possible that in the samples which did not show Y-specific amplicons, the inherent cffDNA fraction was below the detectable limits or may be present in fragmented form in contrast to the spiked fetal DNA.

Our last trial further confirmed the variability of results with Y-specific PCR when we used different DNA extraction methods for samples obtained from cows 181-240 days in gestation. The spiked fetal DNA could be successfully re-extracted from all the samples when the same set of spiked plasma was subjected to extraction using all three kits. However, when neat plasma samples from the same pregnant cows were used for DNA extraction and Y-specific PCR, successful amplification was observed in 3/5, 1/5, and 0/5 plasma extracts obtained using DNeasy Blood & Tissue Kit, QIAamp DSP Virus Kit, and NucleoMag cfDNA Isolation Kit, respectively. Several authors reported similar findings that demonstrated different cfDNA extraction methods to yield different results for prenatal fetal sexing when DNA extraction was performed on the same set of plasma samples obtained from pregnant cows (Davoudi et al., 2011; Ristanic et al., 2018). In addition, we noticed a lack of consistency amongst the DNA extraction method in the present study. The DNA extraction methods used in the study yielded different results on different days, although the performance of the DNeasy Blood & Tissue Kit was comparatively consistent than its counterparts. The fundamental differences in extraction methods and highly fragmented nature of cffDNA (K. C. A. Chan et al., 2004; Li et al., 2004) along with the transient release of fetal DNA in cows (Turin et al., 2007) might somewhat explain the lack of consistency of results between the kits (K. C. A. Chan et al., 2004; Dar et al., 2016; Li et al., 2004). However, further studies are warranted on a larger number of animals to identify extraction methods with higher accuracy and repeatability.

In summary, Y-specific sequence PCR assay had a lower limit of detection than the bAML PCR assay for prenatal fetal sexing. Furthermore, the sensitivity of fetal sexing by Y-specific PCR was higher during the second half of gestation due to relatively higher fetal DNA concentrations.

3.6. Conclusions

Fetal serum and cfDNA from aborted male fetii spiked in plasma of their respective dams could be successfully re-extracted for Y-specific PCR even at the lowest proportion (0.5%, v/v). Under our laboratory conditions, the minimum threshold of cffDNA needed for successful extraction and amplification were ≥31.25 pg/ml and ≥2 ng/ml of maternal plasma for Y-specific PCR and bAML PCR, respectively. In other terms, at least 10 and 648 copies of the whole fetal genome must be present per milliliter of maternal plasma for successful extraction, followed by Y-specific and bAML PCR, respectively. Moreover, the chances of successfully amplifying male fetal gene sequences increased in advanced stages of pregnancy in cattle; however, the cffDNA might be highly variable between animals.

4. GENERAL DISCUSSION AND FUTURE DIRECTIONS

4.1. General discussion

Since the discovery of cffDNA in the peripheral blood circulation of pregnant women (Dennis Lo et al., 1997), various assays have been developed to use fetal DNA for prenatal fetal sex determination (Dennis Lo, Tein, et al., 1998; Sekizawa, Kondo, et al., 2001) and genetic screening of the heritable diseases (Bianchi, 2012; Ferrari et al., 2015; Mennuti et al., 2015; Skrzypek & Hui, 2017). Although the synepitheliochorial placenta of cattle is least invasive due to lack of direct contact between maternal bloodstream and fetal trophoblast (P. Wooding & Burton, 2008), the phenomenon of transplacental passage of fetal DNA has been demonstrated in cattle (Turin et al., 2007). Considering the low quantities of fetal DNA in the blood circulation of pregnant cows, sample handling and DNA extraction methodologies play a critical role in determining the success of PCR-based prenatal sex determination assay (Davoudi et al., 2012; Ristanic et al., 2018). So, the first experiment of the present study was aimed to identify the ideal sample type and DNA extraction methodology for PCR-based prenatal fetal sex determination in pregnant cows. The second experiment was focused on determining the lower limits of cffDNA in bovine plasma samples needed for successful PCR detection.

Our first trial failed to amplify fetal gene sequences in the PCR with frozen-thawed plasma samples from pregnant cows. In contrast, fresh plasma samples from pregnant cows resulted in the successful amplification of fetal gene sequences. Previously studies in pregnant cattle have used fresh plasma and blood for DNA extraction and prenatal fetal sex determination (Da Cruz et al., 2012; Ristanic et al., 2018), whereas others have successfully used frozen-thawed plasma for the same purpose (Davoudi et al., 2011; G. Wang et al., 2010). The contrasting results between frozen and fresh plasma samples in the present study could be attributed to the fragmentation of cffDNA in samples subjected to the freeze-thaw cycles (K. A. Chan et al., 2005; El Messaoudi et al., 2013) owing to the existence of cfDNA in a highly degradable form in the biological samples (Sanchez et al., 2018; Zandvakili & Lazaridis, 2019). Further, it is possible that a very low fetal fraction of cfDNA resulted in the amplification of the maternal cfDNA similar to previous reports in samples from pregnant women (Dennis Lo, Tein, et al., 1998). The exceedingly high proportion of maternal DNA (93.8-96.6%) compared to fetal DNA (3.4-6.2%) in pregnant women (Dennis Lo, Tein, et al., 1998) has been proposed to overshadow the smaller fetal DNA molecules (Wright & Burton,

2009), making the extraction of fetal DNA even more challenging. While 467 bp bAML sequence (from X-chromosome) served as the internal control for every DNA extraction, the lack of amplification of maternal cfDNA from some samples processed with the phenol-chloroform method was perplexing. The persistence of PCR inhibitors (Lemarchand et al., 2005; Nolan et al., 2006) or samples contamination due to multiple tube transfer steps in the phenol-chloroform method could be the likely reasons for the lack of amplification of maternal cfDNA (Köchl et al., 2005). In contrast, DNeasy Blood and Tissue and MagMAX cfDNA isolation kits amplified the internal control bAML sequence at all instances, possibly due to additional washing steps for these kits. Although the lower number of samples in the present study did not allow a statistically meaningful comparison regarding the relative efficiency of seven different DNA extraction methodologies, our findings supported earlier evidence of wide variability in the yield of cffDNA amongst the extraction protocols (Davoudi et al., 2011; Ristanic et al., 2018).

The results of our second experiment indicated that spiked cffDNA could be re-extracted for PCR if the fetal fractions in maternal plasma are around 0.5%, which is lower than the previous report of 1% fetal fraction needed for a successful PCR for fetal sexing in pregnant cattle (Cruz et al., 2011; Da Cruz et al., 2012). Further, we observed that the simultaneously processed neat samples from the same pregnant cows did not yield Y-specific amplicons, suggesting either absence or very low quantities of cffDNA in the maternal plasma. Some studies in humans have shown that cffDNA is highly fragmented (Dennis Lo et al., 2010; Sun et al., 2019) that could be one of the most important reasons for its extremely challenging extraction from the maternal plasma (Keshavarz et al., 2015). Studies need to be carried out comparing manually fragmented fetal DNA with intact fetal DNA and cffDNA in the maternal plasma to confirm or refute this notion in cattle. Our trials also provided the minimum threshold of cffDNA for the Y-specific PCR and bAML PCR in pregnant cattle to be >31.25 pg/ml and 2 ng/ml of maternal plasma, respectively. Based on the size of the whole bovine genome (Zimin et al., 2009), these values of cffDNA would reflect into approximately 10 and 648 copies of whole fetal genome per ml of maternal plasma for successful extraction of cffDNA for Y-specific sequence and bAML PCR. Our trials also indicated that the likely differences of PCR efficiencies between bAML and Y-specific primers should be considered for future experiments.

Subsequently, we used the minimum threshold levels of cffDNA for further testing of Y-specific sequence-based PCR to compare the effect of stage of gestation on the sensitivity of PCR in

detecting fetal sex. Interestingly, despite the low number of animals recruited for the study, we observed significant differences in PCR results for cows between the first and second half of gestation. These difference in results of PCR-based fetal sexing with the advancement of pregnancy needs to be further studied as our result corroborates with the findings of some studies (Da Cruz et al., 2012; Davoudi et al., 2011; Turin et al., 2007) but not others (Lemos et al., 2011; Ristanic et al., 2018). Considering the higher relative efficacy of PCR-based prenatal fetal sex determination in the later stage of gestation as observed in the present study, the use of this technique might not align with fall pregnancy checks in beef herds of western Canada because most of the cows are more than 90 but less than 150 days pregnant at that time.

In summary, our preliminary studies on the development of PCR-based fetal sexing method in pregnant cattle have provided crucial information about the minimum threshold of cffDNA required for successful PCR and highlighted the limited success with the current approaches for the field adaptability.

4.2. Future directions

Determine extraction efficiency of fragmented cffDNA

To date, few reports in humans have shown that cffDNA is highly fragmented (Dennis Lo et al., 2010; Sun et al., 2019), which could be one of the most important reasons for its poor extractability from the maternal plasma (Keshavarz et al., 2015). Future experiments may be designed using manually fragmented cffDNA from blood cells/tissues of aborted male fetii to determine the extraction efficiency from the plasma samples. More specifically, after manual fragmentation, fetal DNA fragments may be selectively picked based upon their size to test re-extraction efficiency.

Determine fetal DNA distribution in different trimesters of pregnancy

Besides, a few reports suggest that the concentration of fetal DNA in maternal plasma gradually increases with advancement in gestation both in humans (E. Wang et al., 2013) and cattle (Da Cruz et al., 2012; Davoudi et al., 2011). An experiment may be designed to extract cffDNA from the plasma of pregnant cows grouped in different stages of gestation (first-trimester group; second-trimester group; third-trimester group). The extracted DNA could be subjected to gel fractionation

and PCR to determine the prevalence size of fetal DNA fragments in maternal plasma and test the accuracy of PCR-based fetal sex determination.

Magnetic beads-based approach to obtain fetal DNA from plasma/serum of pregnant cows

Since it has been reported that significantly higher yields of maternal DNA in the serum/plasma samples prevent the retention of minutely present fetal DNA (Suzuki et al., 2008), alternative approaches to specifically target male fetal DNA sequences in maternal serum may be used. One such method is based upon the use of capture probes (oligonucleotides) modified and specifically designed to hybridize to a desired DNA sequence which may ultimately be eluted through magnetic beads-based technology. This technology has been earlier used to extract scarcely present *Toxoplasma gondii* DNA in meat samples (Opsteegh et al., 2010) and extraction of urine cfDNA (Oreskovic et al., 2019) for diagnostic purposes. We hypothesize that the same oligonucleotide-based magnetic capture technique may be used for obtaining the male fetus-specific DNA sequences for sex-determination purposes with slight modifications. Yet, this technique is relatively new and is still evolving, so not much data is available to support its feasibility. The high cost/sample might be one major limiting factor hindering the adoption of this technique because it has never been used for fetal sex determination to the best of our knowledge.

5. REFERENCES

Adams, D., Clark, R., Coady, S., Lamb, J., & Nielsen, M. (1994). Extended grazing systems for improving economic returns from Nebraska sandhills cow/calf operations. *Journal of Range Management*, *47*(4), 258–263.

Aiello, D., Giambona, A., Leto, F., & Passarello, C. (2018). Human coelomic fluid investigation: A MS-based analytical approach to prenatal screening. *Scientific Reports*, *8*(1), 1–11.

Akbariqomi, M., Heidari, R., Gargari, S. S., Omrani, M. D., Rigi, G., Sanikhani, N. S., Kooshki, H., Mahmoudian, F., Mazlomi, M. A., & Tavoosidana, G. (2019). Evaluation and statistical optimization of a method for methylated cell-free fetal DNA extraction from maternal plasma. *Journal of Assisted Reproduction and Genetics*, *36*(5), 1029–1038.

Akolekar, R., Farkas, D. H., VanAgtmael, A. L., Bombard, A. T., & Nicolaides, K. H. (2010). Fetal sex determination using circulating cell-free fetal DNA (ccffDNA) at 11 to 13 weeks of gestation. *Prenatal Diagnosis*, *30*(10), 918–923.

Al-Yatama, M., Mustafa, A., Ali, S., & Abraham, S. (2001). Detection of Y chromosome-specific DNA in the plasma and urine of pregnant women using nested polymerase chain reaction. *Prenatal Diagnosis*, *21*(5), 399–402.

Alberry, M., Maddocks, D., & Jones, M. (2007). Free fetal DNA in maternal plasma in anembryonic pregnancies: confirmation that the origin is the trophoblast. *Prenatal Diagnosis*, *27*(5), 415–418.

Allen, W. R., & Stewart, F. (2001). Equine placentation. *Reproduction, Fertility and Development*, *13*(8), 623–634.

Angert, R., LeShane, E., Lo, Y., & Chan, L. (2003). Fetal cell-free plasma DNA concentrations in maternal blood are stable 24 hours after collection: analysis of first-and third-trimester samples. *Clinical Chemistry*, *49*(1), 195–198.

Angert, R., LeShane, E., & Yarnell, R. (2004). Cell-free fetal DNA in the cerebrospinal fluid of women during the peripartum period. *American Journal of Obstetrics & Gynecology*, *190*(4), 1087–1090.

Anker, P., Stroun, M., & Maurice, P. (1975). Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Research*, *35*(9), 2375–2382.

Aoki, M., Kimura, K., Hirako, M., & Hanafusa, Y. (2002). Cannulation for a bovine fetus in late gestation under regional anesthesia. *Journal of Reproduction and Development*, *48*(5), 455–460.

Asadpour, R., Asadi, M., & Jafari-Joozani, R. (2015). Ovine fetal sex determination using circulating cell-free fetal DNA (ccffDNA) and cervical mucous secretions. *Asian Pacific Journal of Reproduction*, *4*(1), 65–69.

Ashoor, G., Syngelaki, A., & Poon, L. (2013). Fetal fraction in maternal plasma cell-free DNA at 11–13 weeks' gestation: relation to maternal and fetal characteristics. *Ultrasound in Obstetrics & Gynecology*, *41*(1), 26–32.

Assis Neto, A., Pereira, F., & Santos, T. (2010). Morpho-physical Recording of Bovine Conceptus (Bos indicus) and Placenta from Days 20 to 70 of Pregnancy. *Reproduction in Domestic Animals*, *45*(5), 760–772.

Barr, M., & Bertram, E. (1949). A morphological distinction between neurones of the male and female, and the behaviour of the nucleolar satellite during accelerated nucleoprotein synthesis. *Nature*, *163*(4148), 676–677.

Barrau, M. D., Abel, J. H., Torbit, C. A., & Tietz, W. J. (1975). Development of the implantation chamber in the pregnant bitch. *American Journal of Anatomy*, *143*(1), 115–129.

Barrett, A. N., Zimmermann, B. G., Wang, D., Holloway, A., & Chitty, L. S. (2011). Implementing prenatal diagnosis based on cell-free fetal DNA: Accurate identification of factors affecting fetal DNA yield. *PLoS ONE*, *6*(10), e25202.

Bauer, M. K., Harding, J. E., Bassett, N. S., Breier, B. H., Oliver, M. H., Gallaher, B. H., Evans, P. C., Woodall, S. M., & Gluckman, P. D. (1998). Fetal growth and placental function. *Molecular and Cellular Endocrinology*, *140*(1–2), 115–120.

Baur, R. (1977). Morphometry of the placental exchange area. *Advances in Anatomy Embryology and Cell Biology*, *53*, 16–41.

BCRC. (2018). *Costs Of Siring Calves: Artificial Insemination Compared To Natural Service*. Beef Cattle Research Council. https://www.beefresearch.ca/blog/costs-of-siring-calves-artificial-insemination-compared-to-natural-service/

BCRC. (2019a). *Beef Cattle Research Council, Adoption Rates of Recommended Practices by Cow-Calf Operators in Canada*.

BCRC. (2019b). Ontario Cow-Calf Survey of 2018. *Reproductive Management: Adoption Rates of Recommended Practices by BCRC*, 25–33.

BCRC. (2019c). Western Canadian Cow-Calf Survey of 2018. *Reproductive Management: Adoption Rates of Recommended Practices by BCRC*, 25–33.

Berry, S. M., Stone, J., Norton, M. E., Johnson, D., & Berghella, V. (2013). Fetal blood sampling. *American Journal of Obstetrics and Gynecology*, *209*(3), 170–180.

Beutler, E., Gelbart, T., & Kuhl, W. (1990). Interference of heparin with the polymerase chain reaction. *BioTechniques*, *9*(2), 166.

Bianchi, D. W. (1999). Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. *British Journal of Haematology*, *105*(3), 574–583.

Bianchi, D. W. (2012). From prenatal genomic diagnosis to fetal personalized medicine: progress and challenges. *Nature Medicine*, *18*(7), 1041–1051.

Bianchi, D. W., LeShane, E. S., & Cowan, J. M. (2001). Large Amounts of Cell-free Fetal DNA Are Present in Amniotic Fluid. *Clinical Chemistry*, *47*(10), 1867–1869.

Bischoff, F. Z., Hahn, S., Johnson, K. L., Simpson, J. L., Bianchi, D. W., Lewis, D. E., Weber, W. D., Klinger, K., Elias, S., Jackson, L. G., Evans, M. I., Holzgreve, W., & De la Cruz, F. (2003). Intact fetal cells in maternal plasma: are they really there? *The Lancet*, *361*(9352),

139–140.

Bischoff, F. Z., Lewis, D. E., & Simpson, J. L. (2005). Cell-free fetal DNA in maternal blood: kinetics, source and structure. *Human Reproduction Update*, *11*(1), 59–67.

Bongso, T. A., & Basrur, P. K. (1975). Prenatal diagnosis of sex in cattle by amniocentesis. *The Veterinary Record*, *96*(6), 124–126.

Bongso, T. A., Wettimuny, S. G., Edirisinghe, R., & Kumaratileke, L. (1978). Determination of fetal sex in cattle by cytologic examiantion of amniotic fluid. *American Journal of Veterinary Research*, *39*(9), 1545–1546.

Brambati, B., & Simoni, G. (1983). Diagnosis of fetal Trisomy 21 in first trimester. *The Lancet*, *321*(8324), 585–586.

Breveglieri, G., D'aversa, E., Finotti, A., & Borgatti, M. (2019). Non-invasive prenatal testing using fetal DNA. *Molecular Diagnosis & Therapy*, *23*(2), 291–299.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., & Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science*, *303*(5663), 1532–1535.

Brison, N., Neofytou, M., Dehaspe, L., Bayindir, B., Bogaert, K. Van Den, Dardour, L., Peeters, H., Esch, H. Van, Buggenhout, G. Van, Vogels, A., Ravel, T. de, Legius, E., Devriendt, K., & Vermeesch, J. R. (2018). Predicting fetoplacental chromosomal mosaicism during non-invasive prenatal testing. *Prenatal Diagnosis*, *38*(4), 258–266.

Bronkhorst, A. J., Aucamp, J., & Pretorius, P. J. (2015). Cell-free DNA: Preanalytical variables. *Clinica Chimica Acta*, *450*, 243–253.

Burton, G. J., & Fowden, A. L. (2015). The placenta: a multifaceted, transient organ. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1663), 20140066.

Burton, G. J., Kaufmann, P., & Huppertz, B. (2006). Anatomy and genesis of the placenta. In *Knobil and Neill's Physiology of Reproduction* (pp. 189–243).

Canick, J. A., Palomaki, G. E., Kloza, E. M., Lambert-Messerlian, G. M., & Haddow, J. E. (2013). The impact of maternal plasma DNA fetal fraction on next generation sequencing tests for common fetal aneuploidies. *Prenatal Diagnosis*, *33*(7), 667–674.

Carlson, L. M., & Vora, N. L. (2017). Prenatal Diagnosis: Screening and Diagnostic Tools. *Obstetrics and Gynecology Clinics of North America*, *44*(2), 245.

Carter, A. M. (2012). Evolution of Placental Function in Mammals: The Molecular Basis of Gas and Nutrient Transfer, Hormone Secretion, and Immune Responses. *Physiological Reviews*, *92*(4), 1543–1576.

Carter, A. M. (2019). Evolution of placentation in cattle and antelopes. *Animal Reproduction*, *16*(1), 3.

Carter, A. M., & Martin, R. D. (2010). Comparative anatomy and placental evolution. In *Placental Bed Disorders* (pp. 109–126).

Carter, A. M., & Mess, A. M. (2018). The evolution of fetal membranes and placentation in carnivores and ungulates (Ferungulata). *Animal Reproduction*, *14*(1), 124–135.

CCA. (2020). *Beef industry stats*. Canadian Cattlemen's Association. https://www.cattle.ca/cca-resources/industry-stats/#:~:text=Canada produces approximately 1.3 million tonnes of beef,a new record high in beef export value.

Chan, K. A., Yeung, S. W., Lui, W. B., Rainer, T. H., & Lo, Y. D. (2005). Effects of Preanalytical Factors on the Molecular Size of Cell-Free DNA in Blood. *Clinical Chemistry*, *51*(4), 781–784.

Chan, K. C. A., Zhang, J., Hui, A. B. Y., Wong, N., Lau, T. K., Leung, T. N., Lo, K. W., Huang, D. W. S., & Lo, Y. M. D. (2004). Size Distributions of Maternal and Fetal DNA in Maternal Plasma. *Clinical Chemistry*, *50*(1), 88–92.

Chang, C. P. Y., Chia, R. H., Wu, T. L., Tsao, K. C., Sun, C. F., & Wu, J. T. (2003). Elevated cell-free serum DNA detected in patients with myocardial infarction. *Clinica Chimica Acta*, *327*(1–2), 95–101.

Chiu, R. W. K., Poon, L. L. M., Lau, T. K., Leung, T. N., Wong, E. M. C., & Lo, Y. M. D. (2001). Effects of Blood-Processing Protocols on Fetal and Total DNA Quantification in Maternal Plasma. *Clinical Chemistry*, *47*(9), 1607–1613.

Cicchillitti, L., Corrado, G., Angeli, M. De, Mancini, E., Baiocco, E., Patrizi, L., Zampa, A., Merola, R., Martayan, A., Conti, L., Piaggio, G., & Vizza, E. (2017). Circulating cell-free DNA content as blood based biomarker in endometrial cancer. *Oncotarget*, *8*(70), 115230–155243.

Colmant, C., Morin-Surroca, M., Fuchs, F., Fernandez, H., & Senat, M. V. (2013). Non-invasive prenatal testing for fetal sex determination: is ultrasound still relevant? *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *171*(2), 197–204.

Comey, C., Koons, B., Presley, K., Smerick, J., Sobieralski, C., Stanley, D., & Baechtel, F. (1994). DNA Extraction Strategies for Amplified Fragment Length Polymorphism Analysis. *Journal of Forensic Science*, *39*(5), 1254–1269.

Comline, R. S., Hall, L. W., Lavelle, R. B., Nathanielsz, P. W., & Silver, M. (1974). Parturition in the cow: Endocrine changes in animals with chronically implanted catheters in the foetal and maternal circulations. *Journal of Endocrinology*, *63*(3), 451–472.

Comline, R. S., Hall, L. W., Lavelle, R., & Silver, M. (1975). The use of intravascular catheters for long-term studies on the mare and fetus. *Journal of Reproduction and Fertility. Supplement*, *Oct*(23), 583–588.

Cornelis, G., Heidmann, O., Degrelle, S. A., Vernochet, C., Lavialle, C., Letzelter, C., Bernard-Stoecklin, S., Hassanin, A., Mulot, B., Guillomot, M., Hue, I., Heidmann, T., & Dupressoir, A. (2013). Captured retroviral envelope syncytin gene associated with the unique placental structure of higher ruminants. *Proceedings of the National Academy of Sciences*, *110*(9), E828–E837.

Cran, D., Johnson, L., Practitioner, N. M.-T. B., & 1993, U. (1993). Production of Bovine Calves Following Separation of X-and Y-Chromosome Bearing Sperm and In Vitro. *The Bovine*

Practitioner, *132*(2), 143–144.

CRS. (2020). *CanFax Research Services, The cattle cycle-Global impacts and domestic indicators*.

CRS. (2021a). *CanFax Research Services, Statistical briefer*.

CRS. (2021b). *CanFax Research Services, The two-stage cattle cycle in Canada-factsheet*.

Cruz-Lemini, M., Parra-Saavedra, M., Borobio, V., Bennasar, M., Goncégonc´goncé, A., Mart´inez, J. M., Mart´inez, M., & Borrell, A. (2014). How to perform an amniocentesis. *Ultrasound in Obstetrics & Gynecology*, *44*(6), 727–731.

Cruz, A. S., Silva, D. C., Costa, E. O. A., Silva, D. M., Abreu, D. A., Cruz, A. D., Replicon, N. P., & De Biologia, D. (2011). Sensibilidade da PCR na amplificação do DNA bovino em diluição seriada e mistura de amostra macho e fêmea [Sensitivity of PCR to amplify sex-specific bovine DNA in diluted samples]. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*, *63*, 1012–1015.

Cullen, M. T., Reece, E. A., Whetham, J., & Hobbins, J. C. (1990). Embryoscopy: Description and utility of a new technique. *American Journal of Obstetrics & Gynecology*, *162*(1), 82–86.

Curnow, K. J., Gross, S. J., Hall, M. P., Stosic, M., Demko, Z., Zimmermann, B., Hill, M., Sigurjonsson, S., Ryan, A., Banjevic, M., Kolacki, P. L., Koch, S. W., Strom, C. M., Rabinowitz, M., & Benn, P. (2014). Clinical experience and follow-up with large scale single-nucleotide polymorphism–based noninvasive prenatal aneuploidy testing. *American Journal of Obstetrics and Gynecology*, *211*(5), 527.e1-527.e17.

Curran, S. (1992). Fetal sex determination in cattle and horses by ultrasonography. *Theriogenology*, *37*(1), 17–21.

Curran, S., Kastelic, J. P., & Ginther, O. J. (1989). Determining sex of the bovine fetus by ultrasonic assessment of the relative location of the genital tubercle. *Animal Reproduction Science*, *19*(3–4), 217–227.

D'Aversa, E., Breveglieri, G., Pellegatti, P., Guerra, G., Gambari, R., & Borgatti, M. (2018). Non-invasive fetal sex diagnosis in plasma of early weeks pregnants using droplet digital PCR. *Molecular Medicine*, *24*(1), 1–8.

Da Cruz, A. S., Silva, D. C., Costa, E. O. A., De M-Jr., P., Da Silva, C. C., Silva, D. M., & Da Cruz, A. D. (2012). Cattle fetal sex determination by polymerase chain reaction using DNA isolated from maternal plasma. *Animal Reproduction Science*, *131*(1–2), 49–53.

Daffos, F., Capella-Pavlovsky, M., & Forestier, F. (1983). Fetal blood sampling via the umbilical cord using a needle guided by ultrasound report of 66 cases. *Prenatal Diagnosis*, *3*(4), 271–277.

Daffos, F., Capella-Pavlovsky, M., & Forestier, F. (1985). Fetal blood, sampling during pregnancy with use of a needle guided by ultrasound: A study of 606 consecutive cases. *American Journal of Obstetrics and Gynecology*, *153*(6), 655–660.

Dar, P., Shani, H., & Evans, M. I. (2016). Cell-free DNA: Comparison of Technologies. *Clinics in Laboratory Medicine*, *36*(2), 199–211.

Davoudi, A., Aleyasin, A., & Tarang, A. (2011). The application of amplified TSPY and amelogenin genes from maternal plasma as a non-invasive bovine fetal DNA diagnosis. *Eurasian Journal of Biosciences*, *5*(1), 119–126.

Davoudi, A., Tarang, A., Aleyasin, S. A., Salehi, A., Seighalani, R., & Tahmoressi, F. (2012). Evaluation of two DNA extraction methods from maternal plasma for using in non-invasive bovine fetus gender determination. *Iranian Journal of Reproductive Medicine*, *10*(6), 523.

De Graaf, S. P., Leahy, T., & Vishwanath, R. (2014). *Biological and practical lessons associated with the use of sexed semen*.

de Leon, P. M. M., Campos, V. F., Dellagostin, O. A., Deschamps, J. C., Seixas, F. K., & Collares, T. (2012). Equine fetal sex determination using circulating cell-free fetal DNA (ccffDNA). *Theriogenology*, *77*(3), 694–698.

Dennis Lo, Y. M., Chan, K. C. A., Sun, H., Chen, E. Z., Jiang, P., Lun, F. M. F., Zheng, Y. W., Leung, T. Y., Lau, T. K., Cantor, C. R., & Chiu, R. W. K. (2010). Maternal Plasma DNA Sequencing Reveals the Genome-Wide Genetic and Mutational Profile of the Fetus. *Science Translational Medicine*, *2*(61), 61ra91.

Dennis Lo, Y. M., Corbetta, N., Chamberlain, P. F., Rai, V., Sargent, I. L., Redman, C. W. G., & Wainscoat, J. S. (1997). Presence of fetal DNA in maternal plasma and serum. *The Lancet*, *350*(9076), 485–487.

Dennis Lo, Y. M., Hjelm, N. M., Fidler, C., Sargent, I. L., Murphy, M. F., Chamberlain, P. F., Poon, P. M. K., Redman, C. W. G., & Wainscoat, J. S. (1998). Prenatal Diagnosis of Fetal RhD Status by Molecular Analysis of Maternal Plasma. *New England Journal of Medicine*, *339*(24), 1734–1738.

Dennis Lo, Y. M., Patel, P., Sampietro, M., Gillmer, M. D. G., Fleming, K. A., & Wainscoat, J. S. (1990). Detection of single-copy fetal DNA sequence from maternal blood. *The Lancet*, *335*(8703), 1463–1464.

Dennis Lo, Y. M., Tein, M. S. C., Lau, T. K., Haines, C. J., Leung, T. N., Poon, P. M. K., Wainscoat, J. S., Johnson, P. J., Chang, A. M. Z., & Hjelm, N. M. (1998). Quantitative Analysis of Fetal DNA in Maternal Plasma and Serum: Implications for Noninvasive Prenatal Diagnosis. *The American Journal of Human Genetics*, *62*(4), 768–775.

Dennis Lo, Y. M., Wainscoat, J. S., Gillmer, M. D. G., Patel, P., Sampietro, M., & Fleming, K. A. (1989). Prenatal sex determination by DNA amplification from maternal peripheral blood. *The Lancet*, *334*(8676), 1363–1365.

Dennis Lo, Y. M., Zhang, J., Leung, T. N., Lau, T. K., Chang, A. M. Z., & Magnus Hjelm, N. (1999). Rapid Clearance of Fetal DNA from Maternal Plasma. *The American Journal of Human Genetics*, *64*(1), 218–224.

Desai, R. G., Creger, W. P., Gausepohl, N., & Loomis, M. (1963). Maternofetal Passage of Leukocytes and Platelets in Man. *Blood*, *21*(6), 665–673.

DesCoteaux, L., Gnemmi, G., & Colloton, J. (2010). *Practical Atlas of Ruminant and Camelid Reproductive Ultrasonography*.

Devaney, S. A., Palomaki, G. E., Scott, J. A., & Bianchi, D. W. (2011). Noninvasive Fetal Sex Determination Using Cell-Free Fetal DNA: A Systematic Review and Meta-analysis. *JAMA*, *306*(6), 627–636.

Donald, I., Macvicar, J., & Brown, T. G. (1958). Investigation of abdominal masses by pulsed ultrasound. *The Lancet*, *271*(7032), 1188–1195.

Drury, S., Hill, M., & Chitty, L. S. (2016). Cell-Free Fetal DNA Testing for Prenatal Diagnosis. *Advances in Clinical Chemistry*, *76*, 1–35.

Eaglesome, M. D., & Mitchell, D. (1977). Collection and cytogenetics of fetal fluids from heifers during the third month of pregnancy I. Collection. *Theriogenology*, *7*(4), 195–201.

Eetvelde, M. Van, Kamal, M. M., Hostens, M., Vandaele, L., Fiems, L. O., & Opsomer, G. (2016). Evidence for placental compensation in cattle. *Animal*, *10*(8), 1342–1350.

El Messaoudi, S., Rolet, F., Mouliere, F., & Thierry, A. R. (2013). Circulating cell free DNA: Preanalytical considerations. *Clinica Chimica Acta*, *424*, 222–230.

Elshimali, Y. I., Khaddour, H., Sarkissyan, M., Wu, Y., & Vadgama, J. V. (2013). The Clinical Utilization of Circulating Cell Free DNA (CCFDNA) in Blood of Cancer Patients. *International Journal of Molecular Sciences*, *14*(9), 18925–18958.

Emerson, D. S., Felker, R. E., & Brown, D. L. (1989). The sagittal sign. An early second trimester sonographic indicator of fetal gender. *Journal of Ultrasound in Medicine*, *8*(6), 293–297.

Enders, A. C., & Blankenship, T. N. (1999). Comparative placental structure. *Advanced Drug Delivery Reviews*, *38*(1), 3–15.

Enders, A. C., & Carter, A. M. (2006). Comparative Placentation: Some Interesting Modifications for Histotrophic Nutrition – A Review. *Placenta*, *27*(SUPPL.), 11–16.

Enders, A. C., & Liu, I. K. M. (1991). Trophoblast-Uterine interactions during equine chorionic girdle cell maturation, migration, and transformation. *American Journal of Anatomy*, *192*(4), 366–381.

Fan, H. C., Blumenfeld, Y. J., Chitkara, U., Hudgins, L., & Quake, S. R. (2010). Analysis of the Size Distributions of Fetal and Maternal Cell-Free DNA by Paired-End Sequencing. *Clinical Chemistry*, *56*(8), 1279–1286.

Fan, H. C., & Quake, S. R. (2010). Sensitivity of Noninvasive Prenatal Detection of Fetal Aneuploidy from Maternal Plasma Using Shotgun Sequencing Is Limited Only by Counting Statistics. *PLOS ONE*, *5*(5), e10439.

Favaron, P. O., Morini, J. C., Mess, A. M., Miglino, M. A., & Ambrósio, C. E. (2014). Placentation and fetal membrane development in the South American coati, Nasua nasua(Mammalia, Carnivora, Procyonidae). *Reproductive Biology and Endocrinology*, *12*(1), 1–7.

Fernández-Martínez, F. J., Galindo, A., Garcia-Burguillo, A., Vargas-Gallego, C., Nogués, N., Moreno-García, M., & Moreno-Izquierdo, A. (2012). Noninvasive fetal sex determination in maternal plasma: a prospective feasibility study. *Genetics in Medicine*, *14*(1), 101–106.

Fernando, M. R., Chen, K., Norton, S., Krzyzanowski, G., Bourne, D., Hunsley, B., Ryan, W. L.,

& Bassett, C. (2010). A new methodology to preserve the original proportion and integrity of cell-free fetal DNA in maternal plasma during sample processing and storage. *Prenatal Diagnosis*, *30*(5), 418–424.

Ferrari, M., Carrera, P., Lampasona, V., & Galbiati, S. (2015). New trend in non-invasive prenatal diagnosis. *Clinica Chimica Acta*, *451*, 9–13.

Findlay, I., Atkinson, G., Chambers, M., Quirke, P., Campbell, J., & Rutherford, A. (1996). Diagnostic techniques: Rapid genetic diagnosis at 7–9 weeks gestation: diagnosis of sex, single gene defects and DNA fingerprint from coelomic samples. *Human Reproduction*, *11*(11), 2548–2553.

Fleischhacker, M., & Schmidt, B. (2007). Circulating nucleic acids (CNAs) and cancer—A survey. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1775*(1), 181–232.

Flores-Foxworth, G. (2007). Reproductive biotechnologies in the goat. In *Current Therapy in Large Animal Theriogenology* (pp. 603–614).

Fontes, P. L. P., Oosthuizen, N., & Cliff Lamb, G. (2020). Reproductive management of beef cattle. In *Animal Agriculture: Sustainability, Challenges and Innovations* (pp. 57–73). Academic Press.

Frank, M. O. (2016). Circulating cell-free DNA differentiates severity of inflammation. *Biological Research for Nursing*, *18*(5), 477–488.

Freda, V. J., & Adamsons Jr, K. (1964). Exchange transfusion in utero, report of a case. *American Journal of Obstetrics and Gynecology*, *89*(6), 817–821.

Freyer, C., & Renfree, M. B. (2009). The mammalian yolk sac placenta. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *312B*(6), 545–554.

Fricke, P. M., Ricci, A., Giordano, J. O., & Carvalho, P. D. (2016). Methods for and Implementation of Pregnancy Diagnosis in Dairy Cows. *Veterinary Clinics: Food Animal Practice*, *32*(1), 165–180.

Friess, A. E., Sinowatz, F., Skolek-Winnisch, R., & Triutner, W. (1980). Anatomy and Embryology The Placenta of the Pig 1. Finestructural Changes of the Placental Barrier During Pregnancy. *Anatomy and Embryology*, *158*(2), 179–191.

Fuchs, F., & Riis, P. (1956). Antenatal Sex Determination. *Nature*, *177*(4503), 330.

Furukawa, S., Kuroda, Y., & Sugiyama, A. (2014). A Comparison of the Histological Structure of the Placenta in Experimental Animals. *Journal of Toxicologic Pathology*, *27*(1), 11–18.

Garcia, A., & Salaheddine, M. (1997). Bovine ultrasound-guided transvaginal amniocentesis. *Theriogenology*, *47*(5), 1003–1008.

Gardiner, C., Harrison, P., Belting, M., Böing, A., Campello, E., Carter, B. S., Collier, M. E., Coumans, F., Ettelaie, C., Es, N. van, Hochberg, F. H., Mackman, N., Rennert, R. C., Thaler, J., Rak, J., & Nieuwland, R. (2015). Extracellular vesicles, tissue factor, cancer and thrombosis – discussion themes of the ISEV 2014 Educational Day. *Journal of Extracellular Vesicles*, *4*(1), 1–14.

Garner, D. L. (2009). Hoechst 33342: The dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. *Theriogenology*, *71*(1), 11–21.

Garner, D. L., Evans, K. M., & Seidel, G. E. (2013). Sex-Sorting Sperm Using Flow Cytometry/Cell Sorting. *Methods in Molecular Biology*, *927*, 279–295.

Garner, D. L., & Seidel, G. E. (2008). History of commercializing sexed semen for cattle. *Theriogenology*, *69*(7), 886–895.

Ginkel, J. H. van, Broek, D. A. van den, Kuik, J. van, Linders, D., Weger, R. de, Willems, S. M., & Huibers, M. M. H. (2017). Preanalytical blood sample workup for cell-free DNA analysis using Droplet Digital PCR for future molecular cancer diagnostics. *Cancer Medicine*, *6*(10), 2297–2307.

Grabuschnig, S., Bronkhorst, A. J., Holdenrieder, S., Rodriguez, I. R., Schliep, K. P., Schwendenwein, D., Ungerer, V., & Sensen, C. W. (2020). Putative Origins of Cell-Free DNA in Humans: A Review of Active and Passive Nucleic Acid Release Mechanisms. *International Journal of Molecular Sciences*, *21*(21), 8062.

Grace, M. R., Hardisty, E., Dotters-Katz, S. K., Vora, N. L., & Kuller, J. A. (2016). Cell-Free DNA Screening: Complexities and Challenges of Clinical Implementation. *Obstetrical & Gynecological Survey*, *71*(8), 477.

Greenstein, J. S., & Foley, R. C. (1958). Early Embryology of the Cow. I. Gastrula and Primitive Streak Stages,. *Journal of Dairy Science*, *41*(3), 409–421.

Grosser. (1909). *Vergleichende anatomie und entwicklungsgeschichte der eihäute und der placenta: mit besonderer berücksichtigung des menschen. Wilhelm Braumüller.*

Guernsey, M. W., Chuong, E. B., Cornelis, G., Renfree, M. B., & Baker, J. C. (2017). Molecular conservation of marsupial and eutherian placentation and lactation. *ELife*, *6*, e27450.

Guibert, J., Benachi, A., Grebille, A. G., Ernault, P., Zorn, J. R., & Costa, J. M. (2003). Kinetics of SRY gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique. *Human Reproduction*, *18*(8), 1733–1736.

Hall, J. B., & Glaze, J. B. (2014). Sexed semen-How it is produced and how can we use it efficiently. *In Proceedings of the 2014 Applied Reproductive Strategies in Beef Cattle Conference*, 215–230.

Hamilton, W. J., & Boyd, J. D. (1960). Development of the human placenta in the first three months of gestation. *Journal of Anatomy*, *94*(Pt 3), 297.

Hanzen, C., & Delsaux, B. (1987). Use of transrectal B-mode ultrasound imaging in bovine pregnancy diagnosis. *The Veterinary Record*, *121*(9), 200–202.

Hedriana, H., Martin, K., Saltzman, D., Billings, P., Demko, Z., & Benn, P. (2020). Cell-free DNA fetal fraction in twin gestations in single-nucleotide polymorphism-based noninvasive prenatal screening. *Prenatal Diagnosis*, *40*(2), 179–184.

Herzenberg, L. A., Bianchi, D. W., Schröder, J., Cann, H. M., & Iverson, G. M. (1979). Fetal cells in the blood of pregnant women: detection and enrichment by fluorescence-activated cell

sorting. *Proceedings of the National Academy of Sciences*, *76*(3), 1453–1455.

Heuer, C., Kendall, D., Sun, C., Deeb, J., Moreno, J., & Vishwanath, R. (2017). Evaluation of conception rates of sex-sorted semen in commercial dairy farms over the last five years. *In ADSA Annual Meeting. Pittsburgh, Pennsylvania: American Dairy Science Association*.

Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous, S., Vignon, X., & Renard, J. P. (2002). Frequency and Occurrence of Late-Gestation Losses from Cattle Cloned Embryos. *Biology of Reproduction*, *66*(1), 6–13.

Hirayama, H., Kageyama, S., Moriyasu, S., Sawai, K., & Minamihashi, A. (2013). Embryo Sexing and Sex Chromosomal Chimerism Analysis by Loop-Mediated Isothermal Amplification in Cattle and Water Buffaloes. *Journal of Reproduction and Development*, *59*(4), 321–326.

Holden, S. A., & Butler, S. T. (2018). Review: Applications and benefits of sexed semen in dairy and beef herds. *Animal*, *12*(s1), s97–s103.

Holdenrieder, S., Burges, A., Reich, O., Spelsberg, F. W., & Stieber, P. (2008). DNA Integrity in Plasma and Serum of Patients with Malignant and Benign Diseases. *Annals of the New York Academy of Sciences*, *1137*(1), 162–170.

Honda, H., Miharu, N., Ohashi, Y., Samura, O., Kinutani, M., Hara, T., & Ohama, K. (2001). Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Human Genetics*, *110*(1), 75–79.

Hufnagl, C., Stöcher, M., Moik, M., Geisberger, R., & Greil, R. (2013). A modified Phenol-chloroform extraction method for isolating circulating cell free DNA of tumor patients. *Journal of Nucleic Acids Investigation*, *4*(1), e1.

Hui, L., Bethune, M., Weeks, A., Kelley, J., & Hayes, L. (2014). Repeated failed non-invasive prenatal testing owing to low cell-free fetal DNA fraction and increased variance in a woman with severe autoimmune disease. *Ultrasound in Obstetrics & Gynecology*, *44*(2), 242–243.

Hui, L., & Bianchi, D. W. (2020). Fetal fraction and noninvasive prenatal testing: What clinicians need to know. *Prenatal Diagnosis*, *40*(2), 155–163.

Hutchison, J. L., Bickhart, D. M., Souza, A. H., Bessoff, H. J., Danzeisen, E., & Simpson, A. (2016). Sexed-semen usage for Holstein AI in the United States. *Journal of Animal Science*, *94*, 180.

Hylan, D., Giraldo, A. M., Carter, J. A., Gentry, G. T., Bondioli, K. R., & Godke, R. A. (2009). Sex Ratio of Bovine Embryos and Calves Originating from the Left and Right Ovaries. *Biology of Reproduction*, *81*(5), 933–938.

Inoue, T., Miyake, Y., Yamanaka, H., & Kawata, K. (1982). Studies on amniocentesis in cattle. *Japanese Journal of Animal Reproduction*, *28*(4), 179–183.

Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F. O., Hesch, R. D., & Knippers, R. (2001). DNA Fragments in the Blood Plasma of Cancer Patients: Quantitations and Evidence for Their Origin from Apoptotic and Necrotic Cells. *Cancer Research*, *61*(4), 1659–1665.

Jain, M., Balatsky, A. V., Revina, D. B., & Samokhodskaya, L. M. (2019). Direct comparison of

QIAamp DSP Virus Kit and QIAamp Circulating Nucleic Acid Kit regarding cell-free fetal DNA isolation from maternal peripheral blood. *Molecular and Cellular Probes*, *43*, 13–19.

Jauniaux, E., Jurkovic, D., & Gulbis, B. (2003). Coelocentesis: Ultrasound and endoscopic surgery in obstetrics and gynaecology. In *A Combined Approach to Diagnosis and Treatment* (pp. 316–323).

Jelinski, M. (2012). Beef practice models. *American Association of Bovine Practitioners Proceedings of the Annual Conference*, 22–25.

Johnson, L. A., Flook, J. P., & Hawk, H. W. (1989). Sex Preselection in Rabbits: Live Births from X and Y Sperm Separated by DNA and Cell Sorting. *Biology of Reproduction*, *41*(2), 199–203.

Jones, C. J. P., & Jauniaux, E. (1995). Ultrastructure of the materno-embryonic interface in the first trimester of pregnancy. *Micron*, *26*(2), 145–173.

Jorgez, C. J., Dang, D. D., Simpson, J. L., Lewis, D. E., & Bischoff, F. Z. (2006). Quantity versus quality: Optimal methods for cell-free DNA isolation from plasma of pregnant women. *Genetics in Medicine*, *8*(10), 615–619.

Jung, R., Lübcke, C., Wagener, C., & Neumaier, M. (1997). Reversal of RT-PCR inhibition observed in heparinized clinical specimens. *BioTechniques*, *23*(1), 24–28.

Jurkovic, D., Jauniaux, E., Campbell, S., Pandya, P., Nicolaides, K. H., & Cardy, D. L. (1993). Coelocentesis: a new technique for early prenatal diagnosis. *The Lancet*, *341*(8861), 1623–1624.

Jylhävä, J., Kotipelto, T., Raitala, A., Jylhä, M., Hervonen, A., & Hurme, M. (2011). Aging is associated with quantitative and qualitative changes in circulating cell-free DNA: The Vitality 90+ study. *Mechanisms of Ageing and Development*, *132*(1–2), 20–26.

Kadivar, A., Dehghani Tafti, R., Heidari Khoei, H., Heidari Nasirabadi, M., Shams Esfandabadi, N., Cheraghi, N., & Davoodian, N. (2016). Developing a Nested Real-Time PCR Assay for Determining Equine Fetal Sex Prenatally. *Journal of Equine Veterinary Science*, *40*, 74–79.

Kadivar, A., Hassanpour, H., & Amiri Dehcheshmeh, J. (2015). A novel approach to prenatal fetal sex diagnosis by detecting an insertion in the Y-chromosome of ovine Amelogenin gene. *Small Ruminant Research*, *123*(2–3), 218–223.

Kadivar, A., Hassanpour, H., Mirshokraei, P., Azari, M., Gholamhosseini, K., & Karami, A. (2013). Detection and quantification of cell-free fetal DNA in ovine maternal plasma; use it to predict fetal sex. *Theriogenology*, *79*(6), 995–1000.

Kadivar, A., Rashidzadeh, H., Davoodian, N., Nazari, H., Tafti, R. D., Khoei, H. H., Samani, H. S., Modaresi, J., & Ahmadi, E. (2021). Evaluation of the efficiency of TaqMan duplex real-time PCR assay for non-invasive pre-natal assessment of foetal sex in equine. *Reproduction in Domestic Animals*, *56*(2), 287–291.

Kadokawa, H., Takusari, N., Minezawa, M., Takahashi, H., & Kariya, T. (1996). 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*(3), 205–208.

Kamimura, S., Kondoh, S., Enomoto, S., Sameshima, H., Goto, K., & Hamana, K. (1994). Ultrasonic Diagnosis of Bovine Fetal Sex by the Relative Location of the Genital Tubercle in Early Pregnancy. *Journal of Reproduction and Development*, *40*(4), 343–347.

Kamimura, S., Nishiyama, N., Ookutsu, S., Goto, K., & Hamana, K. (1997). Determination of bovine fetal sex by PCR using fetal fluid aspirated by transvaginal ultrasound-guided amniocentesis. *Theriogenology*, *47*(8), 1563–1569.

Kazachkova, N., Gontar, J., Verlinsky, O., & Ilyin, I. (2019). Successful early fetal sex determination using cell-free fetal DNA isolated from maternal capillary blood: A pilot study. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *3*, 100038.

Kearin, M., Pollard, K., & Garbett, I. (2014). Accuracy of sonographic fetal gender determination: predictions made by sonographers during routine obstetric ultrasound scans. *Australasian Journal of Ultrasound in Medicine*, *17*(3), 125–130.

Kelley, K. (2010). Amniocentesis Prior to 1980. In *Embryo Project Encyclopedia*. Arizona State University. School of Life Sciences. Center for Biology and Society. Embryo Project Encyclopedia.

Keshavarz, Z., Moezzi, L., Ranjbaran, R., Aboualizadeh, F., Behzad-Behbahani, A., Abdullahi, M., & Sharifzadeh, S. (2015). Evaluation of a Modified DNA Extraction Method for Isolation of Cell-Free Fetal DNA from Maternal Serum. *Avicenna Journal of Medical Biotechnology*, *7*(2), 85.

King, B. F. (1992). Comparative Studies of Structure and Function in Mammalian Placentas with Special Reference to Maternal-Fetal Transfer of Iron. *Integrative and Comparative Biology*, *32*(2), 331–342.

Kinnings, S. L., Geis, J. A., Almasri, E., Wang, H., Guan, X., McCullough, R. M., Bombard, A. T., Saldivar, J. S., Oeth, P., & Deciu, C. (2015). Factors affecting levels of circulating cell-free fetal DNA in maternal plasma and their implications for noninvasive prenatal testing. *Prenatal Diagnosis*, *35*(8), 816–822.

Köchl, S., Niederstätter, H., & Parson, W. (2005). DNA Extraction and Quantitation of Forensic Samples Using the Phenol-Chloroform Method and Real-Time PCR. *Methods in Molecular Biology*, *297*, 13–29.

Kruse, R., Tess, M., Grings, E., & SHORT, R. (2004). Evaluation of beef cattle operations utilizing different seasons of calving, weaning strategies and post-weaning management. *Journal of Animal Science*, *82*(2), 111.

Lam, N. Y. L., Rainer, T. H., Chiu, R. W. K., & Lo, Y. M. D. (2004). EDTA Is a Better Anticoagulant than Heparin or Citrate for Delayed Blood Processing for Plasma DNA Analysis. *Clinical Chemistry*, *50*(1), 256–257.

Lamb, G. C., Mercadante, V. R. G., Henry, D. D., Fontes, P. L. P., Dahlen, C. R., Larson, J. E., & DiLorenzo, N. (2016). Invited Review: Advantages of current and future reproductive technologies for beef cattle production. *The Professional Animal Scientist*, *32*(2), 162–171.

Lamothe, S. (2018). Northern Beef Study: Northern Ontario and Northern Quebec Beef Cow-Calf Productions. *North Haven Solutions*.

Larson, J. E., & Parish, J. A. (2015). *Embryo transfer in the beef herd*.

Lee, T. H., Montalvo, L., Chrebtow, V., & Busch, M. P. (2001). Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion*, *41*(2), 276–282.

Lee, T. J., Rolnik, D. L., Menezes, M. A., McLennan, A. C., & da Silva Costa, F. (2018). Cell-free fetal DNA testing in singleton IVF conceptions. *Human Reproduction*, *33*(4), 572–578.

Leibo, S. P., & Rall, W. F. (1990). Prenatal diagnosis of sex in bovine fetuses by amniocentesis. *Theriogenology*, *33*(2), 531–552.

Leiser, R., & Koob, B. (1993). Development and characteristics of placentation in a carnivore, the domestic cat. *Journal of Experimental Zoology*, *266*(6), 642–656.

Leiser, R., Pfarrer, C., Abd-Elnaeim, M., & Dantzer, V. (1998). Feto-maternal anchorage in epitheliochorial and endotheliochorial placental types studied by histology and microvascular corrosion casts. *Placenta*, *19*, 21–39.

Lemarchand, K., Berthiaume, F., Maynard, C., Harel, J., Payment, P., Bayardelle, P., Masson, L., & Brousseau, R. (2005). Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. *Journal of Microbiological Methods*, *63*(2), 115–126.

Lemos, D. C., Takeuchi, P. L., Rios, Á. F. L., Araújo, A., Lemos, H. C., & Ramos, E. S. (2011). Bovine fetal DNA in the maternal circulation: Applications and implications. *Placenta*, *32*(11), 912–913.

Lench, N., Barrett, A., Fielding, S., McKay, F., Hill, M., Jenkins, L., White, H., & Chitty, L. S. (2013). The clinical implementation of non-invasive prenatal diagnosis for single-gene disorders: challenges and progress made. *Prenatal Diagnosis*, *33*(6), 555–562.

Leupp, J., Lardy, G., Daly, R., Wright, C., & Paterson, J. (2009). Factors influencing price of North Dakota, South Dakota and Montana feeder calves. *Range/Wildlife*, *7415*(1515), 46.

Lewis, A. R., Valle, J. W., & McNamara, M. G. (2016). Pancreatic cancer: Are "liquid biopsies" ready for prime-time? *World Journal of Gastroenterology*, *22*(32), 7175.

Li, Y., Zimmermann, B., Rusterholz, C., Kang, A., Holzgreve, W., & Hahn, S. (2004). Size Separation of Circulatory DNA in Maternal Plasma Permits Ready Detection of Fetal DNA Polymorphisms. *Clinical Chemistry*, *50*(6), 1002–1011.

Ma, X., Zhu, L., Wu, X., Bao, H., Wang, X., Chang, Z., Shao, Y. W., & Wang, Z. (2017). Cell-Free DNA Provides a Good Representation of the Tumor Genome Despite Its Biased Fragmentation Patterns. *PLOS ONE*, *12*(1), e0169231.

Mair, R., Mouliere, F., Smith, C. G., Chandrananda, D., Gale, D., Marass, F., Tsui, D. W. Y., Massie, C. E., Wright, A. J., Watts, C., Rosenfeld, N., & Brindle, K. M. (2019). Measurement of Plasma Cell-Free Mitochondrial Tumor DNA Improves Detection of Glioblastoma in Patient-Derived Orthotopic Xenograft Models. *Cancer Research*, *79*(1), 220–230.

Malassiné, A., Frendo, J. L., & Evain-Brion, D. (2003). A comparison of placental development

and endocrine functions between the human and mouse model. *Human Reproduction Update*, *9*(6), 531–539.

Mandel, P. (1948). Les acides nucleiques du plasma sanguin chez 1 homme. *C R Seances Soc Biol Fil*, *142*, 241–243.

Manokhina, I., Singh, T. K., Peñaherrera, M. S., & Robinson, W. P. (2014). Quantification of Cell-Free DNA in Normal and Complicated Pregnancies: Overcoming Biological and Technical Issues. *PLOS ONE*, *9*(7), e101500.

Manzanares, S., Benítez, A., Naveiro-Fuentes, M., López-Criado, M. S., & Sánchez-Gila, M. (2016). Accuracy of fetal sex determination on ultrasound examination in the first trimester of pregnancy. *Journal of Clinical Ultrasound*, *44*(5), 272–277.

Marcon, H. S., Coscrato, V. E., Selivon, D., Perondini, A. L. P., & Marino, C. L. (2011). Variations in the sensitivity of different primers for detecting Wolbachia in Anastrepha (diptera: tephritidae). *Brazilian Journal of Microbiology*, *42*(2), 778–785.

Mascio, D., Khalil, A., Rizzo, G., Buca, D., Liberati, M., Martellucci, C. A., Flacco, M. E., Manzoli, L., & D'Antonio, F. (2020). Risk of fetal loss following amniocentesis or chorionic villus sampling in twin pregnancy: systematic review and meta-analysis. *Ultrasound in Obstetrics & Gynecology*, *56*(5), 647–655.

McKiernan, H. E., & Danielson, P. B. (2017). Molecular Diagnostic Applications in Forensic Science. In *Molecular Diagnostics: Third Edition* (pp. 371–394). Academic Press.

Meddeb, R., Pisareva, E., & Thierry, A. R. (2019). Guidelines for the Preanalytical Conditions for Analyzing Circulating Cell-Free DNA. *Clinical Chemistry*, *65*(5), 623–633.

Menees, T. O., Millar, J. D., & Holly, L. E. (1930). Amniography : Preliminary report. *American Journal of Roentgenology*, *24*, 353–366.

Mennuti, M. T., Chandrasekaran, S., Khalek, N., & Dugoff, L. (2015). Cell-free DNA screening and sex chromosome aneuploidies. *Prenatal Diagnosis*, *35*(10), 980–985.

Meschia, G., Barron, D. H., Cotter, J. R., & Breathnach, C. S. (1965). The Hemoglobin, Oxygen, Carbon dioxide and Hydrogen ion concentrations in the umbilical bloods of sheep and goats as sampled via indwelling plastic catheters. *Quarterly Journal of Experimental Physiology and Cognate Medical Sciences*, *50*(2), 185–195.

Miglino, M. A., Ambrósio, C. E., Martins, D. dos S., Wenceslau, C. V., Pfarrer, C., & Leiser, R. (2006). The carnivore pregnancy: The development of the embryo and fetal membranes. *Theriogenology*, *66*(6–7), 1699–1702.

Mihaly, G. W., & Morgan, D. J. (1983). Placental drug transfer: Effects of gestational age and species. *Pharmacology & Therapeutics*, *23*(2), 253–266.

Miller, R., Koszalka, T., & Brent, R. (1976). The transport of molecules across placental membranes. In *The Cell Surface in Animal Embryogenesis and Development* (pp. 145–222).

Mohr, J. (1968). Foetal genetic diagnosis: Development of techniques for early sampling of foetal cells. *Acta Pathologica Microbiologica Scandinavica*, *73*(1), 73–77.

Mortimer, R., & Hansen, T. (2006). The future of pregnancy testing in beef cattle. *Proceedings, Applied Reproductive Strategies in Beef Cattle*, 281–288.

Müller, E., & Wittkowski, G. (1986). Visualization of male and female characteristics of bovine fetuses by real-time ultrasonics. *Theriogenology*, *25*(4), 571–574.

Muzzin, A., & Ben-Ezra, E. (2015). *Developing an economic model for cow-calf producers*.

Nakano, H., Takahashi, T., Imai, K., & Hashizume, K. (2001). Expression of placental lactogen and cytokeratin in bovine placental binucleate cells in culture. *Cell and Tissue Research*, *303*(2), 263–270.

Naniwa, Y., Sakamoto, Y., Toda, S., & Uchiyama, K. (2019). Bovine sperm sex-selection technology in Japan. *Reproductive Medicine and Biology*, *18*(1), 17–26.

Nayyar, R. P., & Barr, M. L. (1966). Sex chromatin in rodents. *Canadian Journal of Genetics and Cytology*, *8*(4), 654–660.

Ng, E. K. O., Tsui, N. B. Y., Lau, T. K., Leung, T. N., Chiu, R. W. K., Panesar, N. S., Lit, L. C. W., Chan, K. W., & Lo, Y. M. D. (2003). mRNA of placental origin is readily detectable in maternal plasma. *Proceedings of the National Academy of Sciences*, *100*(8), 4748–4753.

Nicolaides, K. H., Syngelaki, A., Gil, M. M., Quezada, M. S., & Zinevich, Y. (2014). Prenatal Detection of Fetal Triploidy from Cell-Free DNA Testing in Maternal Blood. *Fetal Diagnosis and Therapy*, *35*(3), 212–217.

Nishimura, F., Uno, N., Chiang, P. C., Kaku, N., Morinaga, Y., Hasegawa, H., & Yanagihara, K. (2019). The Effect of In Vitro Hemolysis on Measurement of Cell-Free DNA. *The Journal of Applied Laboratory Medicine*, *4*(2), 235–240.

Noden, D., & Lahunta, A. (1985). *The embryology of domestic animals. Developmental mechanisms and malformations.*

Nolan, T., Hands, R. E., Ogunkolade, W., & Bustin, S. A. (2006). SPUD: A quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. *Analytical Biochemistry*, *351*, 308–310.

Northrop, E. J., Rich, J. J. J., Rhoades, J. R., & Perry, G. A. (2019). Comparison of two bovine serum pregnancy tests in detection of artificial insemination pregnancies and pregnancy loss in beef cattle. *PLOS ONE*, *14*(1), e0211179.

OECD-FAO. (2020). *OECD-FAO Agricultural Outlook 2020-2029*. Food and Agriculture Organization of the United Nations. https://www.fao.org/documents/card/en/c/ca8861en

Ohashi, Y., Miharu, N., Honda, H., Samura, O., & Ohama, K. (2002). Correlation of Fetal DNA and Human Chorionic Gonadotropin Concentrations in Second-Trimester Maternal Serum. *Clinical Chemistry*, *48*(2), 386–388.

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., & VanDer Giessen, J. (2010). Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR. *International Journal of Food Microbiology*, *139*(3), 193–201.

Oreskovic, A., Brault, N. D., Panpradist, N., Lai, J. J., & Lutz, B. R. (2019). Analytical Comparison of Methods for Extraction of Short Cell-Free DNA from Urine. *The Journal of Molecular Diagnostics*, *21*(6), 1067–1078.

Page, K., Hava, N., Ward, B., Brown, J., Guttery, D. S., Ruangpratheep, C., Blighe, K., Sharma, A., Walker, R. A., Coombes, R. C., & Shaw, J. A. (2011). Detection of HER2 amplification in circulating free DNA in patients with breast cancer. *British Journal of Cancer*, *104*(8), 1342–1348.

Pandya, P. P., Cardy, D. L. N., Jauniaux, E., Campbell, S., & Nicolaides, K. H. (1995). Rapid Determination of Fetal Sex in Coelomic and Amniotic Fluid by Fluorescence in situ Hybridisation. *Fetal Diagnosis and Therapy*, *10*(1), 66–70.

Park, J. L., Kim, H. J., Choi, B. Y., Lee, H. C., Jang, H. R., Song, K. S., Noh, S. M., Kim, S. Y., Han, D. S., & Kim, Y. S. (2012). Quantitative analysis of cell-free DNA in the plasma of gastric cancer patients. *Oncology Letters*, *3*(4), 921–926.

Paschopoulos, M., Meridis, E. N., Tanos, V., O'Donovan, P. J., & Paraskevaidis, E. (2006). Embryofetoscopy: a new "old" tool. *Gynecological Surgery*, *3*(2), 79–83.

Peddi, N. C., Avanthika, C., Vuppalapati, S., Balasubramanian, R., Kaur, J., & N, C. D. (2021). A Review of Cordocentesis: Percutaneous Umbilical Cord Blood Sampling. *Cureus*, *13*(7), e16423.

Père, M. C. (2003). Materno-foetal exchanges and utilisation of nutrients by the foetus: comparison between species. *Reproduction Nutrition Development*, *43*(1), 1–15.

Perry, J. S. (1981). The mammalian fetal membranes. *Reproduction*, *62*(2), 321–335.

Phillippe, M., & Adeli, S. (2017). Cell-free DNA release by mouse placental explants. *PLOS ONE*, *12*(6), e0178845.

Pietropolli, A., Vicario, R., Peconi, C., Zampatti, S., Quitadamo, M. C., Capogna, M. V., Ragazzo, M., Nardone, A. M., Postorivo, D., Spitalieri, P., Sarta, S., Ratto, F., Novelli, G., Sangiuolo, F., Piccione, E., & Giardina, E. (2014). Transabdominal coelocentesis as early source of fetal DNA for chromosomal and molecular diagnosis. *The Journal of Maternal-Fetal & Neonatal Medicine*, *27*(16), 1656–1660.

Poon, L. L. M., Leung, T. N., Lau, T. K., & Lo, Y. M. D. (2000). Prenatal detection of fetal Down's syndrome from maternal plasma. *The Lancet*, *356*(9244), 1819–1820.

Pozor, M. (2016). Equine placenta – A clinician's perspective. Part 1: Normal placenta – Physiology and evaluation. *Equine Veterinary Education*, *28*(6), 327–334.

Prevatt, C. (2019). *Have you figured your winter feeding costs yet? Here's one example*. The Beef Magazine. https://www.beefmagazine.com/feed/have-you-figured-your-winter-feeding-costs-yet-here-s-one-example

Prugnard, C., Lamia, A. B., Cherel, Y., Babarit, C., Guintard, C., Betti, E., Tainturier, D., & Bencharif, D. (2016). Early sex determination in the canine foetus by ultrasound and PCR. *Animal Reproduction Science*, *165*, 56–68.

Quintela, L. A., Becerra, J. J., Pérez-Marín, C. C., Barrio, M., Cainzos, J., Prieto, A., Díaz, C., & Herradón, P. G. (2011). Fetal gender determination by first-trimester ultrasound in dairy cows under routine herd management in Northwest Spain. *Animal Reproduction Science*, *125*(1–4), 13–19.

Ramsey, E. (1975). *The placenta of laboratory animals and man*.

Randall, G. C. B. (1977). Daily changes in the blood composition of conscious pigs with catheters in foetal and uterine vessels during late gestation. *The Journal of Physiology*, *270*(3), 719–731.

Ranucci, R. (2019). Cell-Free DNA: Applications in Different Diseases. *Methods in Molecular Biology*, *1909*, 3–12.

Ristanic, M., Stanisic, L., Maletic, M., Glavinic, U., Draskovic, V., Aleksic, N., & Stanimirovic, Z. (2018). Bovine foetal sex determination—Different DNA extraction and amplification approaches for efficient livestock production. *Reproduction in Domestic Animals*, *53*(4), 947–954.

Rita, T. H. S., Nobre, C. S., Jácomo, R. H., Nery, L. F. A., & Barra, G. B. (2018). Noninvasive fetal sex determination by analysis of cell-free fetal DNA in maternal capillary blood obtained by fingertip puncture. *Prenatal Diagnosis*, *38*(8), 620–623.

Rodgers, J. C., Bird, S. L., Larson, J. E., Dilorenzo, N., Dahlen, C. R., DiCostanzo, A., & Lamb, G. C. (2012). An economic evaluation of estrous synchronization and timed artificial insemination in suckled beef cows. *Journal of Animal Science*, *90*(11), 4055–4062.

Romano, J. E., Thompson, J. A., Forrest, D. W., Westhusin, M. E., Tomaszweski, M. A., & Kraemer, D. C. (2006). Early pregnancy diagnosis by transrectal ultrasonography in dairy cattle. *Theriogenology*, *66*(4), 1034–1041.

Ross, J. A., Jurkovic, D., & Nicolaides, K. (1997). Coelocentesis: A study of short-term safety. *Prenatal Diagnosis*, *17*(10), 913–917.

Ryan, G., & Rodeck, C. H. (1993). Fetal blood sampling. In *Essentials of Prenatal Diagnosis* (p. 63).

Sanchez, C., Snyder, M. W., Tanos, R., Shendure, J., & Thierry, A. R. (2018). New insights into structural features and optimal detection of circulating tumor DNA determined by single-strand DNA analysis. *NPJ Genomic Medicine*, *3*(1), 1–12.

Schmidt, M., Sangild, P. T., Jacobsen, H., & Greve, T. (2004). A method to reduce the invasiveness of fetal catheterization in the cow. *Animal Reproduction Science*, *80*(3–4), 193–200.

Seidel, G. E., Allen, C. H., Johnson, L. A., Holland, M. D., Brink, Z., Welch, G. R., Graham, J. K., & Cattell, M. B. (1997). Uterine horn insemination of heifers with very low numbers of nonfrozen and sexed spermatozoa. *Theriogenology*, *48*(8), 1255–1264.

Seidel Jr, G. E. (2012). Sexing Mammalian Sperm – Where Do We Go from Here? *Journal of Reproduction and Development*, *58*(5), 505–509.

Sekizawa, A., Kondo, T., Iwasaki, M., Watanabe, A., Jimbo, M., Saito, H., & Okai, T. (2001).

Accuracy of Fetal Gender Determination by Analysis of DNA in Maternal Plasma. *Clinical Chemistry*, *47*(10), 1856–1858.

Sekizawa, A., Samura, O., Zhen, D., Falco, V., Farina, A., & Bianchi, D. W. (2000). Apoptosis in fetal nucleated erythrocytes circulating in maternal blood. *Prenatal Diagnosis*, *20*(11), 886–889.

Sekizawa, A., Sugito, Y., Iwasaki, M., Watanabe, A., Jimbo, M., Hoshi, S., Saito, H., & Okai, T. (2001). Cell-free fetal DNA is increased in plasma of women with hyperemesis gravidarum. *Clinical Chemistry*, *47*(12), 2164–2166.

Senger, P. L. (2003). Placentation, the endocrinology of gestation and parturition. In *Pathways to Pregnancy and Parturition* (pp. 304–325).

Shah, V. C., & Smart, V. (1996). Human chromosome Y and SRY. *Cell Biology International*, *20*(1), 3–6.

Sharma, M., Singh, A., Sharma, N., & Rawat, S. (2017). Embryo sexing in cattle: Review. *International Journal of Current Innovation Research*, *3*(12), 955–960.

Shokrzadeh, M., & Mohammadpour, A. (2018). Evaluation of a modified salt-out method for DNA extraction from whole blood lymphocytes: A simple and economical method for gene polymorphism. *Pharmaceutical and Biomedical Research*, *4*(2), 28–32.

Shrivastava, S., Naik, R., Suryawanshi, H., & Gupta, N. (2019). Microchimerism: A new concept. *Journal of Oral and Maxillofacial Pathology*, *23*(2), 311.

Shulman, L. P., Simpson, J. L., & Elias, S. (2008). Invasive prenatal genetic techniques. In *The global library of women's medicine*.

Skrzypek, H., & Hui, L. (2017). Noninvasive prenatal testing for fetal aneuploidy and single gene disorders. *Best Practice & Research Clinical Obstetrics & Gynaecology*, *42*, 26–38.

Stanghellini, I., Bertorelli, R., Capone, L., Mazza, V., Neri, C., Percesepe, A., & Forabosco, A. (2006). Quantitation of fetal DNA in maternal serum during the first trimester of pregnancy by the use of a DAZ repetitive probe. *Molecular Human Reproduction*, *12*(9), 587–591.

Steemers, N. Y., Rop, C. De, & Assche, A. Van. (1995). Zonary placenta. *International Journal of Gynecology & Obstetrics*, *51*(3), 251–253.

Stemmer, C., Beau-Faller, M., Pencreac'h, E., Guerin, E., Schneider, A., Jaqmin, D., Quoix, E., Gaub, M. P., & Oudet, P. (2003). Use of Magnetic Beads for Plasma Cell-free DNA Extraction: Toward Automation of Plasma DNA Analysis for Molecular Diagnostics. *Clinical Chemistry*, *49*(11), 1953–1955.

Stephanie, C. Y., Chan, K. A., Zheng, Y. W., Jiang, P., Liao, G. J. W., Sun, H., Akolekar, R., Leung, T. Y., Go, A. T. J. I., Vugt, J. M. G. van, Minekawa, R., Oudejans, C. B. M., Nicolaides, K. H., Chiu, R. W. K., & Lo, Y. M. D. (2014). Size-based molecular diagnostics using plasma DNA for noninvasive prenatal testing. *Proceedings of the National Academy of Sciences*, *111*(23), 8583–8588.

Steven, D. (1975). Anatomy of the placental barrier. In *Comparative Placentation; Essays in*

Structure and Function (pp. 25–57). American Press.

Stocker, J., & Evens, L. (1977). Fetal sex determination by ultrasound. *Obstetrics and Gynecology*, *50*(4), 462–466.

Stokowski, R., White, K., Hacker, C., Doshi, J., & Schmid, M. (2020). Hemolysis and Fetal Fraction in Cell-Free DNA Blood Collection Tubes for Noninvasive Prenatal Testing. *Molecular Diagnosis & Therapy*, *24*, 185–190.

Stoops, M. A., Winget, G. D., DeChant, C. J., Ball, R. L., & Roth, T. L. (2018). Early fetal sexing in the rhinoceros by detection of male-specific genes in maternal serum. *Molecular Reproduction and Development*, *85*(3), 197–204.

Stroun, M., Lyautey, J., Lederrey, C., Mulcahy, H., & Anker, P. (2001). Alu Repeat Sequences Are Present in Increased Proportions Compared to a Unique Gene in Plasma/Serum DNA. *Annals of the New York Academy of Sciences*, *945*(1), 258–264.

Stroun, M., Maurice, P., & Vasioukhin, V. (2000). The origin and mechanism of circulating DNA. *Annals of the New York Academy of Sciences*, *906*(1), 161–168.

Sun, K., Jiang, P., Cheng, S. H., Cheng, T. H. T., Wong, J., Wong, V. W. S., Ng, S. S. M., Ma, B. B. Y., Leung, T. Y., Chan, S. L., Mok, T. S. K., Lai, P. B. S., Chan, H. L. Y., Sun, H., Chan, K. C. A., Chiu, R. W. K., & Lo, Y. M. D. (2019). Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Research*, *29*(3), 418–427.

Suzuki, N., Kamataki, A., Yamaki, J., & Homma, Y. (2008). Characterization of circulating DNA in healthy human plasma. *Clinica Chimica Acta*, *387*(1–2), 55–58.

Takata, K., Fujikura, K., & Shin, B.-C. (1997). Ultrastructure of the Rodent Placental Labyrinth: A Site of Barrier and Transport. *Journal of Reproduction and Development*, *43*(1), 13–24.

Teksen, F., Kaymaz, M., & Ertugrul, O. (2005). Chorionic Villus Sampling (CVS) in Cows. *Turkish Journal of Veterinary and Animal Sciences*, *29*(1), 183–187.

Telugu, B. P., & Green, J. A. (2007). Comparative Placentation. In *Comparative Reproductive Biology* (pp. 271–319).

Tonekaboni, F. R., Narenjisani, R., Staji, H., & Ahmadi-hamedani, M. (2020). Comparison of Cell-Free Fetal DNA Plasma Content Used to Sex Determination Between Three Trimesters of Pregnancy in Torkaman Pregnant Mare. *Journal of Equine Veterinary Science*, *95*, 103273.

Trigg, R. M., Martinson, L. J., Parpart-Li, S., & Shaw, J. A. (2018). Factors that influence quality and yield of circulating-free DNA: A systematic review of the methodology literature. *Heliyon*, *4*(7), e00699.

Tsai, N. W., Lin, T. K., Chen, S. Der, Chang, W. N., Wang, H. C., Yang, T. M., Lin, Y. J., Jan, C. R., Huang, C. R., Liou, C. W., & Lu, C. H. (2011). The value of serial plasma nuclear and mitochondrial DNA levels in patients with acute ischemic stroke. *Clinica Chimica Acta*, *412*(5–6), 476–479.

Tsumita, T., & Iwanaga, M. (1963). Fate of Injected Deoxyribonucleic Acid in Mice. *Nature*,

198(4885), 1088–1089.

Tungwiwat, W., Fucharoen, G., Ratanasiri, T., Sanchaisuriya, K., & Fucharoen, S. (2003). Non-invasive fetal sex determination using a conventional nested PCR analysis of fetal DNA in maternal plasma. *Clinica Chimica Acta*, *334*(1–2), 173–177.

Turin, L., Invernizzi, P., Woodcock, M., Grati, F. R., Riva, F., Tribbioli, G., & Laible, G. (2007). Bovine fetal microchimerism in normal and embryo transfer pregnancies and its implications for biotechnology applications in cattle. *Biotechnology Journal*, *2*(4), 486–491.

Ulrich, B. C., & Paweletz, C. P. (2017). Cell-Free DNA in Oncology: Gearing up for Clinic. *Annals of Laboratory Medicine*, *38*(1), 1–8.

Umetani, N., Hiramatsu, S., & Hoon, D. S. B. (2006). Higher Amount of Free Circulating DNA in Serum than in Plasma Is Not Mainly Caused by Contaminated Extraneous DNA during Separation. *Annals of the New York Academy of Sciences*, *1075*(1), 299–307.

Valenti, C. (1973). Antenatal detection of hemoglobinopathies: A preliminary report. *American Journal of Obstetrics and Gynecology*, *115*(6), 851–853.

Van Der Vaart, M., & Pretorius, P. J. (2008). Circulating DNA: Its origin and fluctuation. *Annals of the New York Academy of Sciences*, *1137*(1), 18–26.

van Vliet, R. A., Verrinder Gibbins, A. M., & Walton, J. S. (1989). Livestock embryo sexing: A review of current methods, with emphasis on Y-specific DNA probes. *Theriogenology*, *32*(3), 421–438.

Vicente, A. M. M., Rodriguez, L., Diaz, N. R. G., Salais, S. F., & Simó, J. C. (2020). Hystero-embryoscopy a tool for diagnosis in early pregnancy loss. *Fertility and Sterility*, *114*(3), e472–e473.

Vijaya Lakshmy, K., Manimegalai, J., & Lambe, U. (2018). Different methods of embryo sexing: A review. *The Pharma Innovation Journal*, *170*(10), 170–172.

Ville, Y., Khalil, A., Homphary, T., & Moscoso, G. (1997). Diagnostic embryoscopy and fetoscopy in the first trimester of pregnancy. *Prenatal Diagnosis*, *17*(13), 1237–1246.

Vincze, B., Gáspárdy, A., Biácsi, A., Papp, E. Á., Garamvölgyi, L., Sós, E., Cseh, S., Kovács, G., Pádár, Z., & Zenke, P. (2019). Sex determination using circulating cell-free fetal DNA in small volume of maternal plasma in elephants. *Scientific Reports*, *9*(1), 1–7.

Vishwanath, R., & Moreno, J. F. (2018). Review: Semen sexing – current state of the art with emphasis on bovine species. *Animal*, *12*(s1), s85–s96.

Waldner, C. L. (2005). Serological status for N. caninum, bovine viral diarrhea virus, and infectious bovine rhinotracheitis virus at pregnancy testing and reproductive performance in beef herds. *Animal Reproduction Science*, *90*(3–4), 219–242.

Waldner, C. L., Kennedy, R. I., Rosengren, L. B., Pollock, C. M., & Clark, E. (Ted) G. (2010). Gross postmortem and histologic examination findings from abortion losses and calf mortalities in western Canadian beef herds. *The Canadian Veterinary Journal*, *51*(11), 1227.

Walknowska, J., Conte, F. A., & Grumbach, M. M. (1969). Practical and theoretical implications

of fetal/maternal lymphocyte transfer. *The Lancet*, *293*(7606), 1119–1122.

Wang, E., Batey, A., Struble, C., Musci, T., Song, K., & Oliphant, A. (2013). Gestational age and maternal weight effects on fetal cell-free DNA in maternal plasma. *Prenatal Diagnosis*, *33*(7), 662–666.

Wang, G., Cui, Q., Cheng, K., Zhang, X., Xing, G., & Wu, S. (2010). Prediction of Fetal Sex by Amplification of Fetal DNA Present in Cow Plasma. *Journal of Reproduction and Development*, *56*(6), 1008250297–1008250297.

Wataganara, T., Bui, T., & Choy, K. (2016). Debates on fetal fraction measurement and DNA-based noninvasive prenatal screening: time for standardisation? *Wiley Online Library*, *123*(3), 31–35.

Westin, B. (1954). Hysteroscopy in early pregnancy. *The Lancet*, *264*(6843), 872.

Wichmann, D., Panning, M., Quack, T., Kramme, S., Burchard, G. D., Grevelding, C., & Drosten, C. (2009). Diagnosing Schistosomiasis by Detection of Cell-Free Parasite DNA in Human Plasma. *PLOS Neglected Tropical Diseases*, *3*(4), e422.

Wong, D., Moturi, S., Angkachatchai, V., Mueller, R., DeSantis, G., van den Boom, D., & Ehrich, M. (2013). Optimizing blood collection, transport and storage conditions for cell free DNA increases access to prenatal testing. *Clinical Biochemistry*, *46*(12), 1099–1104.

Wooding, F. B. P. (1980). Electron Microscopic Localization of Binucleate Cells in the Sheep Placenta Using Phosphotungstic Acid. *Biology of Reproduction*, *22*(2), 357–365.

Wooding, F. B. P. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. *American Journal of Anatomy*, *170*(2), 233–250.

Wooding, P., & Burton, G. (2008). Comparative placentation: structures, functions and evolution. In *Springer Science & Bussiness Media* (pp. 133–167).

Wright, C. F., & Burton, H. (2009). The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Human Reproduction Update*, *15*(1), 139–151.

Xi, J. F. (2006). Rapid sex identification of embryos in different developmental stages by blood of pregnant dairy cattle. *J Shihezi Univ Natural Sci*, *24*, 446–449.

Xue, X., Teare, M. D., Holen, I., Zhu, Y. M., & Woll, P. J. (2009). Optimizing the yield and utility of circulating cell-free DNA from plasma and serum. *Clinica Chimica Acta*, *404*(2), 100–104.

Yao, H., Jiang, F., Hu, H., Gao, Y., Zhu, Z., Zhang, H., Wang, Y., Guo, Y., Liu, L., Yuan, Y., Zhou, L., Wang, J., Du, B., Qu, N., Zhang, R., Dong, Y., Xu, H., Chen, F., Jiang, H., … Liang, Z. (2014). Detection of fetal sex chromosome aneuploidy by massively parallel sequencing of maternal plasma DNA: initial experience in a Chinese hospital. *Ultrasound in Obstetrics & Gynecology*, *44*(1), 17–24.

Zandvakili, I., & Lazaridis, K. N. (2019). Cell-free DNA testing: future applications in gastroenterology and hepatology: *Therapeutic Advances in Gastroenterology*, *11*, 1–13.

Zhu, J., Zhang, F., Du, M., Zhang, P., Fu, S., & Wang, L. (2017). Molecular characterization of

cell-free eccDNAs in human plasma. *Scientific Reports*, *7*(1), 1–11.

Zimin, A. V, Delcher, A. L., Florea, L., Kelley, D. R., Schatz, M. C., Puiu, D., Hanrahan, F., Pertea, G., Van Tassell, C. P., Sonstegard, T. S., Marçais, G., Roberts, M., Subramanian, P., Yorke, J. A., & Salzberg, S. L. (2009). A whole-genome assembly of the domestic cow, Bos taurus. *Genome Biology*, *10*(4), 1–10.