SEXING THE BOVINE FETUS USING FETAL FLUID CELLS RECOVERED BY TRANSVAGINAL ULTRASOUND-GUIDED UTERINE PUNCTURE.

by

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A thesis submitted for the degree of Masters in Veterinary Medicine in the Faculty of Veterinary Medicine, University of Glasgow.

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and
Veterinary Anatomy

March, 1996

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ACKNOWLEDGEMENTS

This work was carried out in the Departments of Veterinary Physiology and Veterinary Anatomy. I wish to thank Professor P. H. Holmes and Professor J. S. Boyd for allowing me to work in their respective departments.

My sincere thanks to my supervisors, Dr. L. Robertson and Dr. I. A. Jeffcoate for their support, encouragement and constructive criticism throughout my studies. In addition, I wish to extend my heartfelt indebtness to Dr. L. Robertson, my friend and colleague Y. S. Amiridis and Mr. C. Paterson for sharing every bit of the most trying moments of this study. Their involvement, support and encouragement was very instrumental to this work. I also wish to thank Professor J. S. Boyd for the immense ultrasonographic knowledge and the untold effort to confirm my sexing results.

My acknowledgements also with thanks to Dr. P. O'Shaughnessy for the vast molecular biology knowledge and PCR, otherwise PCR would have just been another acronym. Many thanks also to the members of staff in the Departments of Veterinary Physiology and Veterinary Anatomy for the help, encouragement and friendly atmosphere I will always remember. Also remembered is Ms Liz Dunlop for the many trips to and from the abattoir, thank you. Special thanks also to the staff at the Netherton farm, staff at the James Herriot Library and staff at the Paisley Abattoir for their help during the study.

My family for the support, love and encouragement they have ever given me, thank you folks!

Acknowledged with thanks also are my sponsors, The British Council for according me the finances to undertake this study.

Above all thanks to the Almighty God for the Guidance and Inspiration throughout my study.

DECLARATION

I Kennedy Makondo do declare that this work is original, was carried out by the undersigned and has not previously been presented in any form to any university for an award of a degree.

Signature:

Date: 22.04.96.

DEDICATION

Dedicated to

My late parents, Buumba V. Makondo and Chinyama M. H. Makondo,

and to

My late brothers, Himweemba N. Makondo and Nkuupi C. Makondo

May Their Souls Ever Rest In Peace.

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Abstract

This study was aimed at using high quality ultrasonography to recover fetal fluids in early gestation (50-120 days) for the purpose of fetal sex diagnosis using the polymerase chain reaction (PCR). A transvaginal puncture technique developed for ovum pick-up was modified for fetal fluid aspiration. Where possible, and preferably, fluid was recovered from the nonpregnant horn.

Fetal fluid samples were shown to contain cells and genomic DNA was extracted for sex determination by a nested, allele-specific amplification of the bovine *zfx* and *zfy* gene fragments by PCR. The accuracy and applicability of this technique was verified using abattoir material from animals of known gender.

Repeated transvaginal ultrasound-guided fetal fluid recovery proved traumatic to the fetus causing fetal death and/or spontaneous pregnancy loss in six animals, five out of five cows and one out of three heifers. However, two heifers have retained live fetuses for over two weeks since the procedure. Sex diagnosis by PCR was highly accurate despite obvious maternal cell contamination of some samples. The fetal gender of all the pregnancies tested agreed with fetal sex obtained using ultrasonography or after examining the genitalia of aborted fetuses.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The idea of altering the sex-ratio in mammals with particular reference to farm animals was once described as ".....an old dream" of livestock producers (Ortavant, 1976). The interest in developing sexing techniques, however, called for a symposium devoted to the subject (Kiddy and Hafs, 1971) and over the years advanced and highly accurate techniques have been developed to the extent of sex determination of an embryo prior to its transfer (Hare and Betteridge, 1978; King, 1984; Ellis et al., 1988; Herr et al., 1990). The possibility of separating sperm bearing X from those bearing Y chromosomes would doubtlessly be the ideal method of controlling sex ratio but it is not an immediate prospect despite abundant claims to the contrary (Anderson, 1991).

Amniotic cells were first used to determine fetal sex in humans by Fuchs and Riis (1956) following the discovery of the chromatin (Barr) body in XX cells (Barr and Bertram, 1949, cited by Fuchs and Cederqvist, 1978). Efforts to apply the method of Barr body detection for sexing domestic animals have since proven futile due to the granular nature of the cell cytoplasm. Bongso and Basrur (1975) however, karyotyped amniotic cells from cows at 70-90 days of gestation for sex determination. After reports from the medical field, they applied cytological criteria to amniotic cells in order to predict the sex of the bovine fetus by quantifying eosinophilic, orangeophilic and cyanophilic cells following Papanicolaou staining (Bongso et al., 1978). Using a different approach, fetal androgens were observed to predict sex in humans (Younglai, 1973). Bongso et al. (1976) also reported that

testosterone levels in allantoic fluid between day 90 and 150 of gestation in cattle predicted fetal sex. In addition, studies on pregnant heifers suggested that testosterone levels in a dam carrying a male fetus were higher than in those carrying a female fetus (Mongkonpunya et al., 1975). However, this group further reported that the hormone increased in both heifers carrying male or female fetuses throughout gestation; making it subjective to account the difference as a function of fetal sex.

In recent times, however, diagnostic ultrasound has become a routine procedure in domestic animal reproduction and obstetrics, with applications ranging from transabdominal to transrectal and transvaginal scanning for various diagnostic procedures. It is now possible to sex a bovine fetus using transrectal ultrasound to image the fetal genitalia (Muller and Wittkowski, 1986; Curran et al., 1989; Kahn, 1990; De Moura et al., 1993). Using a different approach, a commercial PCR amplification technique has been developed to determine the sex of an embryo from a single blastomere prior to embryo transfer (Herr et al., 1990; Peura, 1991; Kirkpatrick and Monson, 1993; Rao et al., 1994; Peippo et al., 1995).

The present study reports the use of ultrasound-guided uterine puncture in cattle to recover fetal fluids for the purpose of early sex diagnosis by PCR. Fetal fluid has been repeatedly and successfully recovered from pregnant cows using the same approach (Vos et al., 1990) and also recently without the benefit of ultrasound (Sprecher and Kaneene, 1992). However, these investigators experienced a considerable rate of abortion following the procedure. At the time

of writing there are no published reports of sex diagnosis using PCR on bovine fetal fluids.

1.2 Objectives.

1. To develop a level of competence examining pregnant and nonpregnant reproductive tracts using ultrasonography.
2. To recover fetal fluids early in gestation (i.e. between day 50-120) using ultrasound-guided uterine puncture.
3. To examine the use of fetal fluids for early sex determination using PCR.

1.3 Development of the Bovine Embryo and Fetus

The development of a bovine conceptus from fertilisation to parturition is arbitrarily divided into three stages: 1) that of the ovum from 0 to 13 days, 2) that of the embryo from 14 to 45 days, and 3) that of the fetus from 46 days until parturition (Winter et al., 1942, cited by Peters and Ball, 1987). Following fertilisation in the oviduct the fertilised ovum moves down into the uterus while cell numbers increase by mitotic division, a process known as cleavage. Cell division continues resulting in a solid cluster of cells or blastomeres known as a morula (mulberry shape), by day 5 or 6, at which point the embryo enters the uterus (Hamilton and Laing, 1946, cited by Peters and Ball, 1987; Flood, 1991).

Up to this time the embryo has retained its spherical shape due to confinement in the zona pellucida but begins to hollow out to become a blastocyst. This consists of a single outer spherical layer of cells, termed the trophoblast, and a hollow centre with an inner cell mass at one pole. At about 8 to 9 days after fertilisation the blastocyst loses the zona pellucida (i.e. hatches) and begins to elongate rapidly. The embryo proper, made up of the inner cell mass, appears as an oval translucent patch near the centre of the elongated blastocyst. Nourishment to this point must be derived from its own stores and from materials absorbed from fluids secreted by the oviduct and the uterus. But due to rapid growth, it soon becomes imperative that the embryo establishes a better source of nutrition. It achieves this through fetal-maternal interaction (placentation).

The embryonic period begins from the 13th to the 45th day; largely characterised by the formation of major tissues, organs and body parts (Noden and De Lahunta, 1985, Peters and Ball, 1987). Its onset is characterised by the formation of three germ layers from the inner cell mass, termed ectoderm, mesoderm and endoderm. The ectoderm gives rise to the external structures such as skin, hair, hooves and mammary glands and also the nervous system. The heart, muscles and bones are formed from the mesoderm whereas the other internal organs are derived from the endoderm. The formation of the primitive organs is complete by the end of the embryonic period (day 45). The heart and circulatory system are formed early in embryo life and by day 21 to 22 the heart has started to beat (Salisbury and VanDemark, 1978). The embryo rapidly changes shape, initially curving into a C-shape and later stretching out into an L-shape while

slowly taking form. The head and tail regions are recognisable by day 22 to 23 and by day 25 to 30 the limb and tail buds are evident (Noden and De Lahunta, 1985). As the embryonic period comes to an end, the embryo averages about 3.3 cm in length and weighs about 2.5 g (Foley et al., 1953, cited by Salisbury and VanDemark, 1978). The placenta is now the sole source of nutrients for the developing embryo.

The third and final stage of development is the fetal period, the interval between the 46th day and term and this is characterised by differential growth of the various organs and body parts that were laid down during the embryonic period. Dennis (1969, cited by Mowa et al., 1994) further subdivided the fetal period into three: the first trimester (46-120 days of gestation), second trimester (120-180 days of gestation) and the third trimester (180 days to term). The fetus increases in size throughout this period but marked growth occurs in the third trimester.

1.3.i Fetal Membranes and Fluids

During the embryonic period extraembryonic membranes are formed from the three germ layers. These are essential auxiliary structures which are necessary for embryonic growth, nutrition, respiration and excretion throughout pregnancy. The first to form is the yolk sac which is an outpouching of the developing gut from the endoderm and vascular mesoderm (splanchnopleure). Its function is only of transitory importance in mammals. Thus in the cow it is fully vascularised by day

16 and its blood vessels absorb nutrients for the embryo (Steven and Morriss, 1975). Soon it is displaced and quickly regresses as the rapidly expanding allantoic sac obliterates the extraembryonic coelom and takes over its functions.

The formation of the amnion and chorion is closely interrelated. They are formed from the extraembryonic ectoderm and avascular mesoderm (somatopleure). In the cow, like other mammals, the amnion forms by folding of the somatopleure between day 13 and 16 (Steven and Morriss, 1975, Perry, 1981) on either side of the embryo to fuse with the trophectoderm and enclose the embryo in a complete sac. By day 18 (Latshaw, 1987) it has fully enveloped the embryo while the chorion forms from the trophoectoderm and surrounds the entire conceptus. Following fusion of the amnion and chorion the amniochorion is formed dorsal to the embryo. Both the amnion and chorion are relatively avascular since their mesodermal component is of the avascular somatic origin.

The allantois is essentially an outgrowth of the embryonic hindgut and is continuous with the urinary bladder. It first appears in the extraembryonic coelom between day 14 and 21 (Latshaw, 1987) as a small diverticulum from the hindgut of the embryo derived from the endoderm and vascular mesoderm (splanchnopleure). The allantois grows rapidly extending cranially and caudally along the lateral and ventral aspect of the embryo. By day 25 the allantois is a tubular-like structure ventral to the embryo with an inverted "T"-shape (Salisbury and VanDemark, 1978). Soon it becomes very large, fills the extraembryonic coelom and fuses with the chorion and amnion to form the allantochorion and allantoamnion by day 28 to 33 (Latshaw, 1987). The allantois is fully extended by

day 35 or 37 to fill the nonpregnant horn (Salisbury and VanDemark, 1978). The fusion of the allantois with the amnion and chorion serves to vascularise the allantochorion, allantoamnion and also the amniochorion. Because of membrane fusion, especially that of the amnion and chorion, the allantoic cavity is not continuous dorsally in ruminant and pig embryos.

Signs of implantation are evident by day 20 to day 24 (Wooding and Flint, 1994) as extraembryonic membranes develop. By day 28 to 30, villus development in the caruncular areas begins and fragile cotyledonary attachment is evident by day 33 (King, et al. 1979, cited by Wooding and Flint, 1994). Embryo attachment continues until day 40 to 45 of gestation (Eley, et al., 1978).

The fluid in the extraembryonic membrane cavities provides a protective cushion vital for the developing embryo. In addition the amnion, which immediately surrounds the embryo, prevents adhesion to the surrounding membranes and the uterine wall. As a result the embryo is free to grow symmetrically and with unrestricted movements in later gestation. In the two fetal sacs, total fluid volume increases progressively throughout gestation with a decrease in the rate of accumulation between the 5th and 7th month of pregnancy. Increases in allantoic and amniotic fluid volume essentially parallels growth of the membranes. Three sharp rises occur in cattle: between 40 and 65 days, 3 to 4 months and 6.5 and 7.5 months (Arthur, Noakes and Pearson, 1989). The first and last increase is attributed to allantoic fluid while the second is to amniotic fluid. Therefore allantoic fluid is greater in volume than amniotic fluid early in gestation and increases faster until about 60 days of gestation (Eley et al., 1978). The sharp

increase in amniotic fluid at this time leads to a temporary amniotic fluid excess but allantoic fluid volume becomes greater later in gestation. In the cow the total volume at term is about 20 litres, two-thirds of which is allantoic fluid (Arthur, 1957; 1965; Bongso and Basrur, 1976; Wahid et al., 1991).

The source of the amniotic and allantoic fluids is not clear. Allantoic fluid in early gestation is thought to be a dialysate of the maternal blood in the allantoic vessels (Latshaw, 1987; Eley, et al., 1978). However, it is generally accepted that urine production by the kidneys at different stages of development may also contribute to the allantoic and amniotic fluids via the urachus and urethra respectively (Windle, 1940, cited by Arthur, 1957; Latshaw, 1987). Thus both fluids resemble dilute urine in early and mid-gestation until about two and half months before term (Arthur, 1957). At this time allantoic fluid becomes more urine-like because of the onset of the fetal kidney function while amniotic fluid becomes colourless and viscous. It is suggested that drainage of urine into the amnion through the urethra ceases due to the onset of urinary bladder sphincter function and amniotic fluid viscosity comes from salivary mucus and intestinal gland secretions (Arthur, 1957). Studies on the ultrastructure of the allantochorion suggest it to be involved in some sort of fluid transport (Latshaw, 1987) and also that amniotic fluid may be a product of amniotic and allantoic epithelial secretion.

1.3.ii Sexual Differentiation of the Fetus

From fertilisation through the three developmental stages, sexual dimorphism can be described at three levels; genetic, gonadal and phenotypic. These levels represent a hierarchy that directs sexual differentiation in the fetal period and finally sexuality in life. Genotypic sex of an individual is determined by the sex chromosomes (X and Y) and is fixed at fertilisation. It directs the gonadal sex (i.e. ovary or testis) during sexual differentiation in the fetal period, which in turn determines phenotypic sex of an individual.

Before sex differentiation, there is a stage of embryonic development during which it is difficult, if not impossible, to tell the sexes apart by either gross or microscopic examination. This period is known as the indifferent stage when all the anlagen required for subsequent differentiation into complete male or female systems are present in rudimentary form (Nalbandov, 1976). Both systems however, have the potential to develop upon receiving the appropriate signal. The development of male structures require an active stimulus whereas female structures develop passively in the absence of male determinants (Jost, 1953, cited by Glover et al., 1990). Basically the genetic material of the fertilising sperm determines whether an individual is to be genetically male or female and, in turn, the specific reproductive structures develop accordingly. The mechanisms responsible for sexual differentiation differ, however, depending upon the stage of development i.e. either at ovum, embryonic or fetal period.

In cattle the indifferent stage persists until around the 6th week of gestation when either male or female structures first appear. This is characterised by the formation of the gonadal ridge by the migration of primordial germ cells from the yolk sac endoderm. Differentiation of the gonads into testes is evident by about 36 days of gestation (Bascom, 1923, cited by Salisbury and VanDemark, 1978). As mentioned above, the Y chromosome signals testicular development and in its absence ovaries develop. It was thought that the Y chromosome exerted this effect by controlling the production of a specific protein called the H-Y antigen (histocompatibility Y antigen). It was said that H-Y antigen was secreted by Sertoli cell precursors early in gestation, and bound to receptors on adjacent gonadal cells causing testicular differentiation. Ovarian cells were also said to have receptors for H-Y antigen but the specific protein was lacking (Page, et al., 1987, cited by Glover et al., 1990). The absence of the H-Y antigen in females meant that cells of the indifferent gonad never received the signal for testicular formation and consequently ovaries developed. However, the hypothesis that H-Y antigen is the primary testis-inducer has long been disputed. Studies in humans and mice have shown that phenotypically normal males occur without the presence of the H-Y antigen (McLaren et al., 1984, 1990, 1991).

Recently the candidate gene for testis-determining factor (TDF), named sex-determining Y gene (*SRY*) has been reported in humans (Sinclair et al., 1990), mice (Gubbay et al., 1990) and most recently in cattle (Daneau et al., 1995). Following its ability to transform XX karyotype mouse embryos into phenotypic male mice, it is now generally accepted that *SRY* controls expression of the testis-

determining factor in mammals (Haqq et al., 1994). Its presence during differentiation invokes a cascade of male developmental changes and in its absence female development proceeds.

At the phenotypic level, the development of genital tract and genitalia is directed by the gonadal sex following differentiation. Unlike the gonads which develop from common anlagen, the reproductive tract develops from different ones. In males, the reproductive tract is derived from embryonic rudimentary structures known as the Wolffian ducts, while the female tract develops from the Mullerian ducts. However, prior to sexual differentiation, both duct systems are present in the embryo which therefore has potential to develop either a male or a female reproductive tract, and associated external genitalia from the genital tubercle. Shortly after differentiation, the location of the genital tubercle is characteristic of fetal gender, being close to the umbilical cord in the male and beneath the tail in the female.

Two hormones, testosterone and Mullerian-inhibiting factor (or anti-Mullerian hormone), secreted by male gonads, determine phenotypic sex. Again, female sex differentiation is a passive process. Testosterone secreted by the interstitial (Leydig) cells, stimulates the development of Wolffian ducts and differentiation of the male genitalia. Mullerian-inhibiting factor secreted by the sustentacular (Sertoli) cells causes regression of the Mullerian ducts. Thus the Mullerian ducts will regress in males while the Wolffian ducts and genitalia develop into the male reproductive tract, penis, and scrotum. In the female, the absence of both testosterone and Mullerian-inhibiting factor lead to Wolffian duct regression

and inevitably the passive development of the Mullerian ducts and external genitalia into uterus, oviducts, clitoris, vagina, and vulval labia.

One aim of the present study was to review the morphology of the early conceptus during the first trimester in cattle using ultrasonography, with emphasis on the fetal membranes and location of the external genitalia. At the same stage of development, the fetus would be sexed using cells recovered from fetal fluids which were to be collected by ultrasound-guided needle puncture while taking extreme care not to disturb fetal viability.

1.4 Ultrasonography

Ultrasound was first used to detect submarines during the second world war (cited by Omran, 1989 and Mowa, 1994). Later it was developed as a diagnostic aid in human obstetrics (King, 1973). In the veterinary field it has long been used to estimate fat thickness in cattle (Temple et al. 1956) and later as a diagnostic aid for detection of pregnancy in sheep (Lindahl, 1966). This work was followed by the application of the technique in dogs (Kaene, 1969; Helper, 1970), primates (O'Grady, 1978), cattle and horses (Fraser, 1971). These early workers either used A-mode (amplitude mode) (Lindahl, 1966; Koch and Rubin, 1969) or Doppler (Fraser, 1967, 1971 and 1973; Lindahl, 1969 and Helper, 1978). Nowadays, however, the B-mode display is most popularly used in veterinary reproductive ultrasound.

1.4.i Principles of Ultrasound Imaging and Interpretation

Ultrasound is very high frequency sound, generally in the range 1.0 to 10 MHz, beyond the audible range (330 to 20,000 Hz). Like audible sound, ultrasound is a mechanical wave of compressions and rarefactions transmitted through medium at a constant velocity [1540m/s in tissues (Herring and Bjornton, 1985)] but varying in frequency and wavelength. It is produced by specialised crystals called piezo-electric (pressure-electric) crystals. These crystals, housed in a

transducer can convert electric energy into mechanical energy or vice versa thereby acting both as receiver and transmitter of ultrasonic waves.

Ultrasonic imaging operates on a pulse-echo principle where sound waves are transmitted into tissues and are continuously reflected back at different acoustic interfaces. The level of energy reflected is determined by differences in the mechanical properties of the tissues which form the reflecting interface (i.e. by the acoustic impedance). Thus the greater the difference between tissues in acoustic impedance, the higher the reflection.

Reflected ultrasonic waves are converted at the transducer face to an electrical signal of the same frequency as the echo. Instead of representing the actual distance travelled by the sound beam from transducer to object surface as a number, the ultrasound scanner converts the information into dots of light (pixels) on a black background in the B-mode (brightness mode) display most popularly used in the veterinary field. The strength of the echo, represented by the brightness of the dots, and the position of each dot is proportional to the distance travelled. This allows the construction of an image of the tissues and organs traversed by the ultrasound beam. Characteristically, the image appears in varying shades of grey ranging from white to black. Highly reflective interfaces are seen as white (bright) areas whereas poorly reflective ones are depicted as black (dull) areas. For the purpose of image interpretation, structure appearance is described by ability to reflect ultrasound waves (termed echogenicity).

Structures which reflect sound are termed echogenic while those without this ability are known as nonechogenic and produce an anechoic image. Non-

viscous fluid filled structures such as follicles, cysts, and urinary bladder are good examples of nonechogenic structures. If, on the other hand, the fluid is viscous, such as an abscess, a small portion of the beam is reflected giving a relatively weak echo. The image appears grey and is known as hypoechoic; the structure is known as hypoechogenic.

Structures which reflect relatively strongly are termed echogenic, producing an echoic image. Soft tissues like the corpus luteum and the endometrium are good examples. Highly reflective interfaces that return strong echoes are known as hyperechogenic, producing a hyperechoic image. Fetal bones, the pelvis and tissue-gas interfaces are good examples where most if not all of the sound beam is reflected back.

Usually in ultrasonography, nonechogenic and hypoechogenic structures are used as acoustic windows that permit relatively free passage of the ultrasound beam into deeper structures. This enables imaging of deeper organs with greater intensity. A full urinary bladder and soft tissues such as the spleen are good examples. Occasionally there are circumstances when surrounding tissues give comparable echoes making it difficult for the sonographer to interpret the displayed image. Such structures are termed isoechogenic, producing an isoechoic image.

Ultrasound waves progressively weaken (attenuate) as they travel from source (transducer) through tissues for various reasons. The rate of loss of mechanical energy in the sound wave is generally about 1 decibel(db) per cm per MHz for soft tissues (Powis, 1986).

There are basically three principle causes of attenuation: absorption, reflection and scattering. Absorption is where minute proportions of the beam are trapped or absorbed by the tissue and mostly converted into heat energy. Absorption increases with distance and frequency, i.e. the higher ultrasound frequencies are absorbed faster and thus can not penetrate as deep as lower frequencies.

Progressive reflection of the ultrasound beam occurs when it strikes tissues of different acoustic impedance resulting in progressive weakening of the beam. Ultrasound beam reflection can either be specular or non-specular depending upon the angle of incidence of the beam relative to the insonated tissue and the tissue size. Specular reflection occurs when the reflecting surface is larger than the sound beam and is perpendicular to the beam axis while non-specular reflection occurs when the beam strikes an irregular interface smaller than its wavelength. Upon reflection the beam is spread in all directions and this is known as scattering. Normally in any single image there is a mixture of specular and non-specular reflections.

One of the important factors in ultrasonography is whether it is possible to identify separate structures or organs, known by the term resolution. Resolution has two components; axial and lateral with the axial component being the ability to separate structures lying close together along the path of the ultrasound beam (beam axis). Lateral resolution refers to the ability of ultrasound to distinguish structures lying close together in a plane perpendicular to the beam axis.

Axial resolution is dependent upon the pulse length of the ultrasound beam, the higher the ultrasound frequency the higher the axial resolution obtained. Lateral resolution is determined by three factors; ultrasound beam width, distance from the interface to the transducer and size of the transducer face i.e. essentially the shape of ultrasound beam. Near the transducer, the beam has the same width as the transducer but the beam tends to diverge as it travels away. This enlarges the image leading to ambiguity in the ultrasound image. Generally the narrower the beam the better the resolution and in most ultrasound designs, the beams are focused to reduce divergence.

Correct interpretation of the ultrasonic image is an obvious requirement for a correct diagnosis. Inherent in every sonogram are ultrasound image artifacts which are echo distortions caused by ultrasound matter interaction. Numerous factors cause such distortions ranging from equipment quality to scanning technique. In some instances these artifacts can be helpful in making an accurate diagnosis while in others, they can lead to serious confusion and mis-diagnosis, especially for inexperienced sonographers. Helpful artifacts include acoustic shadows and enhancement whereas troublesome artifacts are reverberation, mirror-images and refraction.

Acoustic shadows are caused by diminished transmission of sound due to attenuation and/or reflection at an acoustic interface. The appearance of acoustic shadows depends upon the reflecting surface. Acoustic shadows are helpful in the sense that they indicate the presence of a highly reflective structure interacting with the beam and restricting visualisation of the deeper tissues.

Distance enhancement may occur if there is little sound attenuation as it passes through a relatively homogenous medium such as a fluid filled structure. When sound strikes the far wall of such a structure, the echoes appear brighter than the surrounding structures. This enhances the image of the anechoic (fluid filled) structure.

Reverberation occurs when a sound beam strikes a highly reflective interface and the echoes bounce off the transducer face and re-enter the tissue. This causes a second echo to be displayed twice as far from the original acoustic interface and this process is usually repeated many times. Thus mirror-images of a strongly reflective interface will appear in sequentially deeper positions.

Refraction occurs when a sound beam strikes the periphery of a large spherical structure such as a follicle. A shadow appears distal to the structure due to absence of an echo beam as it is turned or refracted away from the transducer.

1.4.ii Technique of Ultrasonography in the Cow

The ultrasonographic examination of the bovine reproductive tract is normally performed by transrectal ultrasound. Technically the procedure is largely performed in a way similar to rectal palpation. With the cow restrained and the rectum evacuated of faecal matter, a hand-held transducer is introduced through the anus. It is advanced cranially along the floor of the rectum while manipulating the tract. Generally linear array transducers are used for transrectal ultrasound although other modes have been used.

Transvaginal ultrasound has become increasingly popular in the cow especially in conjunction with puncture techniques for ovum collection for in-vitro maturation and fertilisation. This technique requires chemical restraint of the animal and awareness of hygiene. The rectum is evacuated of faecal matter and the perineal and vulval area scrubbed before introducing the transducer. Specially designed transducers incorporating a biopsy guide-line and needle guide are used. With one hand in the rectum the transducer is introduced into the vagina and directed to the point of interest while manipulating the tract per rectum. In some cases, this approach offers a great deal of detail due to simultaneous rectal manipulation. Usually sector or microcurvilinear transducers are used in the transvaginal ultrasound.

Transabdominal ultrasound is not popular in the study of bovine reproductive tract except in late gestation. It is more commonly used for small animals.

1.4.iii Ultrasonic Appearance of the Bovine Conceptus

Several researchers have reported use of transrectal ultrasonography of the uterus for early pregnancy diagnosis (Chaffeux, 1982; Body, 1988; Willemse and Taverne, 1989; Badtram et al., 1991); to describe the morphological appearance of the conceptus in early gestation (Pierson and Ginther, 1984; Reeves et al., 1984; Curran et al., 1986; Kastelic et al., 1988), and fetal development (White et al.,

1985; Kahn, 1989, 1990; Mowa, 1994) and for sex determination (Muller and Wittkowski, 1986; Curran et al. 1989, 1992; De Moura, 1993).

It is reported that pregnancy can be detected by ultrasound from as early as day 9 (Boyd et al. 1988) to day 12 (Pierson and Ginther, 1984; Reeves et al., 1984; Kastelic et al., 1991) post-insemination when the conceptus appears as a non-echoic or echogenic vesicle in the horn ipsilateral to the corpus luteum (Curran et al., 1986; Body et al., 1988). It has also been reported that the embryo itself can be imaged on day 11 (Boyd et al., 1988) and day 13 (Curran et al., 1986; Kastelic et al., 1989) contained within the fluid vesicle. However, Kastelic et al. (1991) disagreed, reporting that the embryonic vesicle seen on days 10 and 12 cannot be reliably distinguished from fluid which normally collects in the uterus during the cycle and also in certain pathological conditions. On this basis it may be preferable to wait until day 20 to 21 when the heart beat can be detected (Pierson and Ginther, 1984; Curran et al., 1986; Kastelic et al., 1988).

At day 8 or 9 the embryonic vesicle is spherical with a diameter of 0.2 mm. By days 12 to 13, the embryo is oblong with a diameter of 3 mm (Pierson and Ginther, 1984; Curran et al., 1986). Between days 10 to 18 the embryo elongates rapidly, becoming filamentous (Kahn, 1994) and reaching the contralateral horn. By this stage, the amnion has fully enveloped the conceptus although it is not detectable by ultrasound until day 28 (Curran et al., 1986; Boyd et al., 1988). Between days 21 and 24 the amount of fluid inside the embryonic vesicle increases greatly with the greatest expansion around the amnion (Kahn, 1994). The amnion now appears as a fine, slightly hyperechogenic band surrounding the embryo with

diameter of 3 to 5 mm and a length of 1 cm (Kahn, 1994) and can be visible up to day 60 (Curran et al., 1986). Within the amnion the embryo is seen as a small echogenic line with a pulsating heart. The embryo rapidly changes into a prominent C-shape by day 30 and then into an L-shape by day 39 (Curran et al., 1986)

The allantois can be detected on day 23 (Curran et al., 1986; Boyd et al., 1988; Kastelic et al., 1988) as an attachment to the mid-ventral portion of the embryo. It appears as a thin, hyperechoic, floating membrane. It is often only briefly visible from day 23 to 26, sometimes up to day 30, before it is no longer visible as a separate structure. By day 30 the diameter of the embryonic vesicle is 18 to 20 mm at the point of greatest expansion (Chaffeux et al., 1982; Curran et al., 1986). On average the embryo increases from 5 to 12 mm long over the period from day 25 to 30 of pregnancy (Pierson and Ginther, 1984; Reeves et al. 1984, Curran et al., 1986). During this time the cephalic and anal regions and also the limb buds of the embryo are visible. By day 35 the outline of the fetal form can be detected with the head, extremities and umbilical cord all visible (Boyd et al., 1988; Kahn, 1990). The changes which transform the undifferentiated, primitive embryo into a fetus with a distinct body form continue from day 35 to 40 (Curran et al., 1986; Body et al., 1988). The cross-sectional diameter of the placental vesicle also increases to reach 25 mm by day 40, 35 to 40 mm by day 50 and 50 to 60 mm by day 70 (Kahn, 1994; Curran et al., 1986). The embryo continues to grow at a rate of 1 mm per day until about day 50 so that crown-rump-length (CRL) is 15 mm at day 35 and 20 mm by day 40 (Pierson and Ginther, 1984).

The volume of fluid in the extra embryonic sacs increases rapidly causing marked dilation of the uterus around the embryo but with less distension in the contralateral horn. The embryo floats freely in the amnion while its allantochorion expands rapidly. However, it is not possible to estimate the volume of fluid in the sacs by ultrasound as it becomes increasingly difficult to image the conceptus in one sonogram. After day 40, sonographic examination can be used to demonstrate various embryonic or fetal structures. Thus ossification centres of various bones can be detected ultrasonically at about this time (Kahn, 1990; Mowa, 1994). Another important external feature detected at about day 48 is the genital tubercle which is the forerunner of the penis and clitoris. By monitoring its position fetal gender can be determined at about day 53 in the female and 56 in the male (Curran, 1989, 1992; De Moura, 1993).

The ultrasonic appearance of the amnion and allantois and their fluids also changes. The amniotic fluid remains anechogenic during the first trimester although it subsequently becomes more reflective due to increased cellular content and viscosity (Kahn, 1990). It becomes more dense and gives a snow-storm-like appearance as the pregnancy advances to mid gestation. The allantoic fluid on the other hand remains anechoic until the 6th month when slight echoes appear and its density starts to increase rapidly. From the 8th month until shortly before term the echogenicity of the allantoic fluid may be very similar to that of the amniotic fluid. The amniotic membrane in some cases can be recognised as a prominent, thin echogenic line floating within the nonechogenic placental fluids although it can be very difficult to visualise the membranes in later gestation.

1.5 Embryo/ Fetal Sexing Techniques

The desire to control sex appears to be as old as man himself and certainly dates back to the first records of Greek authors connected with veterinary science (Betteridge, 1984). Early prediction of embryo/fetal sex has recently graduated from mere mythical speculation to a period of intense and scientific investigation. In both human and farm animals, several approaches have thus far been reported. It is important to note that sexing in veterinary science like many other disciplines has greatly benefited from human medicine.

Riis & Fuchs, (1956) reported a cytogenetic histological procedure using amniotic cells to determine sex in humans based on the observation of sex chromatin or Barr body. The Barr body is a remnant of the inactivated X chromosome in female cells. This report sparked a lot of interest in animal studies and led eventually to successful gender prediction in rabbits (Gardner and Edwards, 1968, cited by Miller, 1991). Observation of the Barr body is not possible in other domestic species due to the granular nature of the cell cytoplasm. However, using cultured amniotic cells recovered from cows at 70 to 100 days of gestation, Bongso and Basrur (1975) were able to determine sex of the fetus by chromosomal analysis. Recently, cytogenetic methods have been applied to amniotic cells to determine fetal sex (Singh et al., 1977; Leibo and Rall, 1990). The cytogenetic technique of sexing is highly accurate and reliable such that with the

advent of embryo transfer technology in the early 1970s the technique gained widespread use. Bovine embryos have been sexed at various stages of development including day 12 to 15 trophoblasts by biopsy (Hare et al., 1976) and whole or demi-embryos on day 6 or 7 (Moustafa et al., 1978; cited by King, 1984). The low numbers of cells in metaphase and the time taken to arrive at a diagnosis tend to limit the use of chromosome analysis for embryo sexing. Day 6 to 7 coincides with the period when bovine embryos can be collected, frozen and transferred non-surgically (King, 1984). This allows time to culture cells to generate sufficient metaphase spread for diagnosis. On the other hand, once the blastocyst has hatched it cannot be frozen, thus sexing and transfer must be performed on the same day. Hare et al., (1976) designed a method to hasten the technique by treating a cell biopsy from 14- and 15-day old embryos for 3.5 hours before analysing for sex. However, they still found that cytogenetic analysis was costly of time and experienced technicians. Consequently Bongso et al., (1978) evaluated amniotic smears as an alternative. Staining the cell smear by the Papanicolaou method yielded separate populations of eosinophilic, orangeophilic, and cyanophilic cells. Female amniotic cell smears showed a significantly higher count of cyanophilic cells allowing this group to determine fetal sex within hours.

Analysis of steroid hormone levels, especially testosterone in fetal fluids in humans, showed that levels are higher in the male fetus (Younglai, 1972, 1973). A similar study conducted from day 45 to 193 of gestation in cattle showed significant differences in the concentration of testosterone in allantoic fluid of male and female fetuses, but not in amniotic fluid (Bongso et al., 1976). According to

their findings it was possible to predict fetal sex during the period of study by measuring testosterone levels in allantoic fluid. However, later reports on steroid hormone levels in fetal fluid compartments showed that amniotic fluid levels of testosterone in male fetuses appeared to fluctuate with peaks on days 50, 85, and 115 to 120 (Dominguez et al., 1990). These workers also reported that the testosterone levels in amniotic fluid correlated well with those produced by cultured gonads of the same fetus. In addition the allantoic levels of the hormone did not suggest fetal gender and did not show a correlation to the levels in cultured gonads. They finally concluded that amniotic testosterone levels were more suggestive of fetal gender than those of allantoic fluid. While the hormonal approach can predict sex at particular times of gestation it can only be employed after gonadal sex has been established, i.e. on the 42nd day of gestation in cattle (Noden & De Lahunta, 1985) and probably not until much later when the hormone is present at detectable levels.

Although cytogenetic sexing techniques are highly accurate, they tend to be highly invasive thereby reducing embryo viability. Also when applied to amniotic cells the method increased the calving interval for cows that carry fetuses of the unwanted sex. The primary aim of sex determination is to modify the calf crop as early as possible in gestation, but there is a paucity of culturable amniotic cells in the first trimester. In addition a minimum culture period of 13 days (Leibo and Rall, 1990) is required to obtain sufficient cells for chromosomal analysis, during which time the pregnancy advances. Similarly the testosterone analytical approach is only applicable after the establishment of gonadal sex.

Alternatively, non-invasive, biochemical and immunological methods applied to embryos prior to transfer have been described. It was the rejection of male skin grafts by females in highly inbred mice that led to the discovery of the H-Y antigen (Eichwald and Silmer, 1955, cited by Miller, 1991). Using cytotoxicity and immunofluorescent assays, immunological sexing has been achieved by detection of H-Y antigen in male bovine embryos (Wachtel, 1984; Booman et al., 1989) and in other species (Krco and Goldberg, 1976; Allen and Wright, 1983). Both polyclonal and monoclonal antibodies have been employed against the H-Y antigen but the accuracy of the technique still does not appear to exceed 87% (van Vliet, 1989). Efforts to improve assay specificity and reduce cytotoxic damage of embryos other than the unwanted (male) ones are yet to be fully successful.

The biochemical method is based on the hypothesis that the ratio of X-linked enzyme activity to autosomal enzyme activity will be higher in female than male embryos before the onset of X-inactivation early in development (Monk and Kathuria, 1977). The X-linked enzymes used in this approach are glucose-6-phosphate dehydrogenase and hypoxanthine phosphoribosyltransferase (HPRT) (Biggers and Stern, 1973; Monk and Handyside, 1988; Kita and Imai, 1993). Successful sexing of pre-implantation mouse embryos has been achieved using the technique. The approach was also used in the bovine to evaluate embryo viability and also to determine the sex (Rieger, 1984). Interestingly, these largely non-invasive methods which might have provided an attractive alternative to more invasive techniques have not been taken up by the cattle industry.

Ultrasonographic sex determination is one area which has gained more widespread use, especially in bovine reproductive research. Muller and Wittkowski, (1986) reported ultrasonic detection of the scrotal sac, in later stages with testes, and also the mammary gland to sex the bovine fetus from day 70 to 120 of gestation. Recently it has been reported that fetal sex can be determined in equines and bovines by ultrasonic detection of the relative location of the genital tubercle (Curran, 1986, 1992; De Moura, 1993). This is the forerunner of the male glans penis and the female analogue, the clitoris. The structure slowly migrates from its position when first detected between the hindlegs on day 48 (Curran et al., 1986) either towards the umbilical cord in the male or towards the anal region in the female (Noden and De Lahunta, 1985). By day 56 the genital tubercle lies just caudal to the umbilicus in the male and by day 53, ventral to the anus in females (Figure 1.1). It is possible to differentiate fetal sex by ultrasound from day 53 to 56 with an accuracy approaching 100% in experienced hands (De Moura, 1993).

The identification of bovine Y chromosome specific DNA probes (Leonard et al., 1987; Ellis et al., 1988) transformed sexing of embryos from theory to reality. Using *in-situ* hybridisation and a biotinylated Y-specific DNA probe they determined bovine embryo sex prior to implantation. Coupled with *in-vitro* amplification of the target DNA by the polymerase chain reaction (PCR), accuracy and speed of embryo sexing was greatly enhanced. Several studies have since reported successful sexing of bovine embryos by chromosomal hybridisation of the Y chromosome (Leonard et al., 1987; Ellis et al., 1988; Bondioli et al., 1989). However, according to Bondioli et al., (1989) Y chromosome-specific probe

hybridisation requires a minimum of 8 days to reach a diagnosis. This would necessitate freezing the embryo after biopsy just as in the cytogenetic methods. The in-vitro amplification of pre-selected target DNA by PCR technology (Saiki et al., 1985; 1986; 1988) enabled Herr et al., (1990) to sex bovine embryos using Y chromosome-specific primers. This technique has greatly improved the accuracy and speed of embryo sexing and it can now be performed in a matter of hours. To date, several reports have demonstrated the versatility of the PCR technique, enabling the mapping of the sex chromosomes to determine the sex determining region of the Y chromosome. The *zfy* region was once thought to determine male sex, although this is now disputed (Palmer, et al., 1989, cited by Goodfellow, et al., 1993) because a homologue (*zfx*) was reported on the X chromosome (Scheider-Gadicke, et al., 1988) and has been used to differentiate X from Y bearing DNA samples (Aasen and Medrano, 1990). Using these regions they designed universal primers that enabled amplification and sex DNA samples from humans, cattle, sheep and goats by restriction fragment length polymorphism analysis of the PCR products. Based on the same primers, nested PCR primers that rapidly and theoretically improve sensitivity and accuracy have been reported (Kirkpatrick and Monson, 1993). Thus with the nested PCR technique these workers were able to analyse the products by simple gel electrophoresis and ethidium bromide staining. Detection of the *SRY* gene by PCR has been used for sex determination of pre-implantation embryos in mice and pigs (Kunieda, et al., 1992; Wu, 1993; Pomp, et al., 1995). The *SRY* gene is a highly conserved region in all eutherian mammals and accepted to be the primary candidate controlling

testicular development (Sinclair, et al., 1990; McElreavey, et al., 1993; Goodfellow, et al., 1993). Ongoing projects involving PCR and embryo sexing include the design of techniques for a cow-side test (Thibier and Nibart, 1995; Bredbacka et al., 1995). Nowadays with the advent of DNA microinjection for the production of transgenic animals, manipulation of pre-implantation embryos not only involves sexing but also detection of transgenics. Amniotic and/or allantoic fluid cells are also used to confirm presumptive transgenics later in gestation.

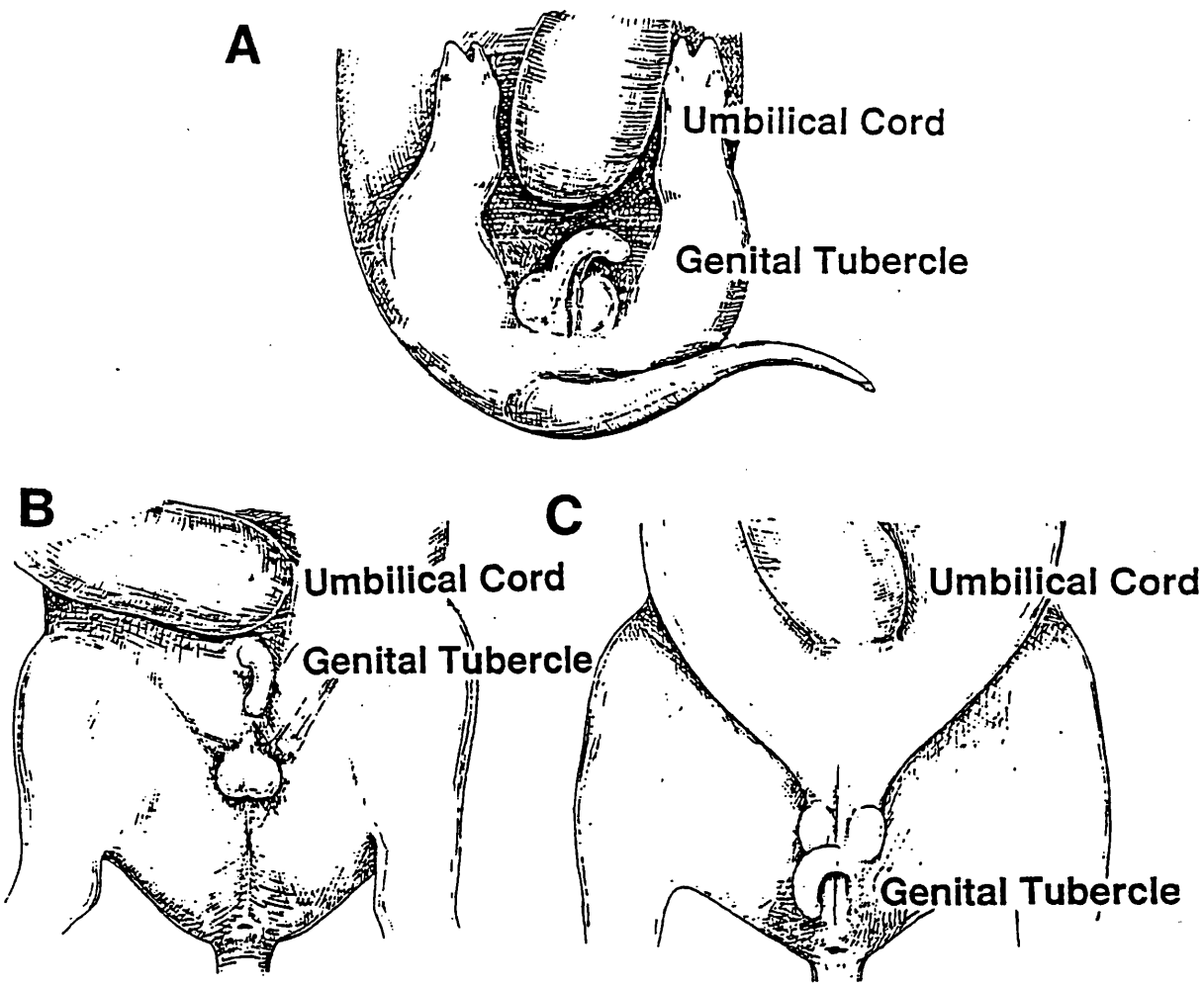
However, efforts to segregate X and Y chromosome-bearing spermatozoa continue with variable success (Johnson et al., 1994; Cran et al., 1994). If ultimately successful, this would provide the means of gender pre-selection in mammals.

Figure 1.1. Diagrams indicating the location of the genital tubercle in the male and female fetus before and after sexual differentiation of the bovine embryo (Re-drawn from Barone, 1990).

A. Undifferentiated embryo (around 42 days). At this developmental stage male and female embryos are identical.

B. Male embryo (around 56 days). Note that the genital tubercle has migrated to a site caudal to the umbilical cord.

C. Female embryo (around 52 days). Note that the genital tubercle is sited beneath the tail.


A
Umbilical Cord
Genital Tubercle
B
Umbilical Cord
Genital Tubercle
C
Umbilical Cord
Genital Tubercle

1.6 Fetal Fluid Recovery (Amniocentesis)

Diagnostic amniocentesis was first advocated by Meness et al. (1930) but it was not until the 1970s that the technique became a routine procedure in human obstetrics (Anandakumar et al., 1992). Among the early studies on fetal fluids were prenatal sex determination (Riis and Fuchs, 1956; Serr et al., 1964) and prenatal diagnosis of chromosome disorders using cultured amniotic cells (Jacobson and Barter, 1969 and Nadler, 1968, cited by Bennet, 1981). Amniocentesis is nowadays a routine procedure in high risk pregnancies and, to date, over 200 different fetal abnormalities have been diagnosed prenatally in human pregnancies (Kurjak et al. 1993). On the other hand studies in domestic animals have been much less extensive.

For a very long time amniocentesis in humans was performed successfully without the benefit of ultrasonography (Meness et al., 1930; Fairweather, 1978; Bennet, 1981). However, the development of B-mode scanning enabled the placenta to be located prior to amniocentesis (Curtis et al., 1972, cited by Fairweather, 1978) and with the use of a special puncture transducer (Holm et al., 1972 and Weiner, 1976, cited by Fairweather, 1978) the use of ultrasound to perform the technique increased. In the late 1970s it became strongly recommended to perform ultrasound before amniocentesis (Bennet, 1981). Nowadays instruments for both transabdominal and transvaginal real-time ultrasound-guided puncture are routinely used.

The debate, however, in humans is the safety of amniocentesis and recently the stage of pregnancy most suitable to perform the procedure. In the early days there was little concern for the safety of the procedure and the only considerations were its diagnostic accuracy and the reasons for performing it (Henry and Miller, 1992). It was also advocated that the procedure be performed as early in gestation as possible in pregnancies at risk. In the late 1970s, however, it became commonly accepted that amniocentesis for genetic studies was best performed at 15 or more gestational weeks (Henry and Miller, 1992). More recently, however, several reports have evaluated earlier ultrasound-guided amniocentesis and reported routine amniocentesis without significant risk (Henry and Miller, 1992). Generally the risk rate in routine amniocentesis with or without ulurasound guidance is less than 1% (Fairweather, 1978; Anandakumar et al., 1992; Kurjak et al., 1993) with a slight increase in inexperienced hands (Hanson et al., 1987; 1992; Henry and Miller, 1992). However, early amniocentesis in humans has highlighted the increased risk of complications and contamination of the sample brought about by multiple needle puncture (Hanson et al. 1992). Also there seems to be a lack of relationship between alpha-fetoprotein (AFP) and acetylcholinesterases (AChE) and the neural tube defects in early samples as these are not normally expressed until the second trimester (Wathen et al., 1991). This tends to reduce the diagnostic value of early amniocentesis thereby favouring the routine later procedures.

Surprisingly there has been great inertia in the veterinary field to benefit from the amniocentesis procedure despite the vast information source and

experience available from human medicine. In the prenatal diagnosis of sex in cattle by amniocentesis, Bongso and Basrur (1975) recovered the fetal fluids by a blind transvaginal route. In another report, Leibo and Rall (1990) collected the fluid surgically from cows at 7 to 22 weeks of gestation for prenatal sexing of pregnancies from embryo transfer. In the former the risk of abortion and induction of congenital malformations was ignored as the animals were bound for the abattoir; while in the latter, spontaneous abortions were recorded. Successful and repeated recovery of bovine fetal fluids through transvaginal ultrasound-guided puncture has been reported by Vos et al. (1990) who successfully aspirated amniotic fluid on day 44 and allantoic fluid on day 32 of pregnancy. Evidence from their study suggested that repeated punctures increased the risk of intrauterine death despite one cow calving at term after sampling five times at weekly intervals. In another study, designed to evaluate the diagnostic quality and effects of transvaginal-transuterine aspiration of bovine fetal fluids on normal fetuses, Sprecher and Kaneene (1992) used blind direct and indirect methods for multiparous and primiparous cows respectively. Using a direct fingertip manipulation of the needle intravaginally they successfully aspirated fetal fluid between 55 and 75 days of pregnancy. In the indirect technique the intravaginal fingertip manipulation of the needle was impossible so a plastic infusion pipette altered by attachment of a needle at its tip was used instead. They successfully aspirated fluid from day 50 to 65. Both techniques were associated with abortion rates that were thought to preclude prospective use of the technique in diagnostic strategies.

The diagnostic value of amniocentesis has also been assessed in mares (Kahn, 1992; Schmidt et al., 1991). Transvaginal ultrasound-guided punctures on singleton pregnancies were performed between 19 and 75 days of gestation but most of the conceptuses died after puncture except for one performed after day 54 (Kahn, 1994). Attempts to apply the same technique in the reduction of twin pregnancies resulted in death of both embryos (Bracher et al., 1993; Khan, 1994) thereby reducing the applicability of the technique. Using the transabdominal approach Williams et al., (1988) determined pulmonary maturity of horses in the third trimester. Bennet et al., (1989) also reported single transabdominal ultrasound-guided amniocenteses of horses in second trimester pregnancies to determine fetal sex and hormonal concentration in amniotic fluid. In both of these studies, transabdominal ultrasonography was used to locate the amniotic fluid, but needle insertion was performed without observation of the needle path. Incidence of fetal death, abortion or any complication following the procedure was not indicated. In a recent study, mares were subjected to serial transabdominal ultrasound-guided amniocentesis performed at 12 to 15 day intervals in late gestation until foaling (Schmidt et al., 1991). This study ruled out the use of the technique clinically due to complications of abortion and placentitis.

It seems that amniocentesis is slowly being applied in the livestock industry especially in genetic engineering of cattle to identify presumptive transgenic calves *in utero* as suggested by Leibo and Rall (1990). Several research workers are using transvaginal ultrasound-guided amniocentesis for preimplantation testing to confirm transgenesis (Bowen et al., 1994; Hyttinen, et al., 1994). It is hoped that

with consistent efforts the technique may be routinely used in domestic animal diagnostics.

1.7 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is an ingenious new tool in molecular biology which provides a faster alternative to the traditional methods of DNA cloning. It involves enzymatic amplification of selected nucleic acids (DNA). The technique utilises a three step cycle of heat denaturation of the double-stranded target DNA, primer annealing and polymerase mediated primer extension. Repeated cycles result in an exponential increase in mass of the original sample.

The pioneers of this enzymatic amplification technique used the thermosensitive Klenow fragment of Escherichia coli (E. coli.) DNA polymerase I (Saiki et al., 1985; Mullis and Faloona, 1986). This required replenishment of enzyme after every cycle. However, following the discovery of the thermostable DNA poylmerase isolate in a thermophilic bacterium, *Thermus aquaticus* (Taq) (Saiki et al., 1988; Innis et al., 1988), the need to replenish the enzyme was eliminated. Taq polymerase survives incubation at 94 to 95°C and PCR has since become automated, gaining wider application in molecular biology research, disease diagnostics and forensics.

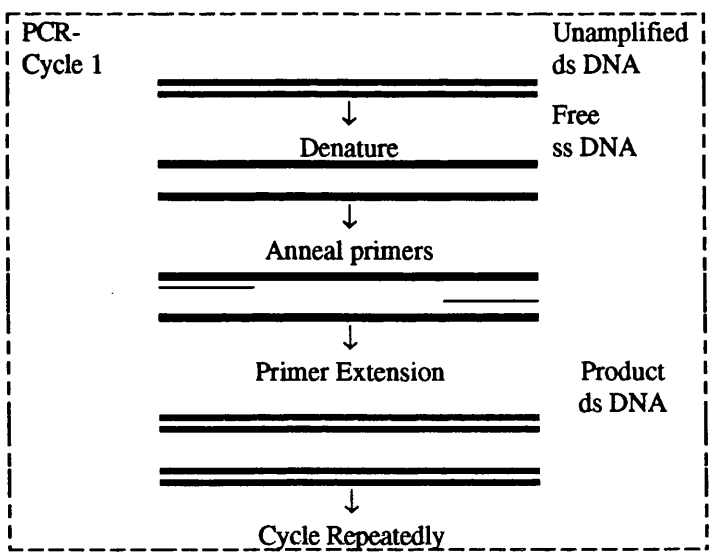
1.7.i Principle of the Technique

PCR exponentially amplifies copies of a pre-selected segment of DNA (template). The amplification process involves a set of synthetic oligodeoxynucleotide primers, thermostable DNA polymerases and four deoxyribonucleotide triphosphates (dNTP) acting on the template DNA. The primers match or anneal to their respective strand of the template either in the sense or the anti-sense orientation. The process basically involves three steps: heat denaturation, primer annealing and strand extension. Heat denaturation of the template double-stranded DNA into single strands is effected by subjecting it to temperatures of 94 to 95°C. The two dissociated strands remain free in solution until the temperature is lowered enough to allow re-annealing. Lowering the temperature, however, allows the primers to compete with the template strand in the process of re-annealing to the respective complementary strand. And since the primer concentration is high compared to the template, the reaction favours primer annealing. Annealing occurs at the 5' end of the template and the enzyme DNA polymerase catalyses extension of the primers toward the 3', end forming a new double-stranded template of the selected sequence. Repeated cycles (usually 30) of heat denaturation, primer annealing and strand extension result in exponential increase of the target DNA sequence. The increase in the amount of target DNA will be 2^n, where n is the number of cycles, if the reactants are optimal.

Despite the straight forwardness of the procedure the reaction mixture, cycle temperature and the number of cycles must be optimised for each PCR. The problems most often encountered include: 1) no detectable products or a low yield of the desired product, 2) the presence of non-specific background bands due to mis-priming or mis-extension of the primers, 3) formation of "primer-dimers" that compete for amplification with the desired product, and 4) mutation or heterogeneity due to mis-incorporation.

It is recommended that parameters that influence specificity, fidelity and yield of the desired product should be kept within certain concentration ranges. For instance, the enzyme behaviour should be tested at concentrations ranging from 0.5 to 5 units/100μl and to minimise mis-priming, dNTP concentration should be as low as possible. The magnesium ion concentration is important as it can affect many aspects of the PCR including enzyme activity. Each PCR should contain 0.5 to 2.5 mM Mg over the total concentration of dNTPs. Other reaction components might be optional but the buffer should be within pH 8.3 to 8.8 at 20°C.

Figure 1.2. An illustrative diagram showing the principle of Polymerase Chain Reaction. Repeated cycles of denaturation, primer annealing and polymerase mediated primer extension result in an exponetial increase of the original sample (target sequence). ds = double stranded, ss = single-stranded.



Chapter 2

Materials and Methods

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2.1.ii Housing and Feeding

2.1.iii Oestrus Synchronisation and Artificial Insemination

2.1.iv Pregnancy Diagnosis

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2.1 Overview

The Materials and Methods section covers a description of the handling and housing of experimental animals, basic and advanced ultrasonographic techniques used in this work and the molecular techniques developed and employed for bovine fetal sexing during this project.

2.1.i Experimental Animals

In total 6 cows and 5 heifers were inducted into the study. Pluriparous Holstein/Friesian cows were bought from the 'cast cow market' (numbers 61, 100, 156, 71, 112 and 131). Five heifers were also purchased. A single, pregnant Holstein/Friesian heifer (heifer 01) and 4 unserved dairy/cross Hereford heifers (numbers 4, 12, 36, and 99). Selection was based upon examination of a normal genital tract and identification of functional ovaries. Because of failure to become pregnant during the timespan of the study, Cow 156 and Heifer 4 were subsequently dropped from the study.

2.1.ii Housing and Feeding

For the duration of the study all animals were housed at the Glasgow Veterinary School Netherton Farm. Accommodation consisted of byre stalls with automatic drinkers. Animals were bedded on straw and fed silage *ad lib* supplemented with cattle cubes.

2.1.iii Oestrus Synchronisation and Artificial Insemination

Oestrus synchronisation was carried out using the progestagen ear implant/ oestradiol injection combination 'Crestar' (3 mg Norgestomet subcutaneous ear

implant and 5 mg Oestradiol valerate in 2 ml intramuscular injection; Intervet, Science Park, Milton Road, Cambridge, UK). Eight days after insertion of Crestar implants, the animals were given an intramuscular injection of 500 mg Cloprostenol, a synthetic prostaglandin analogue (Estrumate, Coopers Animal Health, Crewe Hall, Cheshire, UK) to lyse any active corpus luteum. Implants were removed on day 9 and animals were expected to be in oestrus approximately 48 hours later. Animals were inseminated twice 48 and 72 hours after 'Crestar' implant removal by a technician from Scottish Livestock Services.

2.1.iv Pregnancy Diagnosis

Routine pregnancy diagnosis was carried out by transrectal ultrasonic examination of the reproductive tract about 28 days post insemination. Pregnancy was confirmed by the presence of a mature corpus luteum, of approximately 2.5 cm diameter, on one ovary and a conceptus in the ipsi-lateral uterine horn. The first batch of cows (numbers 61, 100 and 156) were monitored from day 11 onwards, at intervals, in an attempt to identify early features of pregnancy. These preliminary examinations were not carried out in the other animals.

2.2 Ultrasonography - General Training

Ultrasonographic examination of the cow reproductive tract was initially practised on abattoir specimens. Both pregnant and non-pregnant tracts were recovered shortly after slaughter and examined in a water bath to simulate the disposition of the tract in a live animal. This training was augmented with attendance at a reproductive ultrasound course designed for practising veterinarians (Continuing Professional Development). Further skills in transrectal and transvaginal scanning were acquired during pregnancy diagnosis of the study animals.

2.2.i Ultrasound-Guided Follicle Aspiration

The technique of ultrasound-guided transvaginal follicle aspiration provided a basis for training and technique development for the subsequent fetal fluid aspiration. The technique is commonly used for the retrieval of mature and immature ova in both humans and animals for in-vitro maturation and fertilisation (Pieterse et al., 1991; Kruip et al., 1991; Scott et al., 1994; Bols et al., 1995; Bungartz et al., 1995). Details of the technique developed by this Research Group have been published elsewhere (Scott et al., 1994) but a detailed description is given below because of relevance to the fetal fluid aspiration procedure.

2.2.ii Ultrasound Equipment

Two ultrasound scanners were used throughout this study. The first was a real-time B-mode portable scanner equipped with a linear array 7.5 MHz rectal transducer (Concept 2; Dynamic Imaging, Cochrane Square, Brucefield Industrial Park, Livingston, Scotland). This equipment (Figure 2.1) was used for general rectal examinations, pregnancy diagnosis and follow-up examinations after fetal fluid aspiration. The second scanner was a Toshiba Capasee (Model SSA-220A; Toshiba

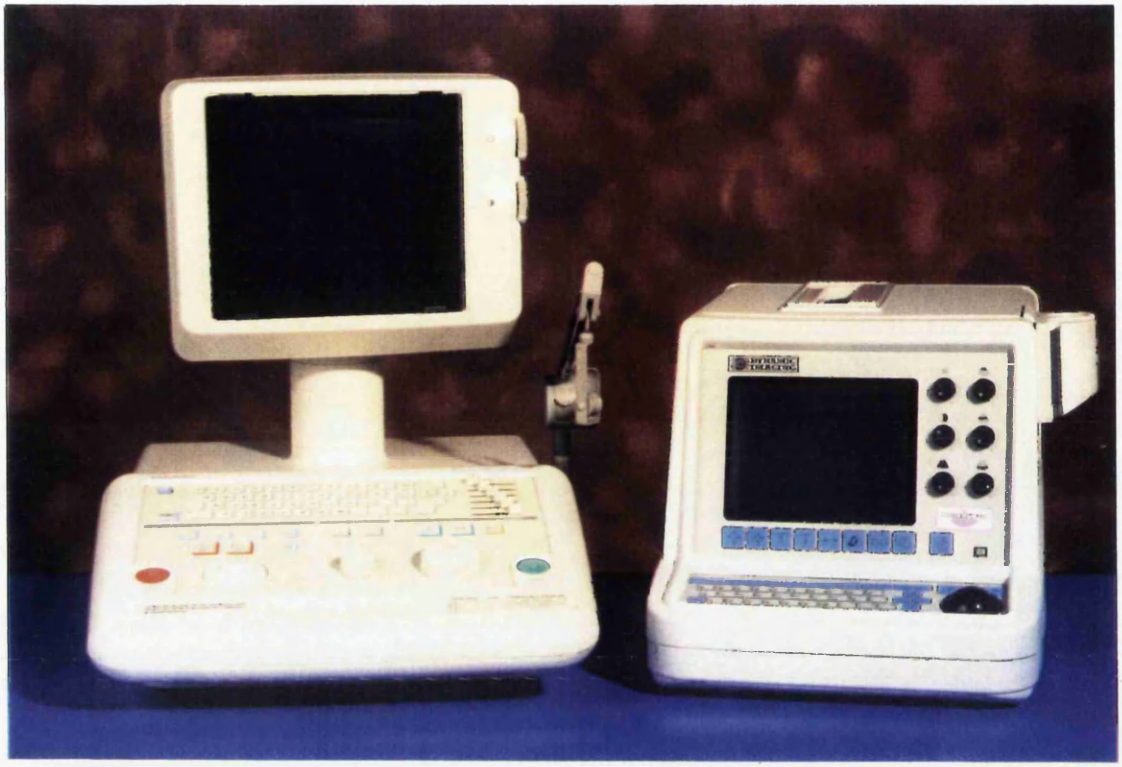
Medical System, Manor Royal, Crawley). This scanner (Figure 2.1) was equipped with a 31 cm long, 6 MHz, microconvex curvilinear human vaginal transducer (Model 601v, Toshiba). The vaginal transducer was equipped with a 21 cm, 14 g needle guide incorporating a biopsy guide-line and was used for follicle and fetal fluid aspiration (Figure 2.2). This scanner was also equipped with a 7.5 MHz microconvex curvilinear rectal transducer. This transducer gave a very high quality image and was most suitable for early pregnancy diagnosis and for visualising fetal structures in detail as required for sex definition.

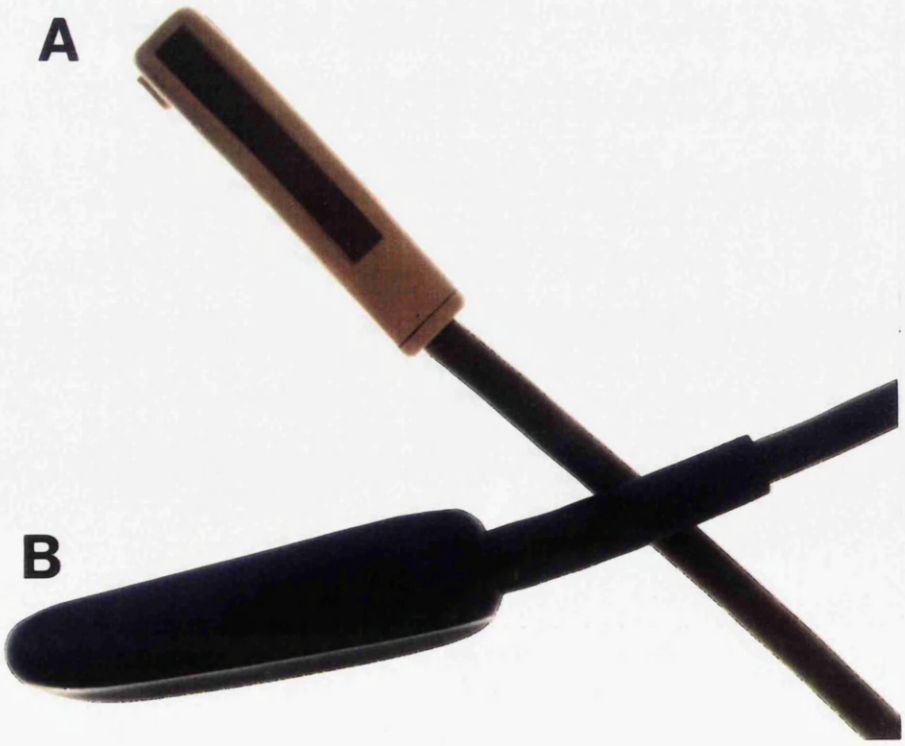
All procedures were recorded using a videorecorder to allow later review. Hardcopy pictures were produced from videotape using a thermal printer (Sony UP850) or a high quality printer (Sony UP3000P, Sony Corporation, Japan).

Figure 2.1. Equipment used for follicle aspiration and fetal fluid recovery.

A. Two microconvex, real-time B-mode scanners were used in the study. On the left of the picture is the Toshiba Capasee, incorporating software for a biopsy guide-line, used for follicle aspirations and fetal fluid recovery. On the right is the Concept MC, utilised for transrectal examinations.

B. Transrectal transducers: (A) 7.5 MHz microconvex, curvilinear transducer used with the Capasee and (B) 7.5 MHz linear-array transducer used with the Concept MC.




A
B

2.2.iii Animal Preparation

During the procedure the animals were restrained in a cattle crush padded to reduce lateral movements. An intravenous injection of 0.3 mg clenbuterol hydrochloride (Planipart, Boehringer Ingelheim, Bracknell, Berkshire) was given to relax the bowel and ease manipulation of the uterus. This was followed 10 minutes later by an intravenous injection of 0.16 mg/kg detomidine hydrochloride (Domosedan, SmithKline Beecham Animal Health, Walton Oaks, Tadworth, Surrey, UK) to achieve sedation. An epidural of 0.1 mg lignocaine hydrochloride in 5 ml (C-vet, Minister House, Bury St Edmunds, Suffolk, UK) was then given to anaesthetise the perineum and this drug treatment allowed 15 to 20 minute working time. The vulva and perineal area were thoroughly scrubbed with 1% chlorhexidine gluconate solution.

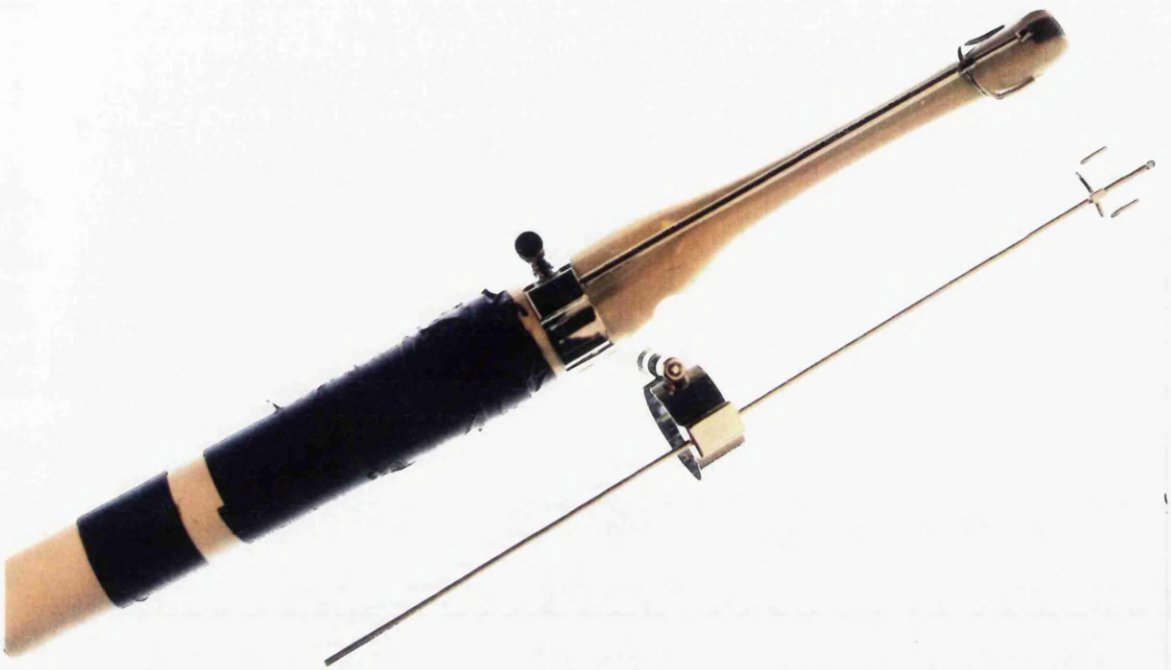
2.2.iv Needles and Aspiration

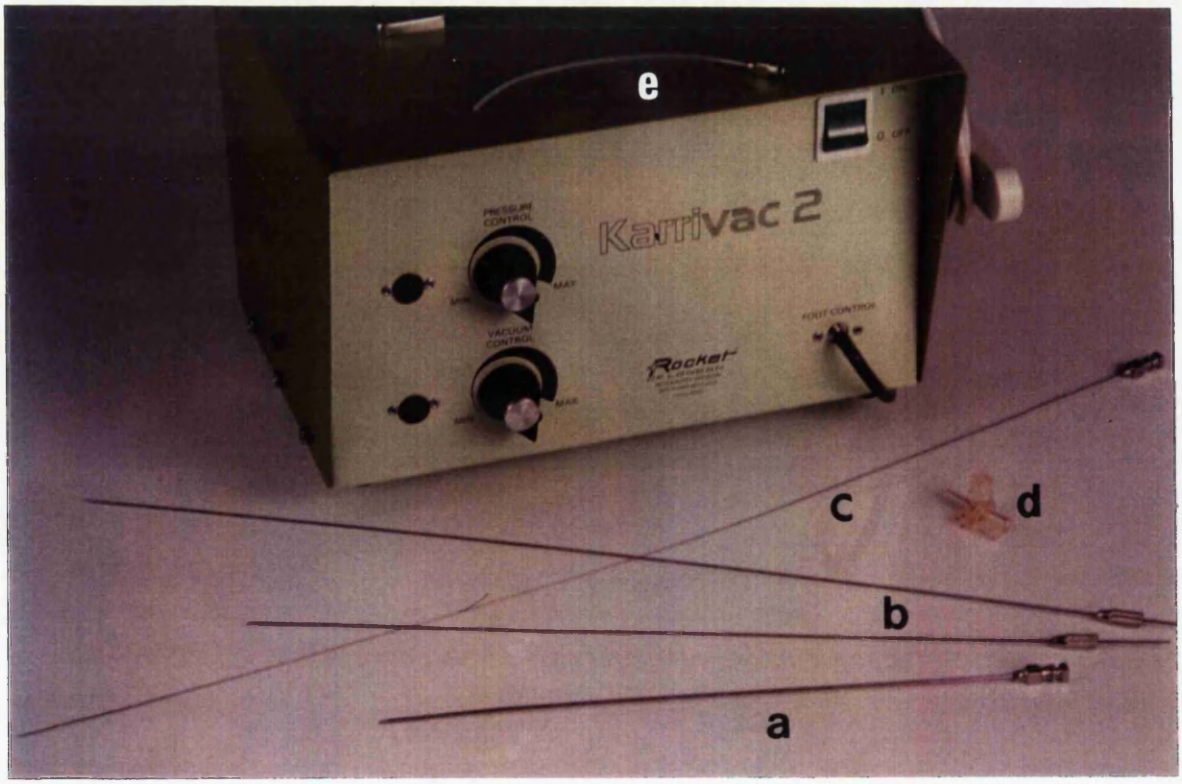
Three needle types were used in the follicle aspirations. A 15 g x 29 cm needle with a central stylet was used as an introducer, to penetrate the vaginal wall. This reduced the incidence of damage to the fragile 18 g needles and prevented blockage of the needles with a plug of vaginal tissue. For aspiration, an 18 g x 32 cm or 45 cm needle, with echogenic tip, were used (Casmed, 36 Kenley Walk, Cheam, Surrey, UK). The aspirate was collected in a centrifuge tube reservoir connected by a 17 g extension tubing to an electric pump activated by a foot pedal (Model-Karri-vac 2, Rocket of London, Imperial way, Watford) (Figure 2.2).

Figure 2.2. Equipment used for follicle aspiration and fetal fluid recovery

A. Transvaginal transducer (6.0 MHz) with original 21 cm needle guide used for follicle aspiration and below it, a customised 36 cm guide, extended for fetal fluid recovery.

B. Foot-pedal activated electric pump mainly used for follicle aspirations. A set of needles used in the aspirations; (a) introducer needle with stylet, (b) 18 g x 32 and 45 cm for aspirations and fluid recovery and (c) 22 g x 50 cm specifically for fetal fluid recovery. Note (d), a 3-way adapter and (e) plastic tube with luer adapter used with a syringe for fetal fluid aspiration.




e
Kartivac 2
PRESSURE CONTROL
VACUUM CONTROL
Rocket
c
d
b
a

2.2.v Follicle Aspiration Procedure

The technique requires an operator and an assistant. The operator was responsible for manipulating the reproductive tract rectally, while ultrasonically scanning *per vaginum.* Needle manipulation were performed by the assistant. Firstly the operator advanced the transducer into the vagina up to the external os of the cervix. The 15 g introducing needle was pushed through the vaginal wall and the stylet removed from this needle. The ovary was manipulated per rectum to lie against the transducer head over the biopsy guideline. Once in that position the 18 g aspiration needle was inserted through the introducer by the assistant. The echo tip of this needle was monitored as it was advanced down the path of the biopsy guide-line into the follicle. Suction was initiated immediately the needle penetrated the follicle. The follicle was seen to collapse on the real-time ultrasound image if the procedure was successful (Figure 2.3) and the follicular contents were then recovered in the centrifuge tube reservoir.

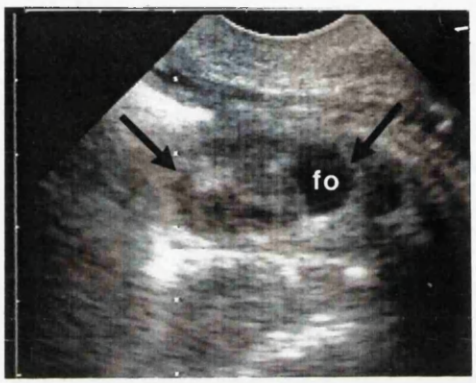
Figure 2.3. Sonographic events during the procedure of follicle aspiration.

A. The entire ovary is imaged using the transvaginal transducer (arrows). This ovary contains one medium-sized follicle (**fo**) and several small follicles.

B. The ovary was manipulated to align the medium-sized follicle (**fo**) with the biopsy guide-line.

C. The needle has been advanced and the echogenic tip is now visible within the follicle (arrow, n).

D. Aspiration was initiated to remove follicular contents. The follicle is no longer visible at the aspiration site although the needle is still visible (arrow).



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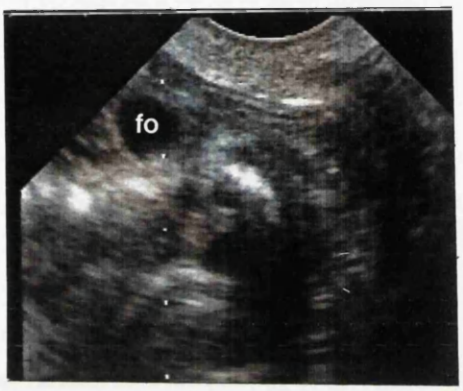

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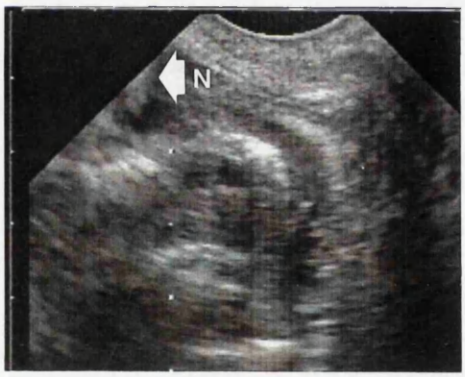

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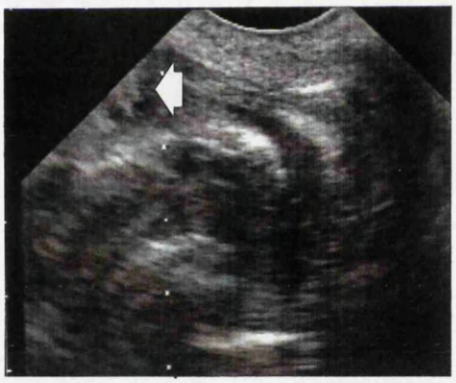

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2.2.vi Ultrasonographic Sexing of the Fetus

Ultrasonic examination was carried out after 52 days of pregnancy for the purpose of sexing the fetuses. This examination was carried out by Professor Boyd or Dr Robertson. The procedure consisted of examination of the reproductive tract per rectum using the high quality Toshiba scanner and a 7.5 MHz transducer. Sedation of the dam was not usually necessary. The fetus was examined and the relative positions of the genital tubercle and the umbilical cord were established. In the male fetus, at this stage of development, the genital tubercle is closely apposed to the umbilical cord whereas in the female fetus, the genital tubercle is seen beneath the tail. The ultrasonographic appearance of the genital tubercle in male and female fetuses is shown in Figure 2.4.

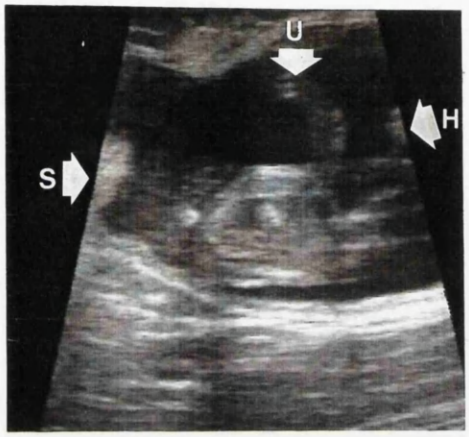
Figure 2.4. Ultrasonographic detection of fetal gender.

A. Orientation of the fetus (s = skull, u = umbilical cord, h = hindlimb). The areas under examination are 1) behind the umbilicus and 2) under the tail.

B. Characteristic appearance of the male. A bright echo is generated by the genital tubercle (white arrow) caudal to the umbilical cord.

C. Same view of the female fetus. No genital tubercle is evident caudal to the umbilical cord.

D. A small bright echo is seen in the female, beneath the tail (at the tip of the white arrow).



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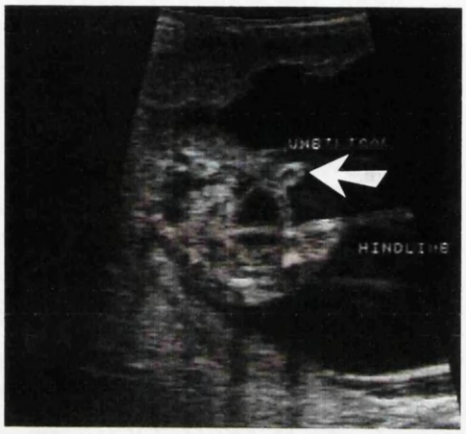

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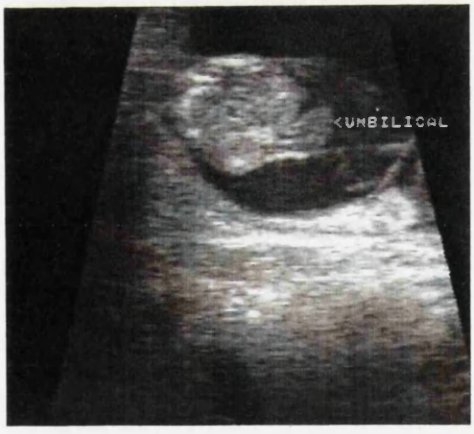

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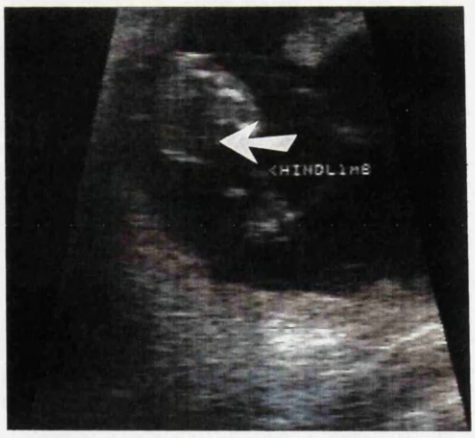

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2.3 Fetal Fluid Aspiration

The method used for fetal fluid aspiration was modified from the follicle aspiration technique to deal with the problems encountered during the study. Equipment and procedural modifications designed to improve the technique are described below. A detailed discussion of the problems encountered and how they were dealt with is given in subsequent chapters.

2.3.i Development of the Technique (Waterbath Specimens)

After a period of training in the technique of ultrasound-guided transvaginal follicle aspiration, fetal fluid aspiration was performed on pregnant tracts recovered from the abattoir. The entire pregnant tract was immersed in a water bath and the transvaginal transducer was introduced into the vulva up to the cervix. The tract was manipulated to localise the fluid-filled embryonic sac before needle insertion was attempted. The vaginal wall was penetrated with a 15 gauge 29 cm introducer needle and uterine puncture was effected with an 18 gauge needle. Suction was applied by the assistant using a 10 ml syringe. Needle advancement through the uterine wall and into the fetal membranes was monitored by ultrasound and the procedure was recorded on videotape (Figure 2.5).

Figure 2.5. Ultrasonographic appearance of the bovine fetus in a waterbath.

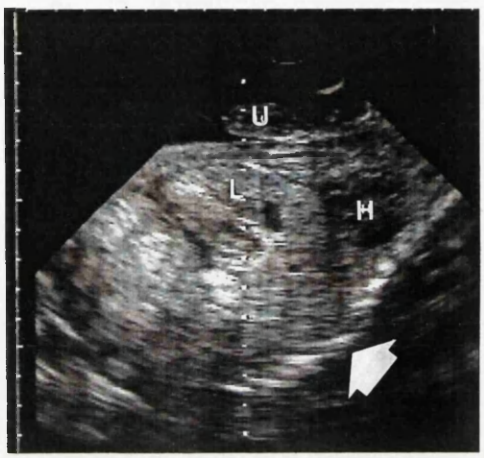
The fetus was examined within the uterus, using the vaginal transducer.

A. Identification of body parts of the fetus. The fetus is lying dorsally, the umbilical cord (U) uppermost. Other structures include the liver (L) and the heart (H) and the spinal column is clearly visible (arrow).

B. The fetal skull is highly echogenic (labelled S).

C. The fetal hind limb (h) and scrotum (s) are clearly visualised.

D. The uterus was manipulated to localise a fluid vesicle. Note the fetus (f) and amnion (a). The echogenic tip of the needle (white arrow) can be visualised pushing on the membranes as it penetrates the fluid vesicle.



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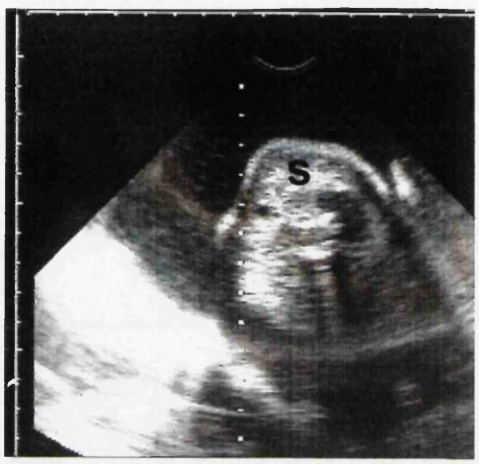

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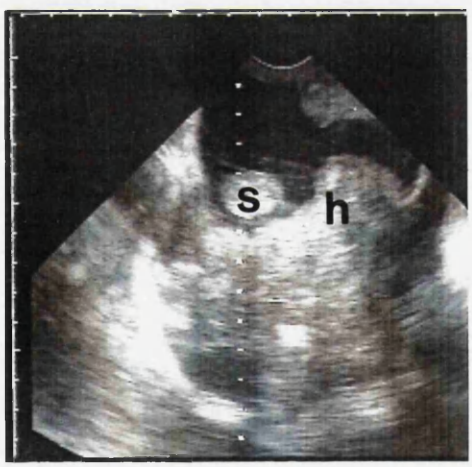

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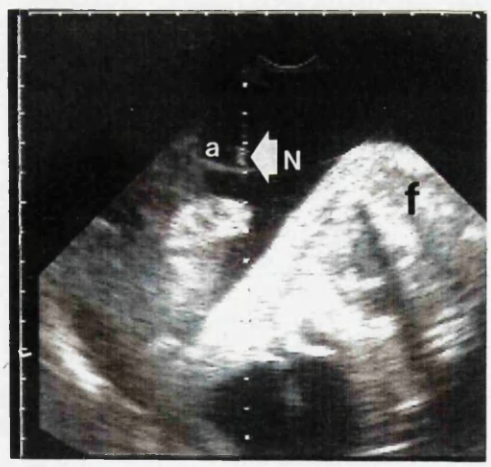

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2.3.ii Equipment Modification

Initially, the equipment used for fetal fluid collection was as applied for follicle aspiration. However, it was immediately realised that this was too short to reach the uterus which is located deeper in the abdomen than the ovaries. The handle of the human transvaginal transducer was extended with a rigid plastic tube to a length of 44 cm (Figure 2.3). Initially a needle guide extension was improvised using 15 g tubing. This was superseded by a customised 36 cm needle guide (Casmed, 36 Kenley Walk, Cheam, Surrey, UK) which allowed sufficient length of needle guide to remain exposed for needle insertion without vulval contamination (Figure 2.2). Longer needles were also required. The optimal needle type was 18 g x 45 cm. A 22 g x 55 cm needle with a stylet and an echogenic tip was also utilised. The 15 g introducing needle which was successful for the follicle aspirations was dropped from this procedure because it was too short. The electric vacuum pump which was initially used to apply suction was superseded by a single syringe connected to the needle by an adapter, with suction applied manually (Figure 2.4).

2.3.iii Fetal Fluid Aspiration Procedure

Animal restraint and chemical preparation was as described for follicle aspiration. Clenbuterol treatment was omitted as this drug could have caused prolonged uterine stasis that might have had detrimental effects on the pregnancy. However, a degree of uterine tone was necessary to adequately manipulate the uterus. In later procedures sedation was achieved using Xylazine (0.2 mg/kg Rompun, Bayer, Eastern Way, Bury St Edmunds, UK). When necessary the urinary bladder was catheterised to void excess urine and provide good contrast for the embryonic vesicle. The rectum was evacuated of faecal matter and the pregnancy was located manually.

This assisted in orientating the pregnancy and assessing fetal viability in repeat procedures.

Upon locating the pregnancy, the operator held the pregnant horn firmly in place close to the pelvic brim while the assistant changed to the transvaginal transducer. Sedative was normally administered during this period and where necessary the assistant cleaned the vulva again. The transvaginal transducer was handed to the operator who advanced it into the vagina up to the external os of the cervix. The hand in the rectum manipulated the uterus to select a fluid vesicle contralateral to the pregnant horn and align it with the biopsy guide-line. Needle insertion by the assistant was initiated upon instruction from the operator. The echogenic tip of the needle was usually monitored by ultrasound as it penetrated the uterus and membranes and advanced into the fluid vesicle, at which time suction was activated. The electric pump could be activated by the operator while manual suction was carried out by the assistant. Recovered fluids were transferred into secure tubes and kept on ice while in transit to the laboratory. Sample tube labelling included animal identity, date of recovery, stage of pregnancy, volume where possible, character (clear or blood-tinged, etc.) and fetal viability at sampling.

Prophylactic antibiotic treatment; 8.75 mg / kg, 'Synulox' containing 35 mg/ml Potassium clavulanate and 140 mg/ml Amoxycillin trihydrate (SmithKline Beecham Animal Health, Walton Oaks, Tadworth, Surrey, UK) was given daily for three days after aspiration. Regular follow-up examinations were conducted until the outcome of the procedure was established.

2.4 Fetal Sexing Using the Polymerase Chain Reaction (PCR)

To sex fetuses by PCR a nested *zfx* and *zfy* gene fragment amplification technique was applied (Kirkpatrick and Monsoon, 1993). Nested PCR is particularly useful when amplifying minute template samples as the target sequence is greatly enriched in the primary reactions. Nested PCR is reported to enhance the sensitivity and specificity of amplification by sequential reaction with primer pairs (70/72 and 71/73) located internally to the initial ones (P68/69) (Table 2.1). The initial primer pair utilised in the assay universally amplifies the 445 (*zfx*) and 447 (*zfy*) bp fragments of male and female genomic DNA in humans, cattle, sheep and goats (Aasen and Medrano, 1990). The nested PCR, on the other hand amplifies the 247 and 167 bp fragments of the *zfx* and *zfy* pseudoautosomal regions respectively. The products are resolved by simple agarose gel electrophoresis and ethidium bromide staining.

In this study the assay was optimised before application using genomic DNA extracted from abattoir collected specimens.

Table 2.1 Primer sequences used in the sexing of bovine fetal fluid cells. Primers 68/69 are derived from Aasen and Medrano (1990) and 70 to 73 from Kirkpatrick and Monson (1993).

Primer	Sequence
68. 5', combined zfx, zfy	ATAATCACATGGAGAGCCACAAGCT
69. 3', combined zfx, zfy	GCACTTCTTTGGTATCTGAGAAAGT
70. 5' zfx, allele-specific	GACAGCTGAACAAGTGTTACTG
71. 5' zfy, allele-specific	GAAGGCCTTCGAATGTGATAAC
72. 3' zfx, allele-specific	AATGTCACACTTGAATCGCATC
73. 3' zfy, allele-specific	CTGACAAAAGGTGGCGATTTCA

2.4.i Training and Validation of the PCR Methodology

To understand the working principles of PCR technique and to optimise the assay conditions, abattoir specimens were used for genomic DNA extraction. The specimens recovered included liver tissue, ovaries for granulosa cells, and fetal fluids.

Extraction of DNA from liver tissue

Samples of liver tissue from animals of known gender were collected shortly after slaughter and transported to the laboratory on ice. In the laboratory DNA extraction was performed immediately using fresh tissues or tissues were stored in a freezer at -20°C for later use.

To liberate DNA from the tissue, cells were lysed in a mixture of detergent and Proteinase K. The lysis mix contained 100 mM KCl, Tris pH 8.2, 1% Triton-X100, 0.45% Tween 20 and Proteinase K. Each 1 ml of lysis mix contained 200 μl Tris/KCl buffer, 200 μl Triton-X100, 20 μl Tween 20, 20 μl Proteinase K and was made up to 1 ml with distilled water.

A tiny piece of tissue sample was cut and put into a 0.5 ml microcentrifuge tube and 125 μl lysis mix was added. The sample was then incubated in a waterbath at 55°C for 1 hour after which the lysis mix was renewed and the sample was incubated for a further one hour at the same temperature. At the end of incubation the temperature was raised to 95°C for 15 minutes to inactivate Proteinase K and nucleases and denature any haemoglobin. The lysate was thoroughly mixed and centrifuged for 5 minutes at a high speed (12,000 g). The supernatant contained liberated genomic DNA ready for use or stored at -20°C for later use.

Extraction of DNA from granulosa cells

Granulosa cells were recovered from ovaries collected from the slaughterhouse. The ovaries were transported to the laboratory on ice where the follicles were dissected free from the ovarian stroma. To recover the cells, follicular fluid was

aspirated using a needle and syringe and the follicle gently flushed with culture medium (DMEM/F12). The follicular fluid samples were transferred into 50 ml plastic tubes and diluted with culture medium (1:20) to avoid clotting. The fluid was then centrifuged at low speed (800-1000 rpm) at 4°C for 5 minutes. The supernatant was discarded and the cell sediments pooled into one tube by re-suspending in 1 ml culture medium. The recovered cells were enumerated live or dead using a haemocytometer. Serial dilutions of the cell suspension were then prepared in 1.5 ml microcentrifuge tubes. These cell suspensions were centrifuged using a bench top microcentrifuge at 1500 rpm for 10 minutes. The recovered cells were used for immediate DNA extraction for immediate use or the cell pellet was stored at -20°C until required.

DNA was liberated from the cells by suspension in water to release the cell contents. The suspension was then incubated in boiling water for 5 minutes and quickly transferred into ice. This ensured DNA liberation, inactivation of the nucleases and denaturation of any haemoglobin.

Extraction of DNA from fetal fluid cells from abattoir specimens

Amniotic and allantoic fluids were recovered from pregnant tracts shortly after slaughter and transported on ice to the laboratory for processing. The fetal gender and estimate of the gestational age from the crown-rump length (CRL) was recorded. Attempts were made to recover amniotic and allantoic fluid separately from each pregnancy. Fetal fluid cells were concentrated by centrifugation for 5 minutes at 4°C and 1000 rpm. The supernatant was discarded and the cell pellets re-suspended in 1 ml of culture medium (DMEM/F12), at the same time pooling those from the same source into one tube. The cell suspensions were transferred into 1.5 ml microcentrifuge tubes and centrifuged at 1500 rpm to pellet the pooled cells for immediate DNA extraction or storage at -20°C in a freezer.

Genomic DNA was extracted in a similar manner as for the granulosa cells.

2.4.ii DNA Extraction and Analysis of Recovered Fetal Fluids

Fetal fluid samples recovered by aspiration were processed in the Laboratory without delay. Fetal fluid cells were recovered and DNA extracted for immediate PCR reactions or stored away in a freezer at -20°C for later use.

Cell recovery

Recovery of cells from samples contaminated with blood was impossible as they were clotted by the time of processing. Also cells could not be recovered in cases where only a small volume of fetal fluid was collected and this had to be flushed with saline from the needle system into the recovery tube. In such situations, entire tube contents were used in the DNA extraction for PCR. Otherwise from successful aspirates, cells were recovered in a manner similar to that described above for fluids from the abattoir.

DNA extraction

Tubes were placed in a boiling waterbath for 5 minutes and quickly cooled under ice to extract total DNA from both re-suspended cell pellets and whole aspirates. Again this process was to ensure DNA extraction and denaturation of any active DNAases and proteins that could interfere with the PCR test. Sex determination by PCR was performed immediately after DNA extraction. DNA samples were stored in a freezer at -20°C for purposes of repeating reactions.

2.4.iii PCR Protocol

PCR procedures were carried out in 0.5 ml microcentrifuge tubes using an automated thermal reactor (Hybaid Ltd, Middlesex, UK). A 30 *ul* reaction mixture consisted of 5 units of thermostable Taq DNA polymerase (AmpliTaq, Strategene Ltd, Cambridge, UK) or DyNAZyme II DNA polymerase (Finnzymes Oy, Epsoo, Finland), 10 mM Tris HCl buffer (pH 8.8 at 25°C), 1.5 to 2 mM $MgCl_2$, 0.2 mM of each dNTPs

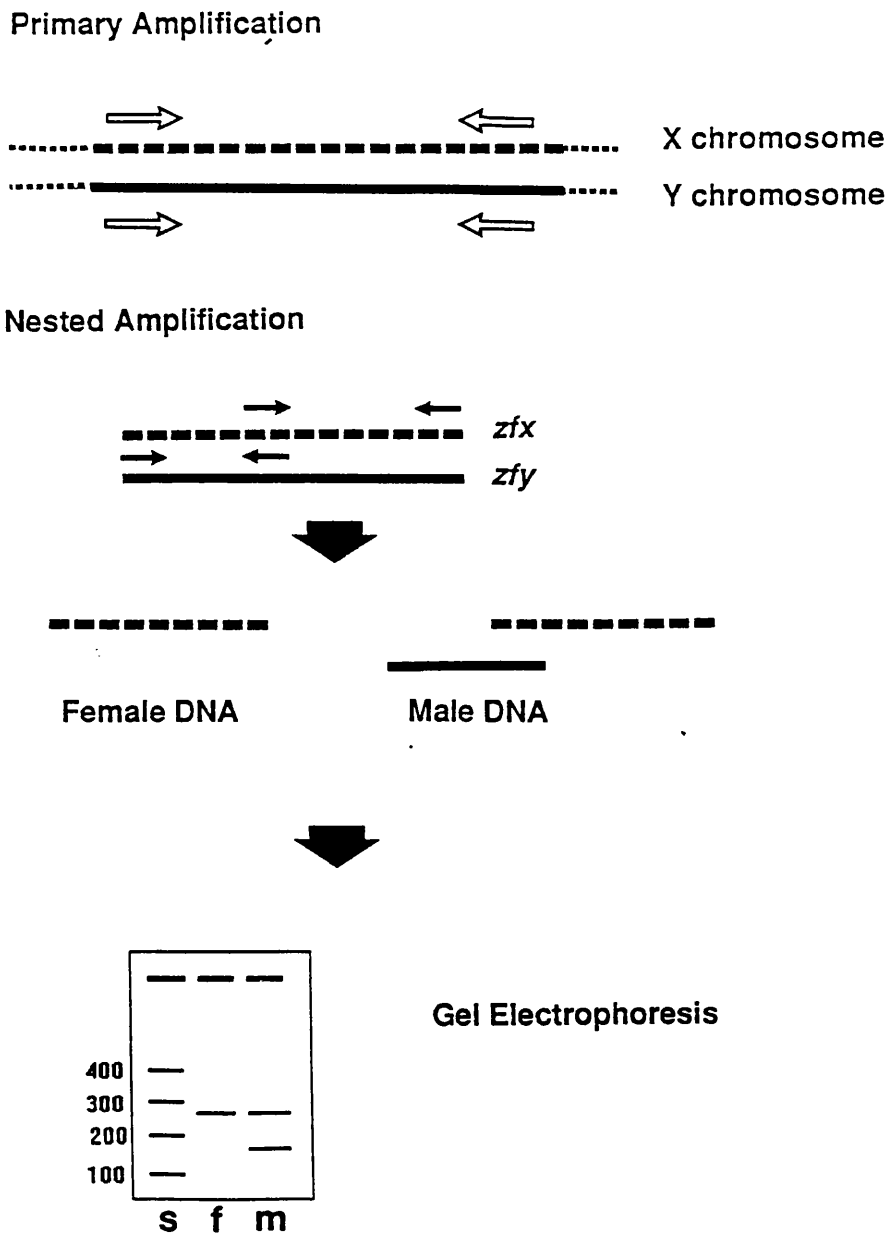
(dTTP, dGTP, dCTP, dATP) and primers. The sample or template was added to the mix and the total volume made to 30 μl with water. The water, buffer and $MgCl_2$ were exposed to ultra violet (u.v.) light to eliminate possible contamination before adding the other reactants. The reaction mixtures were overlaid with mineral oil to prevent evaporation during the thermal cycler programme.

The primary PCR reactions were performed in triphasic cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 45 seconds and extension at 72°C for 1 minute. These cycles were preceded by denaturation at 95°C for 2 minutes, and a total 30 thermal cycles were normally performed.

In the second or nested PCR the same volumes of reaction mixtures were prepared. The thermal reaction programme was also similar from start up and for the first five cycles. Thereafter annealing temperature was raised to 60°C for the same time in the remaining 25 cycles. The PCR strategy is outlined in Figure 2.6.

The products were fractionated on 1.5% agarose gel, stained with ethidium bromide (10 μl / 50 ml gel) against a standard molecular marker (100 bp ladder) (0.3 μl + 25 μl TE buffer pH 7.6). Before loading into the gel wells, samples were stained with loading buffer (Evans blue dye) and about 25 μl of sample and standard were loaded into the wells. Electrophoresis was conducted at a constant voltage of 150 volts for 20 to 30 minutes. The DNA bands were visualised under u.v. light and the results recorded on Polaroid film (Polaroid MP-4, Polaroid Corporation, Cambridge, Mass, USA) or using a video camera connected to a thermal printer (Model 68B, Mitsubishi Electric Corporation, Tokyo, Japan).

Figure 2.6. Nested PCR strategy applied in the sex determination of bovine fetal fluid cells. Primary amplification of the *zfx* and *zfy* regions was effected with primers P68 / P69 indicated in Table 2.1 and nested amplification with allele-specific primers (Table 2.1). Products of the nested PCR are the 167 (*zfy*) and 247 bp (*zfx*) gene fragments. These are easily distinguished by agarose gel electrophoresis (s = standard molecular marker 100bp, f = female DNA and m = male DNA).


Primary Amplification
X chromosome
Y chromosome
Nested Amplification
zfx
zfy
Female DNA
Male DNA
Gel Electrophoresis
400
300
200
100
s f m

Chapter 3

Results

3.1 Overview

3.2 Recovery of Cells From Fetal Fluids

3.3 Optimisation of Conditions for the PCR

3.3.i Application of the PCR Assay to Granulosa Cells

3.3.ii Application of the PCR Assay to Fetal Fluids from Abattoir Specimens

3.4 Ultrasonography

3.4.i Pregnancy Diagnosis

3.4.ii Aspiration of Fetal Fluids From The Live Animal

3.4.iii Record of Fetal Fluid Aspiration

3.4.iv Fluid Aspiration and Pregnancy Outcome

3.5 Sex Determination Using Recovered Fetal Fluid

3.1 Overview

An example of the cells recovered from the fetal fluids of an abattoir specimen is shown in Figure 3.1. The PCR technique was validated using genomic DNA extracted from liver tissue from animals of known gender. An optimal thermal programme for the polymerase chain reaction was derived and is reported. In preliminary experiments the PCR assay was applied on ovarian granulosa cells and on cells recovered from the fetal fluids of abattoir specimens of known gender. Subsequent experiments report the ultrasonographic detection of pregnancy and the procedure of fetal fluid aspiration from the experimental animals. PCR analysis of the DNA extracted from the recovered cells and outcome of the pregnancies in the cows subjected to the aspiration procedure is reported. Finally, the fetal gender obtained using the PCR assay is compared with that derived by ultrasonographic and / or physical examination of the fetuses.

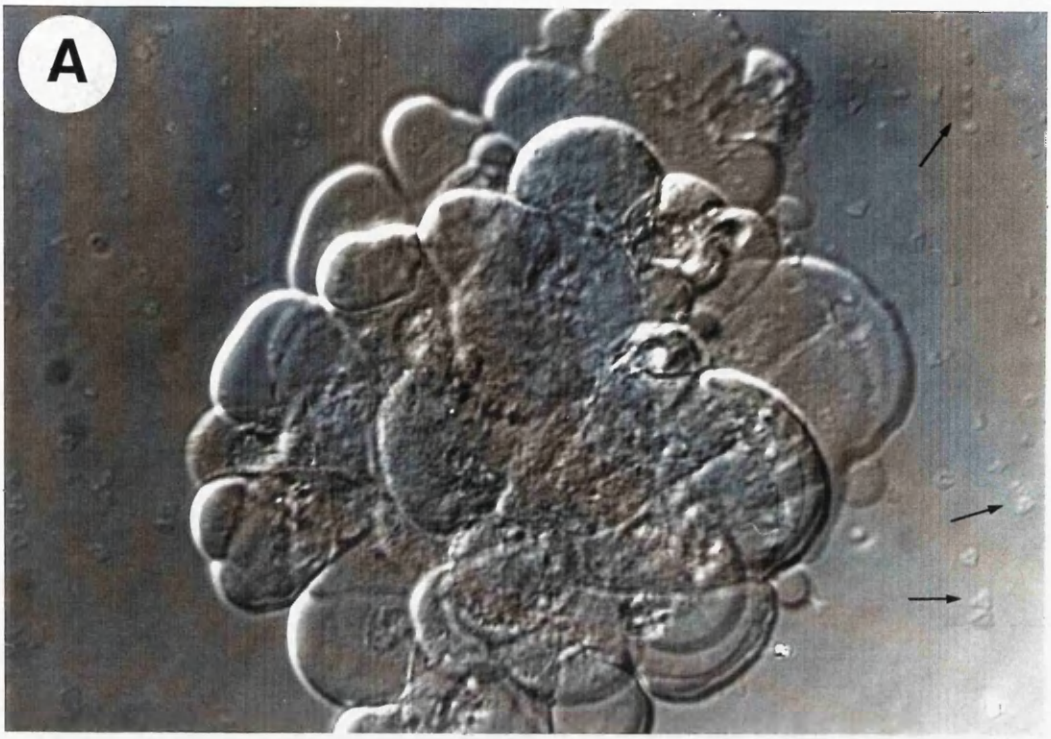
3.2 Recovery of Cells from Fetal Fluids

Cells found in fetal fluids are thought to be those sloughed from fetal surfaces during gestation. To establish the existence of such cells amniotic and allantoic fluid was recovered from a pregnant uterus obtained from the abattoir. The fetal fluids were centrifuged to concentrate the cells and studied under Differential Interference Contrast microscopy. Photographs of the recovered cells are shown in Figure 3.1.

Figure 3.1 Photographs of cells recovered from amniotic and allantoic fluid from an abattoir fetal specimen of crown-rump length of 13 cm (approximate age of 85 days).

A. A cluster of large cells recovered from the amniotic fluid (magnification x4000). Note small cells in the background (arrows).

B. Cells recovered from the allantoic fluid (magnification x4000).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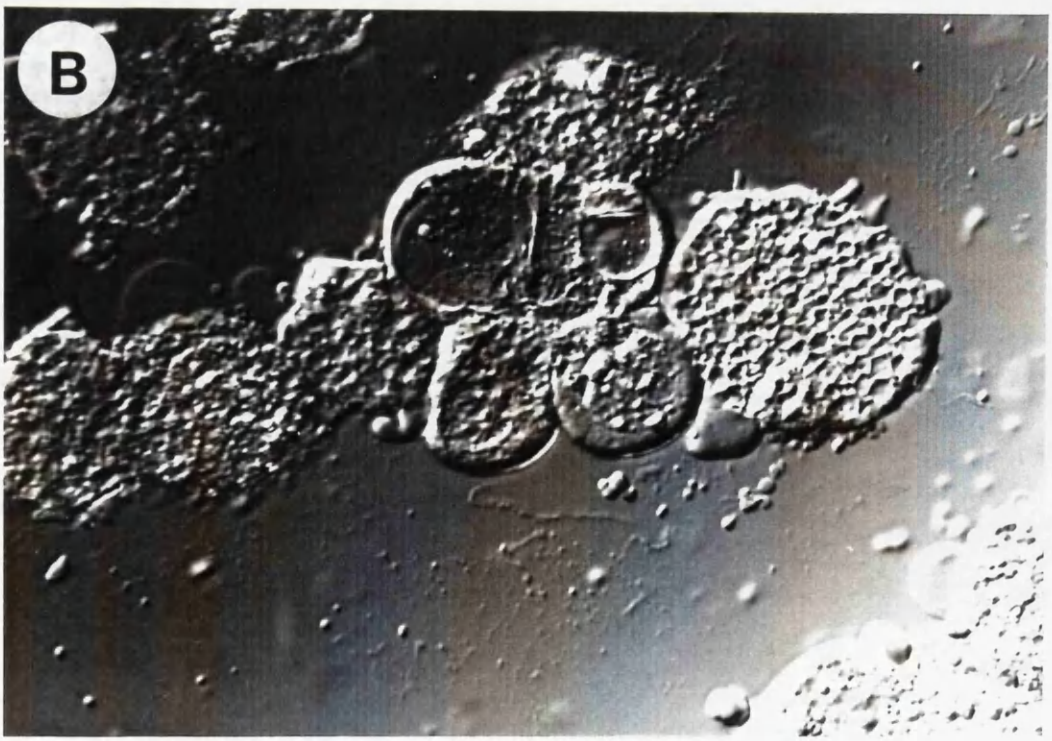
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3.3. Optimisation of Conditions for PCR

In order to derive the optimal thermal programme for the PCR reaction, genomic DNA was extracted from bovine liver tissues of known gender and the PCR was run at various temperatures and holding times.

Primers

The primary PCR assay was carried out with primer pair 68 and 69 (see Table 2.1) selected to amplify the base pair (bp) fragments common to the X and Y regions (*zfx*, a region of 447 bps and *zfy*, a region of 445 bps). Primer pair 68 and 69 was called the common primer pair. The nested PCR assay utilised two primer pairs (70 and 72; 71 and 73, see Table 2.1) designed to amplify the 247 bp specific to the X chromosome and the 167 bp specific to the Y chromosome. In the experiments designed to optimise the thermal programme, primer pairs were used individually and combined. Use of the pairs individually allowed detection of mis-amplifications of either X or Y signal.

Trial thermal programmes

The thermal programme, consisting of the denaturation, annealing and extension cycles, was varied until suitable results were obtained. Modifications of the thermal programme designated as experiments A - D are shown in Table 3.1. Optimal results were achieved using programme D and this programme was used for subsequent assays.

Table 3.1. Derivation of the optimal thermal programme for the amplification of genomic DNA of animals of known gender.

		THERMAL PROGRAMME						
		Denaturation		Annealing		Extension		No. of
		Temp.	Time	Temp.	Time	Temp.	Time	Cycles
EXPT	1° PCR	95°C	1 min	55°C	45 sec	72°C	1 min	30
A	Nested	95°C	1 min	55°C	45 sec	72°C	1 min	5
	PCR	95°C	1 min	60°C	45 sec	72°C	1 min	25
	1°	95°C	2 min					1
EXPT	PCR	95°C	1 min	55°C	45 sec	72°C	1 min	30
B	Nested	95°C	2 min					1
		95°C	1 min	55°C	45 sec	72°C	1 min	5
	PCR	95°C	1 min	60°C	45 sec	72°C	1 min	25
	1°	95°C	2 min					1
EXPT	PCR	95°C	30 sec	50°C	45 sec	72°C	1 min	30
C	Nested	95°C	2 min					1
		95°C	30 sec	50°C	45 sec	72°C	1 min	5
	PCR	95°C	30 sec	60°C	45 sec	72°C	1 min	25
	1°	95°C	2 min					1
EXPT	PCR	95°C	30 sec	52°C	45 sec	72°C	1 min	30
D	Nested	95°C	2 min					1
		95°C	30 sec	52°C	45 sec	72°C	1 min	5
	PCR	95°C	30 sec	60°C	45 sec	72°C	1 min	25

Results

Results of the gel electrophoresis of PCR products using thermal programmes A, B, C and D are shown in Figure 3.2. Using programme A, no bands were detected for the primary PCR products (lanes 7 and 8), as would be expected. For the nested PCR products, single primer pairs amplified male DNA specifically to give both X and Y bands but combined primers only yielded a weak Y signal. Mis-amplification of the Y-specific region in the female sample was observed with single Y- specific primers (Figure 3.2B). However, combined primers gave a specific female band. Using programme B there was still mis-amplification of the Y-specific region in the female DNA after using single Y- specific primers and there was failure to amplify the Y region with combined primers in the male DNA. But a specific X band was detected for both female and male DNA using combined primers.

Programme C failed to amplify detectable bands of both male and female DNA when using single primer pairs for male and female DNA. However, combined primers gave specific bands for both samples. Obviously it was desirable not to be able to amplify female DNA with Y specific primers.

The results recorded with programme D were fairly gender specific. DNA bands were detected with both single and combined primer pairs in the male DNA. In the female there was as expected a failure to detect DNA with Y- specific primers but there was unexpected failure to obtain a signal with single X- specific primers. However, combined primers gave a specific X band. Despite failure to detect female DNA with single X- specific primers, the programme was considered optimum for the sex determination assay and applied in the subsequent experiments.

The optimised thermal programme consisted of a triphasic cycle comprising denaturation at 95°C for 30 seconds, primer annealing at 52°C for 45 seconds and primer extension at 72°C for 1 minute in the primary and first 5 cycles of the nested PCR. Then annealing temperature was raised to 60°C for 45 seconds in the remaining

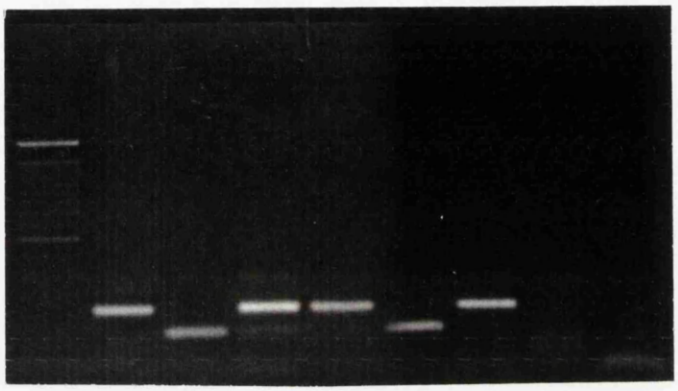
cycles of the nested PCR. The first cycle was preceded by a denaturation step at 95°C for 2 minutes and 30 cycles were performed in the primary and nested PCR.

Figure 3.2. Amplification of genomic DNA from livers of animals of known gender to optimise the PCR reaction conditions. Primer pairs used for each reaction are indicated. Identification of lanes in the figures:
Lane M - Standard molecular marker, 100 bp ladder.
Lanes 1 to 3, male DNA: Lane 1 - *zfx* specific primer pair (70/72), Lane 2 - *zfy* specific primer pair (71/73) and Lane 3 - combined *zfx* and *zfy* primer pairs
Lanes 4 to 6, female DNA: Lane 4 - *zfx* specific primer pair (70/72), Lane 5 - *zfy* specific primer pair (71/73) and Lane 6 - combined *zfx* and *zfy* primer pairs
Lanes 7 and 8 - female and male DNA, respectively, with primary primer pair (68/69).

A. Note detection of the X and Y regions in male DNA with separate primer pairs (lanes 1 and 2), but a weak Y signal with combined primers (lane 3). There was a misamplification of the Y-specific region in the female sample (lane 5), but combined primers and the X-specific primers gave a female band (lane 6 and 4 respectively). Lane 7 and 8, no bands detected for the primary PCR products.

B. Note the misamplification of the Y-specific region in the female DNA (lane 5) with Y specific primers (71/73), and failure to detect the Y region in the male DNA clearly (lane 3) with combined primers. No change in the other lanes.

A.



B.

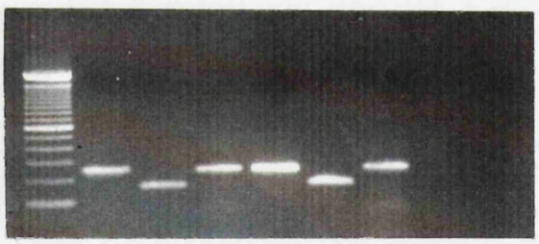
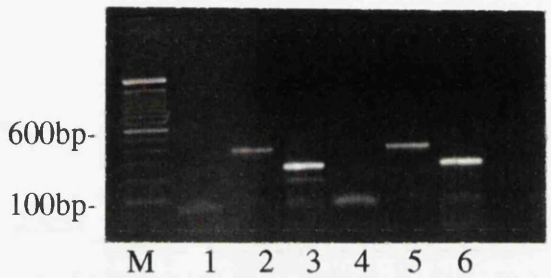


Figure 3.2. (continued) Amplification of genomic DNA from livers of animals of known gender to optimise the PCR reaction conditions. Primer pairs used for each reaction are indicated. Identification of lanes in the figures:
Lane M - Standard molecular marker, 100 bp ladder.
Lanes 1 to 3 male DNA: Lane 1 - *zfx* specific primer pair (70/72), Lane 2 - *zfy* specific primer pair (71/73) and Lane 3 - combined *zfx* and *zfy* primer pairs
Lanes 4 to 6 female DNA: Lane 4 - *zfx* specific primer pair (70/72), Lane 5 - *zfy* specific primer pair (71/73) and Lane 6 - combined *zfx* and *zfy* primer pairs
Lane 7 - negative control (blank). No primary products included.

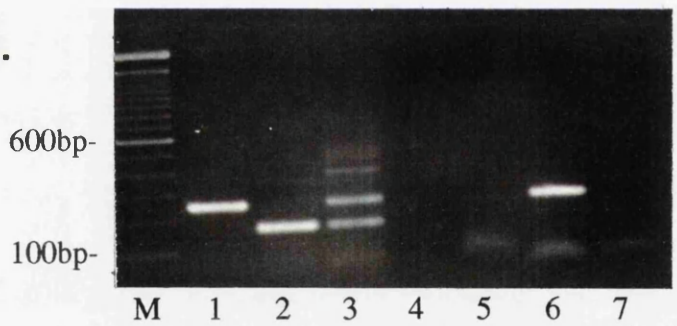
C. Note the failure to detect male DNA with separate primer pairs (lanes 1 and 2), but successful detection of the X and Y regions with combined primers (lane 3). In the female DNA there was no signal with Y-specific primers (71/73)(lane 5) as expected and an unexpected signal with 70/72 (X-specific) (lane 4). Combined primers gave a positive X- specific band (lane 6).

D. Note the specific amplification of the male DNA with both separate and combined primers, bands detected in lane 1 (X), lane 2 (Y) and lane 3 (XY). There was failure to detect female DNA with Y-specific primers (71/73) (lane 4) and an unexpected failure with X-specific primers (70/72) (lane 5) but with combined primers a specific X band is detected (lane 6). Lane 7, negative control is unambiguous. This thermal programme was favourable.

C.



D.



3.3.i Application of the PCR Assay to Granulosa Cells

The nested PCR assay used in these experiments has been used to amplify DNA from a single blastomere (Kirkpartrick and Monson, 1993). However, in the absence of information concerning the quantity of cells recoverable from fetal fluids a preliminary experiment was carried out to establish the sensitivity of the assay when applied to serial dilutions of granulosa cells.

In a preliminary experiment the DNA was extracted from granulosa cells and amplified using thermal programme D selected in the first experiment. Results are shown in Figure 3.3.A. A female signal was detected from the granulosa cell DNA and a male signal was detected from a control sample of male liver DNA.

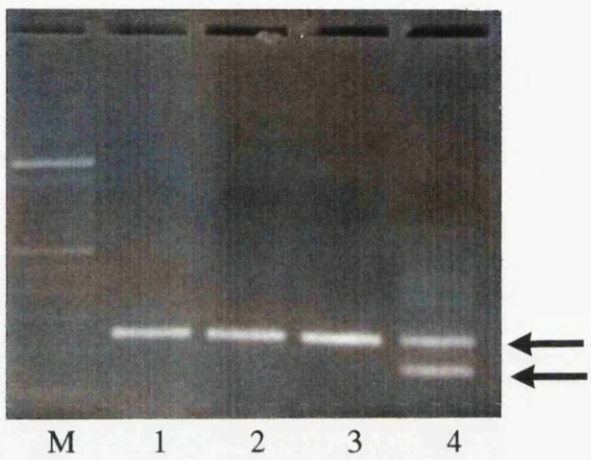
The number of granulosa cells in a follicle sample was counted and the sample serially diluted to give cell numbers ranging from 9.68×10^5, $x10^4$, $x10^3$, $x10^2$, $x10^1$ and $x10^{-1}$ cells / ml. DNA was extracted and amplified as described above. PCR products are shown in Figure 3.3.B. A female signal was detected down to a dilution of 96.8 cells / ml.

Figure 3.3. Preliminary application of the optimised PCR assay on granulosa cell DNA.

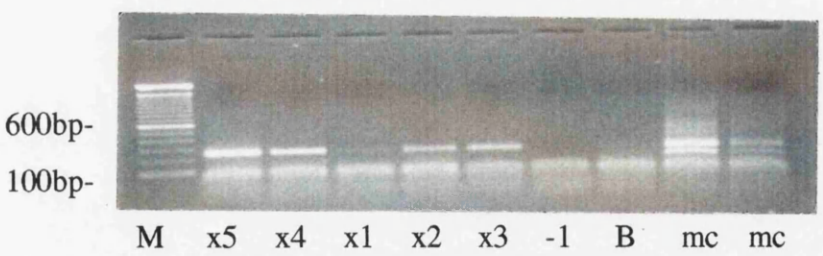
A. Result of gel electrophoresis of PCR products of amplification of granulosa cell DNA. Lane M represents the standard molecular marker, lanes 1 to 3 granulosa cell DNA and lane 4 represents control DNA from male liver tissue. Note the clear 247 bp band for the female DNA in lanes 1 to 3 and a pair of bands at 167 and 247 bp for the male signal in lane 4 (arrows).

B. Determination of the sensitivity of the assay. Granulosa cells were subjected to serial dilution prior to DNA extraction and amplification. Dilutions ranged from 9.68 x 10^5, 10^4, 10^3, 10^2, 10^1 and 10^{-1} cells/ml. Gel lanes 2 to 7 are labelled accordingly. Lane 1 contains molecular marker 100 bp and lane 8 is a blank. Lanes 9 and 10 contain control male DNA (mc) with an extra signal at 167 bp. A faint signal was detected at a concentration of 96.8 cells / ml, and no signal was detected at 9.68 cells / ml.

A.



B.



3.3.ii Application of the PCR Assay to Fetal Fluids from Abattoir Specimens

To test the efficacy of the assay for the detection of fetal gender 10 pregnant uteri were recovered from the abattoir and fetal fluid was either aspirated from the amnion, or the allantois or perhaps both. An estimate of fetal age was made from the crown-rump length (Arthur, Noakes and Pearson, 1989). DNA was extracted as before and amplified in the PCR. Comparison of the results of gender determination using PCR and fetal anatomy is summarised in Table 3.2. Gel electrophoresis of the PCR products is shown in Figure 3.4. In 9 of 10 samples gender was appropriately detected. For 1 sample (sample 7) there was an unexplained failure to detect DNA.

Table 3. 2. Comparison of the genetic sex determined by PCR with anatomical gender of fetuses. Both amniotic (Amnio) and allantoic (Allant) fluids were tested where possible. An estimate of fetal age was made on the basis of crown-rump length. NA= Not available.

Sample	PCR Sex		Anatomical Sex	Age (Days)
	Amnio	Allant		
1	F	NA	F	109
2	F	F	F	105
3	F	F	F	95
4	M	M	M	85
5	NA	F	F	130
6	F	F	F	131
7	NA	NA	M	113
8	F	F	F	135
9	M	M	M	125
10	M	NA	M	77

Figure 3.4. Application of the PCR assay on fetal fluid cells recovered from 10 abattoir specimens at different stages of gestation estimated from the CRL. Amniotic and allantoic fluids were recovered separately where possible and lanes labelled accordingly; a, represents amniotic fluid, b, allantoic fluid and ab, either or both. Lane M is standard molecular marker.

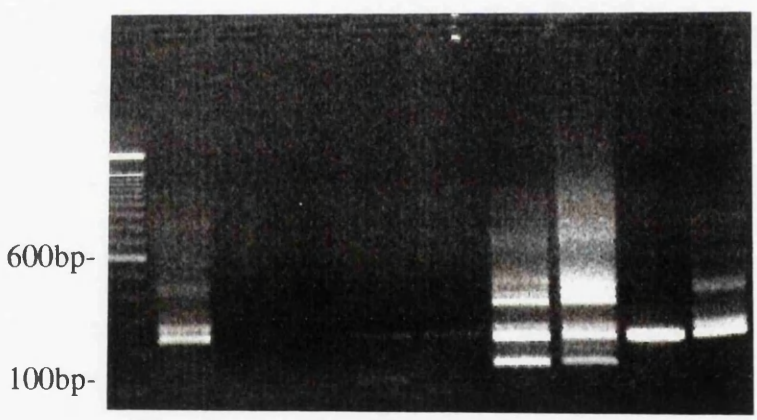
A. Contains samples 1 to 5. Note a very faint X signal DNA in sample 1a and failure to detect DNA in lane 1b. An X-specific band was detected in lane 5b but DNA could not be detected in lane 5a.

B. Contains samples from 6 to 10. DNA was not detected in sample 7a or b. Sample 10 is not included in the picture but DNA was not detected in lane 10b.

A.


600bp-
100bp-
M a b a b ab a b a b
1 2 3 4 5

B.


600bp-
100bp-
M ab a b a b a b F F
6 7 8 9

3.4 Ultrasonography

Pregnancy diagnosis by ultrasound, aspiration procedures and follow-up examinations are described in this section.

3.4.i Pregnancy Diagnosis

For eight of the experimental cows, pregnancy was diagnosed by transrectal ultrasonic examination of the reproductive tract on or after 28 days post-insemination. Pregnancy was confirmed by the presence of a mature corpus luteum, of approximately 2.5 cm diameter, on one ovary and a conceptus in the ipsi-lateral uterine horn. The uterine lumen was filled with fluid. At this stage the heartbeat was visible. The amniotic sac was not distinct. In later examinations the amniotic vesicle became very distinct. By day 35 placentomes were detectable on the uterine wall. Examples of these characteristic features are shown in Figure 3.5. One heifer was found to be non-pregnant and was omitted from further study.

The first batch of cows (numbers 61, 100 and 156) were closely monitored from day 11 onwards, at 3 - 4 days intervals, in an attempt to identify early features of pregnancy. These additional examinations were not carried out in the other animals and examples of the findings are shown in Figure 3.6. At day 11 all cows had a detectable corpus luteum. This would be expected whether pregnant or not. A corpus luteum was located on the right ovary in cows 100 and 156 and on the left ovary in cow 61. Imaging the uterus revealed no significant accumulation of fluid in cow 61 while fluid

was visible in cows 156 and 100 (Figure 3.6). Examination on day 14 post insemination, revealed fluid in the uterine lumen in all the cows. A discrete structure was visible in the lumen of the right horn of the uterus of Cow 156. On day 18 post insemination, all the cows still retained a visible corpus luteum and fluid in the uterine lumen. A careful scan over the uterus showed uterine fluid and a distinct structure within the uterus of cow 156. However, the uterine lumen did not increase in size as it did in the other cows. Cows 61 and 100 both showed considerable amount of fluid in the uterus .

On day 26 post-insemination, cow 100 was diagnosed pregnant with a conceptus in the horn ipsi-lateral to the corpus luteum. The embryo was clearly visible and a strong heart beat was detected. A positive pregnancy diagnosis was made for cow 61 on day 28 post-insemination by imaging an embryo proper with a heartbeat. The discrete fluid vesicle detected earlier in cow 156 was considerably reduced in size by day 28. The cow was not observed to have returned to oestrus over the period of monitoring, however, and a mature corpus luteum was detected at each examination. Unlike the other cows, where the volume of uterine fluid had greatly increased and an embryo had been observed, there was no sign of pregnancy in this animal. The cow was finally diagnosed non-pregnant and it was concluded that the uterine contents originally observed may have been due to uterine infection and it was this which was responsible for the persistence of the corpus luteum.

Fetal death subsequently occurred in cow 61. This was detected around day 40 of gestation when the fetal heartbeat had ceased and the fetal membranes were visible floating loosely in the uterine lumen. The fetus became poorly echogenic as it was reabsorbed. No aborted fetus was ever detected. It was noted that the fetal fluid

remained non-echogenic and remained visible after it had become difficult to visualise the fetus (Figure 3.6). These observations provide a contrast with the findings reported in the next section, where fetal death was caused by infection.

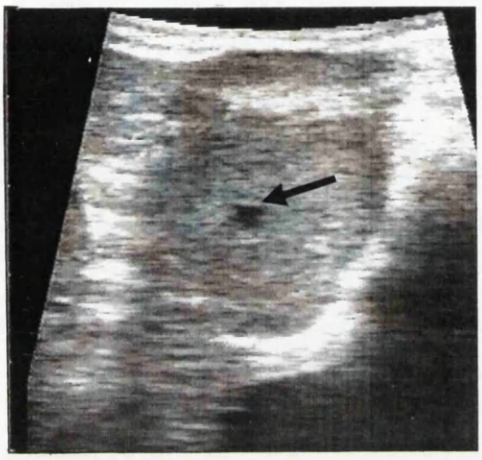
Figure 3.5. Pregnancy diagnosis using ultrasonography.

A. Day 27 post-insemination (cow 61). A mature (2.5 cm diameter) corpus luteum with a central lacuna (arrow) on the left ovary.

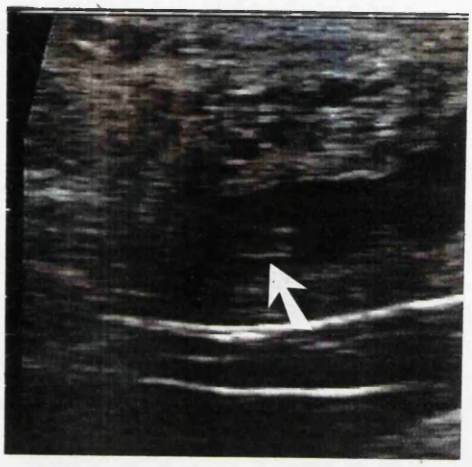
B. Day 27 post-insemination (cow 100). Conceptus is visible (arrow) in the fluid- filled uterine lumen. The heartbeat was detected.

C. Day 31 post-insemination (Cow 12). Conceptus visualised (arrow) in the fluid-filled uterine lumen

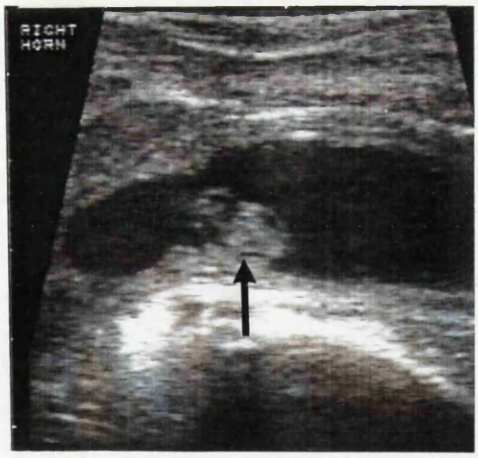
D. Day 42 post-insemination (cow 100). The conceptus is clearly visible within the amniotic sac (white arrow).



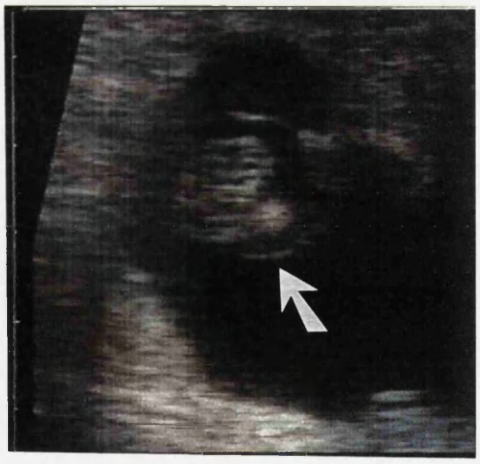
A



B



C



D

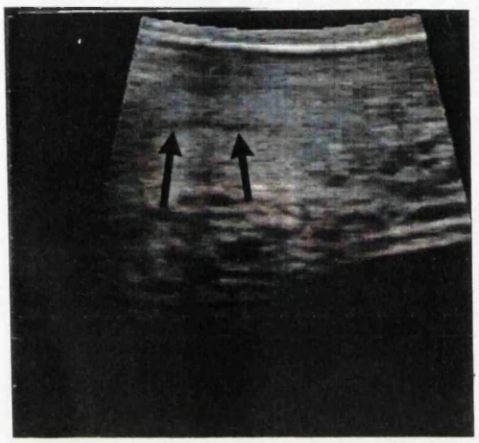
Figure 3.6. Ultrasonographic findings during repeated examination of cows 100, 61 and 156. A corpus luteum was visible on one ovary at each examination but is not illustrated.

A. Cow 100, day 11 post-insemination. Note the fluid visible in the uterine lumen in this longitudinal section of the uterine horn (arrow).

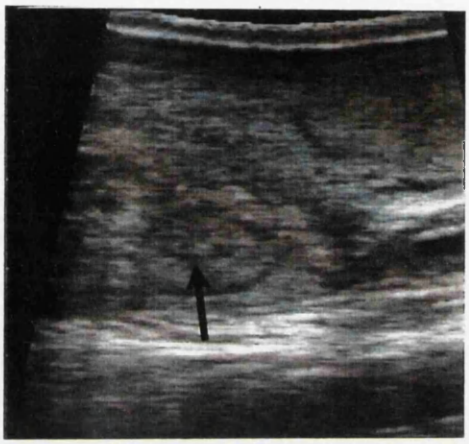
B. Cow 100, day 18 post-insemination. Note fluid visible in the uterine lumen in this transverse section of the uterine horn (arrow).

C. Cow 156, day 18 post-insemination. Fluid is visible in the uterine lumen. An abnormal structure is indicated by the small arrows.

D. Cow 61, day 40 post-insemination. Fetal death has occurred. The fetus is poorly echogenic, heart beat has ceased and fetal re-absorption appears to be taking place. Note clear fetal fluid in comparison with Figure 3.5., where a cloudy appearance is characteristic of infection within the fluid compartments.



A



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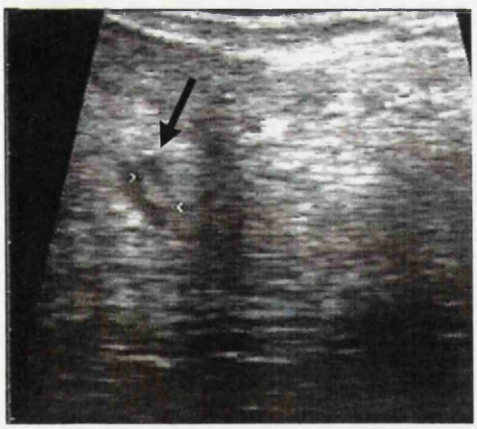

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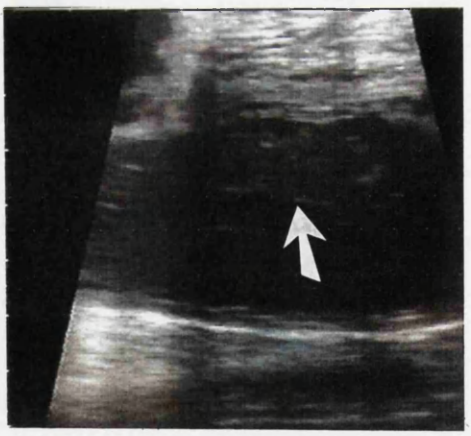


D

3.4.ii Aspiration of Fetal Fluids From The Live Animal

Having established pregnancy, cows were scheduled for fetal sexing to commence 56 days after insemination. By this stage of fetal development the genital tubercle of the male fetus can be visualised as a bright echo, caudal to the umbilical cord. One attempt was made to aspirate fetal fluid at an estimated 49 days of pregnancy. This fetus was never recovered nor sexed by ultrasound so aspiration at this early stage was not repeated. Other cows were aspirated in the range from 60 - 100 days. Ultrasonographic findings during uterine needle puncture for aspiration of fetal recovery are shown in Figure 3.7.

Initially in the procedure, the fetus was visualised within the amnion. Attempts were then made to locate a fluid-filled area remote from the fetus, and the needle was advanced into this fluid area. The echogenic nature of the reflective needle tip caused the needle to produce a bright echo within the fluid. When this was recognised, suction was applied and fetal fluid could be withdrawn. These stages of the procedure are illustrated in Figure 3.7

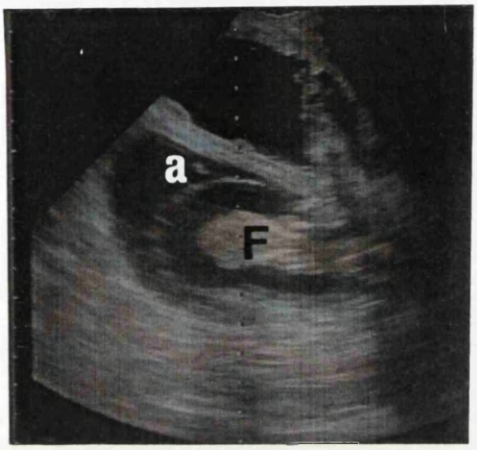
Figure 3.7. Ultrasonographic record of fetal fluid recovery in the live animal.

A. The fetus was located within the amnion using the vaginal transducer. Note the fetus (F) and amniotic membrane (a).

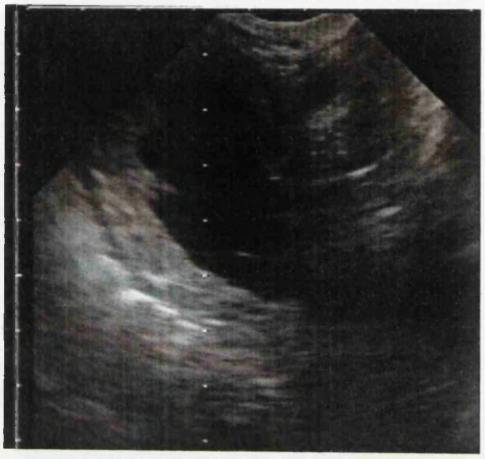
B. A fluid filled portion of the contra-lateral horn was aligned with the biopsy guide-line. Note that the fetus is clear of the selected area.

C. The needle has been advanced into the uterus and is clearly visible within the fluid filled vesicle (n).

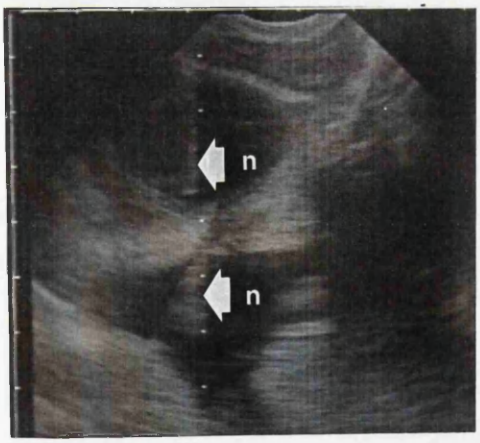
D. Note the passage of the needle (n) through a placentome (p). The needle is within the uterus but clear of the fetus within the amnion (F).



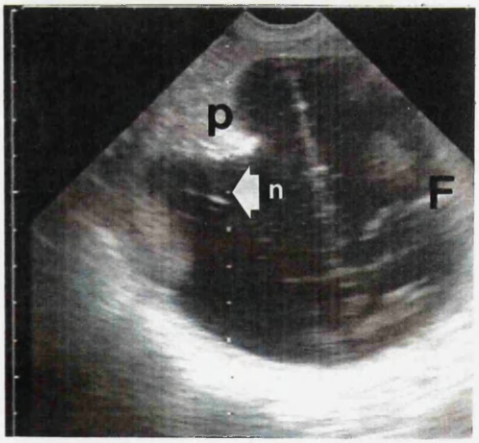
A



B



C



D

3.4.iii Record of Fetal Fluid Aspiration

A summary of the number of aspirations, the stage of gestation, outcome of aspiration and a record of fluid recovered is given in Table 3.3. In total, 14 aspirations were carried out on 9 different cows. Fluid recovery was unsuccessful in 1 cow (61) but aspiration could not be repeated because fetal death had occurred judged by absence of heartbeat. For the other cows, from 1 to 3 aspiration procedures were carried out between days 49 and 97 of gestation.

Attempts were made to recover fluid from the non-pregnant horn which by inference must be allantoic fluid. However, the needle tip was observed within the amnion on more than one occasion. The volume of fluid recovered was variable, from < 0.5 ml to > 5 ml. Where the volume recovered was small, the sample was flushed through the needle system with sterile physiological saline. A good sample was absolutely clear and viscous. More often the sample was blood tinged, although it was not possible to determine whether blood was of fetal or maternal origin. Two distinct types of fluid were collected on different occasions; one being very viscous and difficult to aspirate, the other being more serous.

Table 3.3. Summary of the fetal fluid aspirations, gestational age at the time of aspiration and record of the recovered fetal fluid.

Cow No.	Apirat. No.	Gestation (Days)	Event	Fluid Recovery
100	1	61	Aspiration successful	1ml fluid
	2	66	Aspiration unsuccessful	no aspirate
61	1	61	Aspiration unsuccessful	no aspirate
01	1	49 (est.)	Aspiration unsuccessful	no aspirate
	2	56 (est)	Aspiration successful	2ml fluid
71	1	80	Aspiration unsuccessful	no aspirate
	2	94	Aspiration successful	3ml fluid
	3	97	Aspiration successful	< 0.5ml fluid
112	1	72	Aspiration successful	< 0.5ml fluid
131	1	72	Aspiration unsuccessful	blood contam.
	2	77	Aspiration successful	< 0.5ml fluid
36	1	66	Aspiration successful	< 1ml fluid
99	1	66	Aspiration successful	5 ml fluid
12	1	71	Aspiration successful	< 1 ml fluid

3.4.iv Fluid Aspiration and Pregnancy Outcome

All aspiration procedures were followed by transrectal scanning to monitor progress of the fetus and the pregnancy. Outcome, based on these follow-up examinations, has been summarised in Table 3.4. Fetal death was observed in six out of eight animals aspirated, five cows and one heifer. Two heifers have retained pregnancies for three weeks after the procedure. Where fetal death was recognised by loss of the heartbeat, 500 mg Cloprostenol (Estrumate, Coopers Animal Health, Crewe Hall, Cheshire, UK) was administered. However, prostaglandins did not always cause abortion. Abortion occurred in two cows (01 and 71). The fetus was not recovered from one cow (01) despite diligent observation by the Stockman. This suggested that the fetus was at an advanced stage of maceration and was voided as a muco-purulent discharge. Cow 71 spontaneously aborted three days after the procedure and signs of fetal degeneration were evident (Figure 3.9). A second fetus was recovered after slaughter of the dam (cow 112) and again the fetus showed signs of maceration (Figure 3.9).

With experience it was possible to recognise some abnormalities of the pregnancy even prior to loss of the heartbeat. The main features were flocculence of the fetal fluids, leading to an increase in echogenicity and the finding that membranes were floating in the uterine lumen. Fetal death was recognised because of loss of heartbeat. Loss of pulsation in the umbilicus was also observed. Later stages took the form of reduction in the volume of fetal fluids, leaving only the fetal bones clearly visible a classic ultrasonographic observation in cases of fetal maceration. In others the fluid volume was maintained but the fetus became indistinct perhaps due to a combination of flocculence in the fluid compartments and degeneration of the fetus. Examples of these feature are shown in Figure 3.8.

Table 3.4. Final outcome for the pregnancies after the aspiration procedures.

Cow No.	No. of Asps.	Fetal Scan	Treatment	Outcome
100	1			
	2	Fetal death		re-absorption
61	1	No heartbeat, fetal death prior to asp.	PG	re-absorption
01	1			
	2	Fetal death		re-absorption
71	1			
	2			
	3	Fetal death		aborted
112	1	Fetal death subsequent to asp	slaughter	maceration
131	1			
	2	Fetal death		re-absorption
36	1	Fetus alive		
99	1	Fetal death	PG	aborted
12	1	Fetus alive		

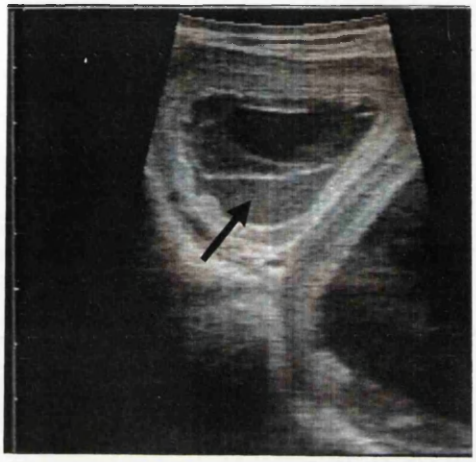
Figure 3.8. Ultrasonographic record of fetal death and re-absorption after the fluid recovery procedure.

A. Flocculence of the fetal fluids was observed (arrow), leading to increased echogenicity (cow 99, 71 days post-insemination and 8 days after fetal fluid recovery).

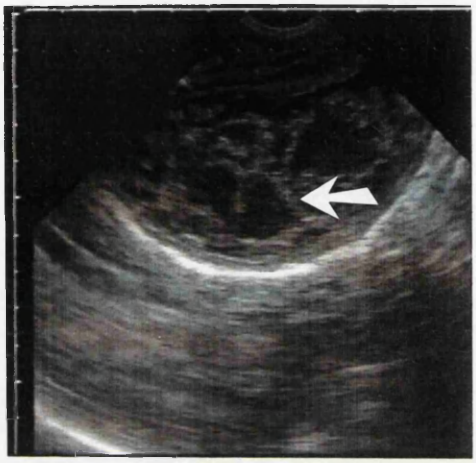
B. Loose membranes are seen floating in the fetal fluid (arrow) indicating the process of detachment following the procedure (cow 112, 72 days post-insemination and 14 days after fetal fluid aspiration).

C. (Cow 99, 71 days). The umbilicus is clearly visible but has ceased to pulsate (u). Examination of the heart confirmed loss of heart beat 8 days after aspiration. (S = skull).

D. Cow 131, 80 days post-insemination. There is loss of fetal fluid though fetal bones are still visible after two successive fetal fluid aspiration. This is the classic ultrasonographic appearance of a macerated fetus.



A



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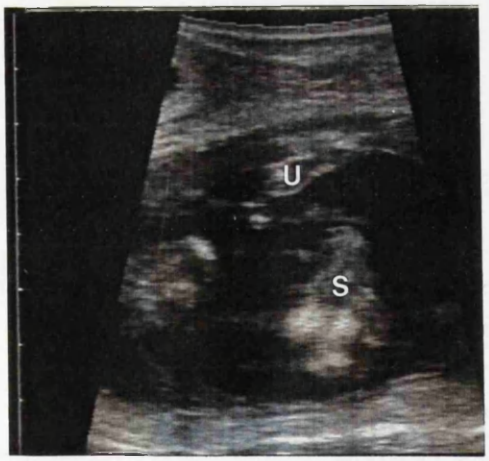
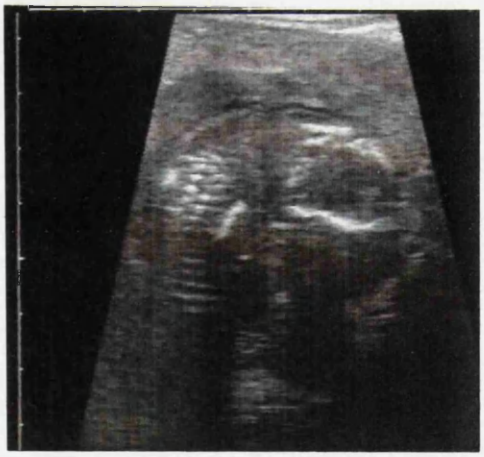


C



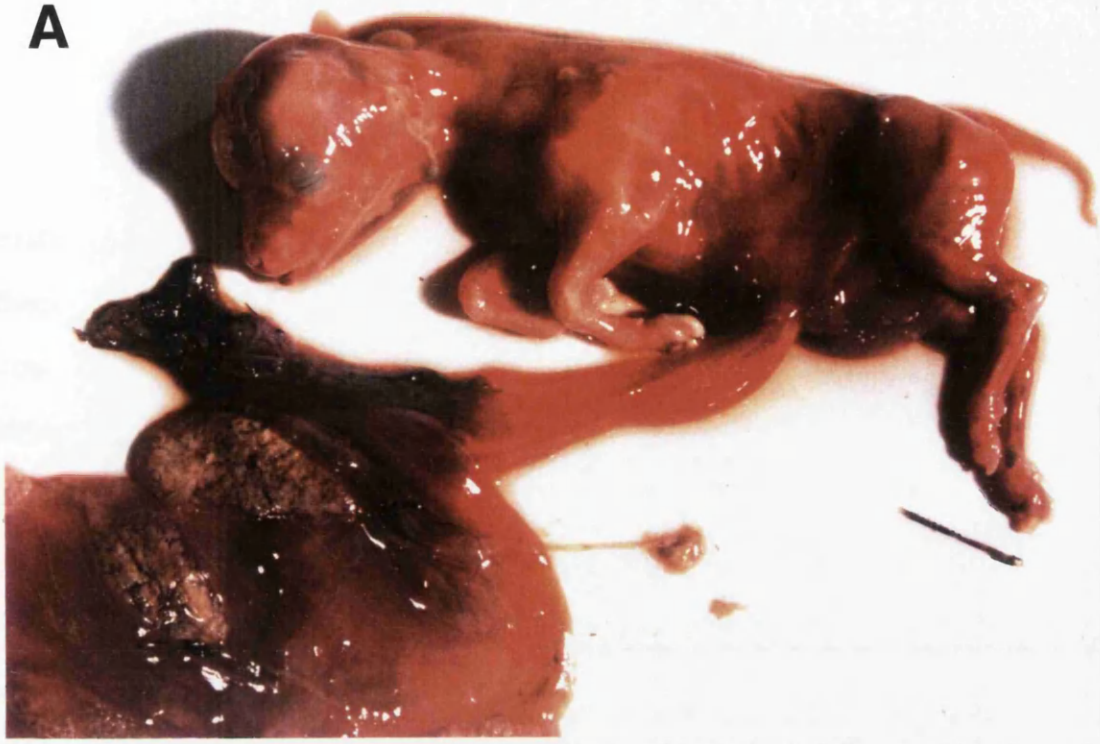
D

Figure 3.9. Examination of fetuses recovered after fetal fluid aspiration.

A. Fetus recovered from Cow 71 after spontaneous abortion. Note the gas bubbles on the fetal surface indicating the presence of infection.

B. Fetus removed from the uterus of Cow 112 after slaughter. Note that the fetus has begun to disintegrate.

A



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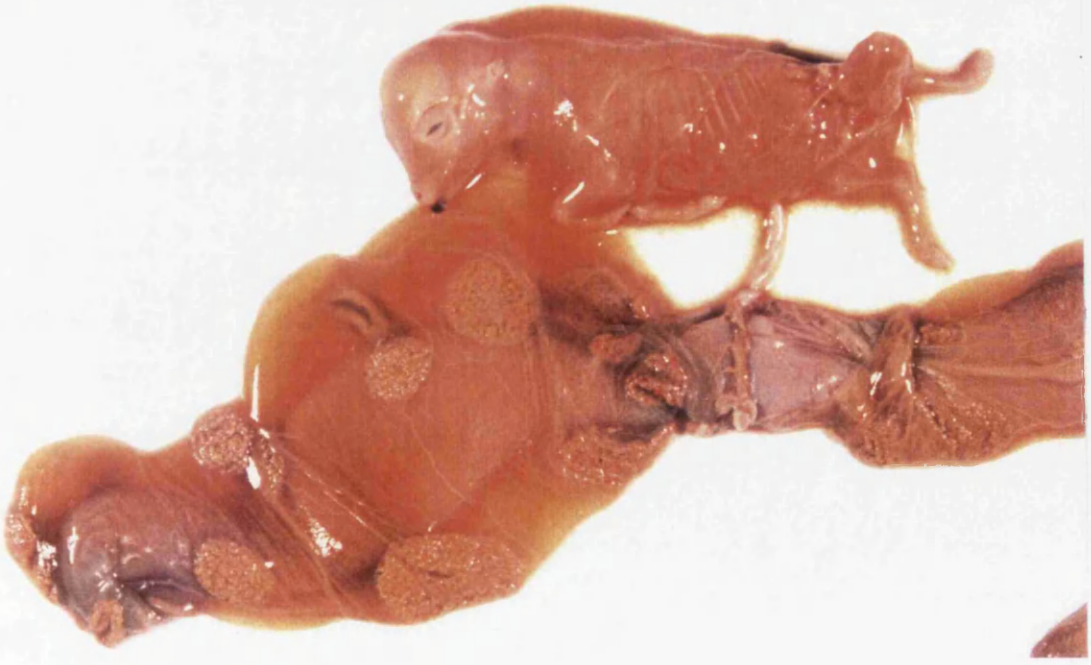


3.5 Sex Determination Using Recovered Fetal Fluid

Fetal gender obtained by the PCR assay is summarised in Table 3.5. These results are compared with fetal anatomical gender assessed by ultrasonography and/or direct observation of the fetus. Of the cows sampled for fetal fluid (see Table 3.3) one (cow 61) was excluded from further analysis after failing to recover fetal fluid. A second (cow 01) was excluded due to failure to establish anatomical gender of the fetus because no intact fetus was recovered after abortion.

PCR reactions were performed shortly after recovery to determine sex on the day of sampling. Gel electrophoresis results were recorded for all the reactions performed and Figures 3.10A - E show representative examples of failure to detect DNA, mis-amplification and successful DNA detection.

In Figure 3.10.A, PCR successfully amplified samples of the first aspirate from cow 100. A female specific band was recorded.

Figure 3.10.B shows failure to detect DNA in all but 1 of the samples, including the male control. The female control gave the appropriate signal and a sample from cow 112 gave male specific bands.

Samples that failed to give a signal were re-run with a new sample from cow 131. PCR products are shown in Figure 3.10.C (samples 1 to 14). Problems were still encountered because there was failure to detect DNA in the new sample from cow 131 and in the repeat samples from cow 71 and 131. For cow 131, carrying a male fetus, only the female band was visible, although for cow 112, also carrying a male fetus, both male and female specific bands were detected as expected.

After these poor results it was decided to change the enzyme from DyNAzyme DNA polymerase to Taq DNA polymerase. This appeared to have the desired effect. All samples were re-run (see Figure 3.10.D) and the results for the assay agreed with the anatomical gender. Most importantly, the positive and negative controls were

unambiguous. Clear signals for both male and female controls were seen (lanes 8 and 9 respectively) with no signal in the blank lane.

Figure 3.10.E shows analysis of the samples from the second batch of animals, the heifers. Correct results were recorded for the two samples from no. 36 carrying a male fetus (lanes 1 and 2) and from no. 99 carrying a female fetus (lanes 3 to 6). The controls were unambiguous.

PCR faithfully amplified the genomic DNA extracted from fetal fluid cells to diagnose genetic sex for all the pregnancies. The poor fluid recovery rate and maternal cell contamination did not adversely affect the outcome.

Table 3.5. Results of the genetic sex determined by PCR and anatomical gender of fetuses based on fluid recovered from the live animal by ultrasound-guided aspiration.

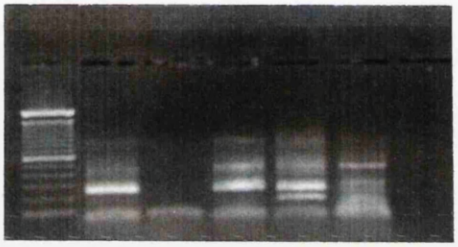
Cow No.	PCR sex	Anatomical sex
100	F	F
71	F	F
131	M	M
112	M	M
36	M	M
99	F	F
12	F	F

Figure 3.10. Application of the PCR assay on fetal fluid cells recovered by ultrasound-guided transvaginal uterine puncture.

A. Analysis of the first aspirate samples from cow 100 at 61 days of gestation. Lane M is the standard molecular marker. Lane 1 contains the sample and lane 2 is the negative control. Lanes 3 and 4 are female and male controls, respectively, while lane 5 contains saline flushings of the sampling needle. A female band was recorded in the sample and the controls are unambiguous.

B. Analysis of samples from cow 71 at 87 days of gestation, and cows 112 and 131 at 72 days of gestation. Lane M is the standard molecular marker. Lanes 1 and 9 contain a sample from cow 71, lanes 2 and 3 from cow 112 and lanes 4 and 5 from cow 131. Lane 6 contains the negative control (blank) and lanes 7 and 8 contain male and female positive controls, respectively. Note failure to detect DNA in nearly all the samples including the male control. Only the sample from cow 112 in lane 3 gave male specific bands. The female control gave the X band.

A.



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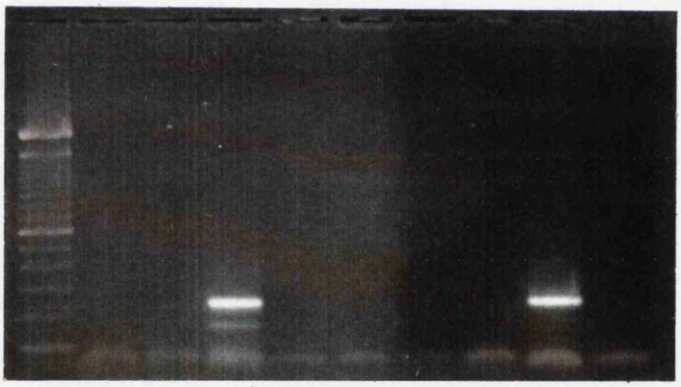
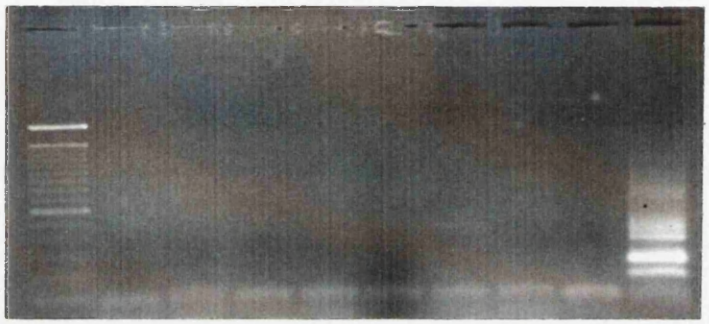


Figure 3.10. (continued)

C. Analysis of new samples from cow 131 at 77 days of gestation and repeat analysis of previous samples from cows 71, 112 and 131. Lane M is the standard molecular marker. Lanes 1 to 3 contain the new samples from cow 131, lanes 4 and 13 contain the blank and lanes 5 and 6 contain male and female DNA controls, respectively. Lanes 7 and 12 contain repeat samples from cow 71, lanes 8, 9 and 10 contain samples from cow 112 and lane 11 contains sample from 131. Note failure to detect DNA in the new samples from cow 131, samples from cow 71 and the control samples. In the previous sample from cow 131 there was failure to amplify the Y region leading to expression of a female specific signal (lane 11). Samples from cow 112 gave male specific bands.

C.



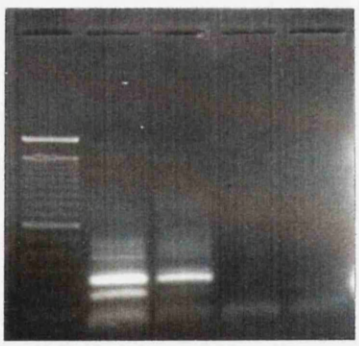
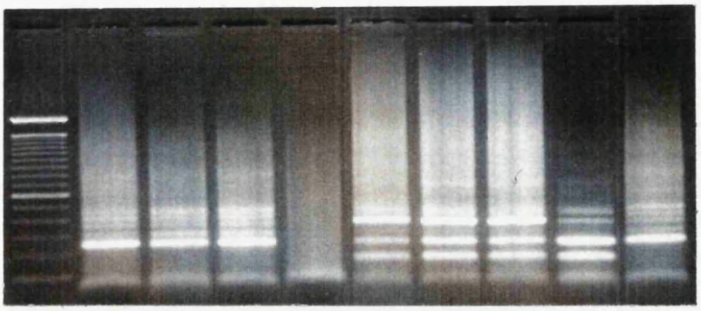


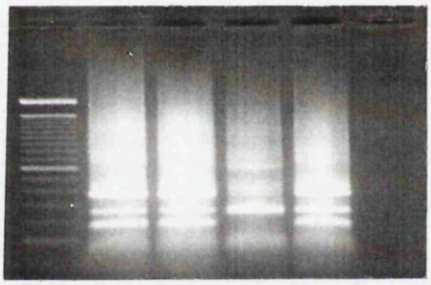
Figure 3.10. (continued).

D. Analysis of new samples from cows 71 and 131 at 95 and 77 days of gestation. All previous samples from cows 71, 112 and 131 were re-analysed. Lane M is the standard molecular marker. Lanes 1 to 3 contain new samples from cow 71 and lane 4 is the blank. Lane 5 to 7 contain samples from cow 131 at 77 days of gestation, lanes 8 and 9 contain male and female DNA controls, respectively. Lanes 10 and 13 contain samples from cow 112 and lane 11 contains sample from cow 71 at 87 days of gestation and lane 12 contains sample from cow 131 at 72 days of gestation. Note specific amplification of samples and positive controls to give detectable bands in all the lanes. The negative control was unambiguous. The results successfully sexed all the pregnancies subjected to ultrasound-guided transvaginal uterine puncture.

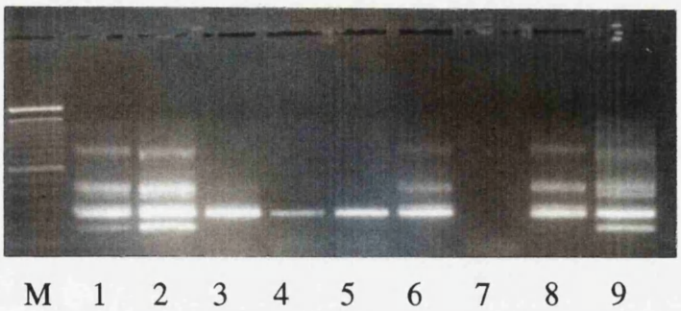
E. Analysis of sample recovered from the heifers (36 and 99). Lane M is the . standard molecular marker. Lanes 1 and 2 contain samples from cow 36 and lanes 3 to 6 contain samples from cow 99. Note specific detection of the male DNA bands (XY) in samples from 36 and the female band (X) in those from cow 99. The outcome of the controls is unambiguous with lane 7 negative control and lanes 8 and 9 contain female and male positive controls respectively.

D.





E.



Chapter 4

Discussion

4.1 Overview

4.2 Aim of the Study

4.3 Fetal Fluid Recovery

4.4 Ultrasonographic Observation of Fetal Death

4.5 Ultrasonographic Fetal Sexing

4.6 DNA Analysis of Fetal Fluid Cells for Sex Determination

4.7 Conclusion

Overview

The discussion consists of three sections. The first section addresses the aims of the study and the extent to which they were achieved.

The second section discusses specific technical problems encountered during the study and the steps taken to solve them. This encompasses the various reproductive related problems encountered with experimental animals and the difficulties with the laboratory based PCR assay.

The third section presents conclusions and puts this work into perspective as regards fetal fluid aspiration and PCR.

Aim of the Study

Nested PCR amplification of allele-specific *zfx* and *zfy* gene fragments was optimised and validated to achieve the primary aim of applying the assay to determining fetal gender using cells recovered by ultrasound-guided puncture. PCR cycle temperatures, concentration of reaction mixtures and number of cycles were optimised after several trials. When the assay was applied to genomic DNA extracted from abattoir collected tissues (liver, ovaries and fetal fluids) from animals of known gender, unambiguous and reproducible results were recorded. Applying the assay for the intended purpose was successful although positive results in some instances were recorded only after several trials.

Fetal Fluid Recovery

During the study fetal fluid was recovered from 9 animals in 14 procedures. Initial results were quite encouraging and provided the experience necessary to improve the technique. The approach was subsequently modified with the aim of improving recovery and pregnancy outcome. When the procedure went smoothly,

fluid recovery was usually successful. However, recovered fluids were sometimes of negligible volume and/or contaminated with maternal blood. With experience, the procedure was performed much more rapidly and with all appearances of sterility.

Before fetal fluid recovery three animals were omitted from further investigation on the grounds of reproductive failure. Cow 156 failed to conceive due to uterine infection that caused persistence of the corpus luteum and failure to return to oestrus after service. Cow 61 was successfully bred but the fetus died from unknown causes, probably due to constant uterine manipulation during the follow-up examinations. Previous studies have already proved that constant digital palpation per rectum for pregnancy diagnosis in the cow increased fetal loss in early gestation (Franco et al., 1987). Finally, heifer 4 was omitted after technical problems associated with synchronisation of oestrus. Even in the remaining animals it was not always possible to obtain a fetal fluid sample for reasons ranging from the design of the cattle handling unit, needles and equipment, to the individual animals used.

During the procedure it was vital for the animal to remain motionless to allow the operator to align the pregnant uterus with the transducer head and for the assistant to advance the needle. Despite sedation and padding of the sides of the cattle crush, the animal could still move slightly from side to side, reducing the working space for the two-man procedure. A different type of cattle crush fitted with removable side panels and sited at floor level might have eased this situation.

The equipment used in the study was of extreme importance. The human vaginal transducer, with a short needle guide, had proven satisfactory for follicle aspiration because ovaries are mobile and can be manipulated towards the transducer head. However, the human equipment was less well suited to uterine puncture because the length and weight of the pregnant uterus demanded deeper insertion of the transducer into the vagina. This caused two problems. Firstly, it was difficult to maintain a good grip of the transducer since the transducer was now inside the vagina. Secondly, the needle guide did not extend beyond the transducer so the needle insertion point was easily contaminated with vaginal fluids and/or faecal matter. This

factor compromised the extreme sterility necessary for pregnancy viability. Extension of the transducer handle with a length of plastic tubing solved the first problem. Several attempts were made before the second problem was solved satisfactorily.

An improvised needle guide was made from a 15 g tubing taped on to the transducer. However, it did not remain aligned with the biopsy guideline during the insertion of the needle through the uterine wall and membranes into the fluid vesicle. Loss of alignment meant the needle did not follow the expected path to the target as indicated on the scanner. This shortcoming was probably the main cause of failure to recover fluid from cow 61 on the first day of attempt. For subsequent aspirations, the original needle guide was extended with a length of metal tubing. This modification improved guidance but did not fully alleviate the contamination problem because there was a space between the guide and the extension. The final solution was the purchase of a custom-built 36 cm long needle guide (Figure 2.2) which improved recovery and greatly reduced multiple punctures in a single procedure. In the later stages of the experiment where the guide was employed, fluid recovery was successful on each occassion, often after a single needle puncture. This greatly improved pregnancy outcome with two out of three animals remaining pregnant several weeks after the procedure.

During follicle aspirations a 15 g x 29 cm needle with stylet was used to penetrate the resilient tissue of the vaginal wall. Extension of the needle guide called for a longer introducing needle such as 18 g x 32 or 45 cm needles. These needles were bent regularly during puncture of the vaginal wall, a problem that was never overcome. More seriously, it was not uncommon to observe the needle tip within a fluid compartment of the uterus yet still fail to recover fluid. In such cases the needle was probably blocked by vaginal or uterine tissue.

In cow 71, after successful introduction of the 18 g needle, the needle tip was observed pushing against the membranes but still failed to penetrate. A 22 g needle was then introduced through the 18 g needle. This penetrated the membranes and fluid was successfully recovered. Attempts to apply this technique routinely failed as there were

cases when the 18 g needle bent after vaginal penetration thereby preventing introduction of a narrower needle.

In retrospect, it is likely that problems associated with needle puncture compromised fetal viability. Repeated punctures were more likely to introduce infections which caused intrauterine death. In some cases, repeated needle punctures were made to obtain fetal fluid. This led to maternal bleeding and contamination of the sample with maternal blood. In addition, since the same needle was used to penetrate the vaginal wall and uterus and finally the fetal membranes, it inevitably contaminated the fetal environment with vaginal flora. Flocculence of fetal fluids seen in follow-up scans was highly suggestive of the presence of infection (Fig 3.10). Fetal death and infection was confirmed by the foul smell on examining the abortus from cow 71 and the recovered tract from cow 112.

The technique of fetal fluid aspiration also had to be modified during the course of procedures. Initially two syringes (2 and 10 ml) were used, connected to the membrane puncture needle by a 3-way tap. The procedure called for aspiration of a small sample into the 2 ml syringe for flushing maternal blood and other contaminants from the system. A clean sample was then obtained in the other syringe. However, negative pressure created along the entire needle-tube system delayed aspiration which needed to be effected immediately after membrane penetration. This led to failure to recover fetal fluid despite clear ultrasound evidence of membrane puncture and multiple punctures were sometimes needed especially if the animal moved suddenly and membrane contact was lost. In addition the 3-way tap made handling of the suction unit cumbersome for the assistant in such a confined working space. An alternative approach was to return to the use of the foot-operated vacuum pump routinely used for follicle aspiration. This was tested on cow 01 at 6 to 7 weeks of gestation. The pump created negative pressure immediately but excessive vacuum pressure constantly caused needles to become blocked by plugs of vaginal or uterine tissue. It was also likely that excessive suction drew the membranes against the needle bevel, again leading to blockage and failure to recover fluid. For example, during an aspiration from

cow 01, the needle tip was clearly visualised in the fetal fluid but no fluid sample could be withdrawn. The best solution to these problem seemed to be a single 10 ml syringe connected to the needle by a 6-8 cm tube. This was light and easily manipulated, and suction could be applied fairly rapidly. This system was used for all later procedures.

The aspirates varied in consistency, colour and degree of contamination. Clear viscous fluid was recovered (eg. cow 71) which was presumed to be amniotic fluid. Less viscous fluid was recovered (eg. heifer 99) which was presumed to be allantoic fluid. Blood tinged fluid was recovered (eg. heifer 01) where it was impossible to determine if the blood was fetal or maternal. In other cases (eg. cow 131) the blood was almost certainly maternal, and as such a contaminant of the sample. In some cases very small volumes were recovered and these could only be removed by flushing the needle and tubing system with physiological saline.

In addition to these equipment problems, the parity of the animal was found to be a factor in the success of the procedure. Fluid was recovered from five pluriparous animals and four heifers. The reproductive tracts of the pluriparous cows were generally located deep in the abdominal cavity and manipulating them when pregnant proved difficult so was retracting the uterus into the pelvic cavity within reach of the needles. Handling of the uterus was a lot easier with the heifers because the tracts were contained in the pelvic cavity. The aspiration procedure was performed most efficiently and with most consistent volume of fluid recovered using heifers, suggesting that a uniparous reproductive tract was well suited to the procedure.

This technique can be used to aspirate fetal fluid and in our hands most attempts were successful. If a single problem has to be highlighted, it is that of repeated needle punctures during a single sampling procedure may endanger fetal viability by introduction of infection and contamination of the sample with maternal blood.

Ultrasonographic Observation of Fetal Death

It was anticipated that during this study fetal death would occur. Fetal maceration or reabsorption rather than abortion was not anticipated, however. In fact, even after diagnosis of fetal death cows injected with Estrumate did not abort. This created a problem because sexing of the fetuses was important to verify the results of PCR. Only one aborted fetus was recovered (cow 71) and one animal (cow 01) was effectively dropped from the study because of failure to recover a fetus after abortion despite careful observation. It can only be assumed that the fetus was extremely degenerated and became lost in the muco-purulent discharge. Unfortunately, ultrasonographic sexing had not been performed before fetal loss. Prostaglandins such as Estrumate are generally considered to act on the corpus luteum of pregnancy, causing luteolysis and decreased plasma progesterone concentration. In this regard they are deemed effective until a minimum of 100 days gestation (Myenuddin, 1988) later than the pregnancies in the present study. Since pregnancy maintenance is critically dependent on progesterone, luteolysis is normally followed by abortion within a few days. Prostaglandins also possess strong and prolonged myometrial effects (Patil et al., 1980) and it is this property which may be required for prompt expulsion of an intact fetus. However, cloprosternol the active prostaglandin analogue in Estrumate was particularly formulated to have little ecbolic effect while still being effective for luteolysis. With this in mind, an analogue with the more pronounced ecbolic effects should perhaps have been considered.

Studying the effect of repeated aspiration procedures from about day 30 onwards was one of the project objectives. Vos et al. (1990) reported repeated recovery of fetal fluid at early stages of pregnancy. They were able to recover allantoic fluid at day 32 and amniotic fluid at day 44. However, one project objective of performing uterine puncture prior to ultrasonographic sexing had to be abandoned on the grounds that fetal maceration would have rendered gender determination impossible by any means.

Ultrasonographic examinations after aspiration yielded some interesting findings. Fetal death was identified by cessation of the heartbeat. However, other

changes to the fetal environment were noted prior to this feature. Characteristically, the fetal fluids developed a flocculated appearance, best likened to a 'snow storm' and the membranes were visible floating loosely in the fetal fluids. A 'snow storm' appearance has been reported elsewhere, in cases of uterine infection and pyometra (Fissore et al., 1986; Kahn and Leidl, 1989) but floating membranes do not appear to have been reported elsewhere. Such a feature may be due to detachment of fetal membranes or perhaps reduced turgidity due to reabsorption of fluid. An interesting contrast can be made between the spontaneous fetal death occurring in cow 61 (see Figure 3.3) and the other fetal deaths perhaps caused by needle puncture and contamination. No flocculence was noted in the fluids of cow 61 and the fetus gradually lost echogenicity as it was reabsorbed. All these fetal changes that indicated loss of pregnancy were observed within two weeks after the procedure.

Ultrasonographic Fetal Sexing

As mentioned above, it was intended to determine fetal gender by ultrasound after fetal fluid aspiration for confirmation of PCR results. Because of the problems advanced already, ultrasound fetal sex detection was carried out before fetal fluid aspiration.

Gender determination by ultrasonographic localisation of the genital tubercle is a fairly simple, non invasive and highly accurate technique in experienced hands (Curran et al., 1989; 1992; de Moura, 1993). It does not usually require chemical restraint although this usually eases uterine manipulation and improves accuracy. The technique has been tried under field conditions with high certainty levels (Curran, 1992) but high quality ultrasound equipment is required for sufficient resolution.

In this study a 7.5 MHz microconvex curvilinear transrectal transducer was used and animals were sedated to ensure correct diagnosis. A male fetus was easily detected by visualising the hyperechoic genital tubercle just behind the umbilical cord after day 56. The umbilical cord pulsations helped in its localisation and in a quick

diagnosis. On the other hand, female gender was not readily detected. The genital tubercle in the female fetus has been described as being located just under the tail by day 53 of gestation (Curran et al., 1989; 1992; de Moura, 1993). Visualisation of the tubercle requires a bit of experience to achieve a scanning plane that differentiates it from surrounding structures. Usually it is sufficient to say that absence of a hyperechoic structure proximal to the umbilical cord is indicative of female gender (Curran, 1992).

DNA Analysis of Fetal Fluid Cells for Sex Determination

Analysis of fetal fluids for sex diagnosis with PCR was first performed on the samples recovered by ultrasound-guided transvaginal puncture at 61 days of gestation from cow 100. In this case, PCR reaction successfully amplified genomic DNA to give a female specific band and controls included yielded unambiguous results. This result was in agreement with ultrasonographic sexing. This was a very satisfactory outcome but various problems with PCR were later to be encountered. Samples obtained from the heifer (cow 01) were analysed on the day of recovery but showed no detectable bands. The controls included, however, yielded expected bands. Repetition of the PCR still did not give a result and, in addition, the controls showed no detectable bands. This suggested an irregularity with the assay or the possibility that the concentration of genomic DNA was insufficient to be detected by the ethidium bromide staining method. The PCR protocol was modified by increasing the number of cycles from 30 to 35 per thermal reaction but still without effect. The problem was later found to be related to the Mg^{2+} concentration of the reaction mixture. In the presence of new thermal enzyme stock,DynaZyme II DNA polymerase, the Mg^{2+} concentration had to be reduced from 3.5 mM to 1.5 mM. Subsequent PCR assays revealed a female band in

the sample from cow 01. Unfortunately, fetal gender was not verified by other means for reasons cited above.

A second problem was encountered with later assays. Samples obtained from cows 71, 112 and 131 were analysed with DynaZyme II DNA polymerase and results recorded on the next day (see Figure 3.10). The sample from cow 112 showed male specific bands which was in agreement with the ultrasound findings. The other samples yielded no result and male positive control did not yield the expected result although the female positive control did. The assay was repeated several times but bands were still not detected. In one reaction, a sample from cow 131 which was known to be carrying a male fetus, showed only a female specific band and both positive controls failed to show any signal. In view of these inconsistencies it was decided to change the polymerases from DynaZyme II DNA polymerase to the previously used Taq DNA polymerase and also prepare new positive controls.

Re-analysis of these various samples, using Taq DNA polymerase gave signals in all samples which were in agreement with the ultrasound scans. The outcome of the negative and positive controls included was unambiguous. These results of DNA analysis suggest that there is difference in activity between the enzymes used. Taq DNA polymerase was found to be more suitable particularly where genomic DNA levels were relatively low. Even then the expression of band intensity under u.v. light varied with the X- band being visible more clearly than the Y- band. This may be an indication of sample contamination with maternal X- DNA contributing to signal strength.

The nested PCR technique used in this study has been proved to be highly sensitive, and is able to amplify DNA from a single blastomere. Resolution of PCR products using agarose gel electrophoresis and viewing under ultra-violet light following ethidium bromide staining does however have its limitations and minute DNA levels still cannot be detected. For example, a clear fluid sample of 2 ml was recovered from cow 01, probably from the amnion yet analysis of this sample failed to

yield a signal. This was disappointing because smaller volumes of fluid (< 0.5 ml) had yielded signals in other cows.

Conclusion

The results of fetal fluid recovery recorded in this study do not differ much from work published previously using the same approach (Vos et al., 1990). This group of workers reported successful aspiration at an earlier stage of gestation than had been attempted in the present study. Bongso and Basrur, (1975), Singh and Hare, (1977) and Sprecher and Kaneene, (1992) all have reported successful fetal fluid aspiration without the benefit of ultrasound guidance but acknowledge that the procedure is easier between 50 - 100 days as was the case in the present study. Likewise, it is generally accepted that second trimester procedures give fetal fluid samples of good diagnostic quality for prenatal diagnoses in humans and cows (Leibo and Rall, 1990).

Spontaneous fetal death followed mostly by reabsorption or abortion occurred in six animals subjected to amniocentesis in this study. Five of the animals were cows aspirated early in the experiments. At this stage the procedures were still being refined and each attempt at aspiration required multiple needle puncture. Two out of three heifers aspirated latterly with the aid of the modified needle-guide have retained live fetuses for two weeks. A two-week index is usually allowed in human pregnancies, within which pregnancy losses associated with amniocentesis are likely to occur (Henry and Miller, 1992; Hanson et al., 1992). The results of this study highlight the importance of a single needle puncture to the survival of the fetus. This was only achieved with the new needle-guide. Another important conclusion from these studies is that amniocentesis is much simpler to perform in heifers due to the intrapelvic location of the pregnant tract and its easier access for needle insertion. Perhaps further refinement of the technique will improve the outcome for pregnancies in older cows. In the animals that lost the pregnancies, signs of intrauterine infection were often

observed before fetal death. This problem was encountered by Vos et al., (1990) and although rare it is has been reported in human transvaginal procedures (Artley et al., 1993). Recommendations for better asepsis by vaginal rinsing prior to the procedure did not improve the outcome in our hands. Jacketing the entire instrument set-up in a sleeve might alleviate the problem.

Fetal fluid volumes recovered in the present study were low compared to previous studies which used fetal fluid cells for sex diagnosis (Bongso and Basrur, 1975; Singh and Hare, 1977; Leibo and Rall, 1990). However, such cytogenetic analysis and microscopic examination requires a considerable number of cells for a correct diagnosis. It is noted that these authors still reported a percentage of disputable results whereas in this study, sex determination with PCR was highly accurate despite small sample volumes. This showed that PCR can be a useful tool in various prenatal diagnoses with minimal sample volume.

However, a female result obtained in the PCR assay was always confounded by the possibility of maternal cell contamination at sampling. This problem could only be resolved if the sampling technique could be so refined as to exclude maternal cell contamination. This was not achieved in the present study. In addition, the PCR assay was previously reported to amplify *zfx* gene fragments across species while the *zfy* was specific to the bovine species (Kirkpatrick and Monson, 1993). This implies that a female result could arise from contamination by human cells. In view of this, if a female result was obtained, this always needed to be confirmed by some other means. However, gender determination is not the only purpose for which fetal cells can be used. For example, the technique is used commercially to monitor incorporation of specific genes in transgenic calves *in utero.* Fetal fluid recovery offers a potentially useful source of cells for various prenatal diagnoses and also for study of feto-maternal interactions. At this stage however, given the risks of fetal viability in older cows and the difficulty of confirming the female gender, collection of fetal fluid for sex detection cannot be considered a routine procedure. However, with future advances in molecular biology techniques, a clear application of sufficient importance to justify the

risk of fetal life may become apparent, and the difficult technique of fluid aspiration may then find a practical application.

This study recommends further investigation to refine the technique of fetal fluid recovery and considerably reduce the risk of fetal death. Further information on the most appropriate gestational age for obtaining fetal fluid samples of good diagnostic quality is also needed. With suitable refinements, fetal fluid aspiration and PCR will be an invaluable tool in farm animal prenatal diagnosis.

CHAPTER 5

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