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EMBRYO SEXING IN CATTLE: REVIEW

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Abstract

Predetermination of sex is of commercial importance since economic value of each livestock species is different for different sex. Embryo sexing enables the producers to run a fewer recipient female and thus quickly increase the size of their herd. The widespread use of embryo transfer technology has created an alternative approach in controlling the sex of offspring. The methods used can be grouped under two main categories: invasive and non-invasive. The latter is generally considered optimal, since embryonic integrity is maintained and the capacity for normal development should be optimized. Non-invasive sexing, however, is not as accurate as invasive techniques and recent advances in DNA probing have minimized the losses in embryonic viability inherent in all invasive techniques. With recent progress in molecular biology, more rapid and reliable embryo sexing techniques are being evolved like PCR (Polymerase Chain Reaction) and fluorescent in-situ hybridization (FISH). A routine method of embryo sexing must include an accurate sexing procedure and survival of embryos. Sexing methods must also be easy to use and inexpensive to operate. The dairy industry eagerly awaits the perfection of this scientific advancement. The sexing of embryo is likely to find a widespread application in embryo transfer industry in near future.

Key Words: Embryo sexing, cow PCR, Invasive and Non-invasive etc

INTRODUCTION

Commercially available sex-sorted sperm can be used for in vitro fertilization (IVF) to generate bovine blastocysts that may lead to pregnancies of the desired sex with an accuracy of approximately 90 % (Rasmussen *et al.* 2013). Unfortunately, frozen sex-sorted semen is expensive and is less efficient for IVF compared to conventional, unsorted semen (Trigal *et al.* 2012b; Xu *et al.* 2009; Bermejo- Alvarez *et al.* 2008a, b; Palma *et al.* 2008; Zhang *et al.* 2003). Furthermore, bull effects notably impact performance of sex-sorted spermatozoa (Xu *et al.* 2006; Bermejo-Alvarez *et al.* 2010a, b; Machado *et al.* 2009); not all bulls are appropriate for flowcytometry sorting. This method is a very slow. i.e. less number of spermatozoa sorted per hour due to sexing of one sperm at a time rather than multiple sperm and thus less number of sperm are being identified for its sex or only less number of straws are being produced (7 – 10 dose/hour) (Seidel, 2007).

The widespread use of embryo transfer technology has created an alternative approach in controlling the sex of offspring. With the birth of the first sexed calf in 1975 and the publication of the technique for examining the chromosome in cells from a piece of trophoblast of day 12 to day 15 bovine embryos in 1976 [9] a feasible means of sex selection in cattle was created. Currently, a number of methods are being employed in embryo sexing eg Immunological assays of male-specific antigens such as H-Y antigen, Quantification of the X-linked enzymes, Analysis of sex chromatin with V-specific DNA probes and Cytogenetic analysis. The first two methods are non-invasive but not always accurate. In contrast, cytogenetic analysis and the use of Y specific probes are highly accurate, but they are invasive methods that are limited by the biopsy. With recent progress in molecular biology, more rapid and reliable embryo sexing techniques are being evolved like PCR (Polymerase Chain Reaction) and fluorescent *in-situ* hybridization (FISH).

The recent development of the polymerase chain reaction allows amplification of Y- chromosome- specific repetitive sequences and thus determination of the sex of the embryo in a relatively short time and with high reliability (Herr *et a/.,* 1990).[10] The FISH technique enables the sensitive detection of specific nucleic acid sequences, or, more simply, identification of individual chromosomes in metaphase and interphase nuclei from many different cell types. It is also able to detect mosaicism and aneuploidy at the same time as determining the embryo's sex (Delhanty *et al.,* 1993).[11] The dairy industry eagerly awaits the perfection of this scientific advancement.

Significance of embryo sexing

There is variety of reasons that determines its importance in animals as well as in human beings.

1. Predetermination of sex is of commercial importance since economic value of each livestock species is different for different sex.
2. It is important for the management and breeding of livestock as well as for the prenatal diagnosis of genetic disorders (in human).
3. Embryo sexing enables the producers to run a fewer recipient female and thus quickly increase the size of their herd.
4. Pre-selection of female has got importance in preserving endangered species.
5. The sexing of embryo is likely to find a widespread application in embryo transfer industry in near future.
6. Necessitates the pre-selection of sex in any animal breeding strategy through embryo transfer allowing procedures to concentrate their genetic improvement on their male or female lines through better utilization of recipient females.

Genetic Basis For Sex

With the penetration of the ovum by the fertilizing spermatozoon, the genetic sex of the zygote is fixed.

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Those ova that are fertilized by spermatozoa bearing a Y-chromosome become genetic males, while those fertilized by X- bearing spermatozoa become genetic females. The gonadal and phenotypic sex is determined later, but only rarely do these differ from the genetic sex. Even though there exist a variation in the morphology of sex chromosomes, they are unidentifiable in each of the domestic species. It is this dimorphism in the sex chromosomes that forms the basis of sex determination by cytological methods.

In most mammals, including humans, mice and marsupials, sexual fate is determined genetically by the presence of the SRY gene (sex-determining region on the Y chromosome), which encodes the testis-determining factor on the Y- chromosome (Sinclair et al.,[12] 1990; Koopman et al., 1991).[13] Several other genes are known to be important fo sex determination in mammals, such as AMH (Anti- Mullerian Hormone), WT1 (Wilm's tumor suppressor gene), SF1 (Steroidogenic factor 1), DAX1 (Dosage sensitive sex-reversal adrenal hypoplasia congenita- critical region of the X- chromosome, gene 1) and DMRT1 (Double sex and mab- 3-related transcription factor 1). Sex determination results from the complex interplay between these genes.

METHODS OF EMBRYO SEXING

Attempts to alter sex ratio by selecting X- or V-bearing spermatozoa ave produced highly variable results, and have attracted only sporadic attention. Embryo technology, on the other hand, is rapidly expanding commercially. Manipulation of embryos *in vitro* to determine their sex prior to transfer, and thus alter the sex ratio of the offspring, is an alternative proposition.

The methods used can be grouped under two main categories: invasive and non-invasive. The latter is generally considered optimal, since embryonic integrity is maintained and the capacity for normal development should be ptimized. Non-invasive sexing, however, is not as accurate as invasive techniques and recent advances in DNA probing have minimized the losses in embryonic viability inherent in all invasive techniques.

A rapid and accurate microarray assay using sandwich mode for sex determination of bovine preimplantation embryos.(Yang *et al*., 2013).[14] Analysis of free fetal DNA in maternal circulation is a noninvasive and useful tool to determine fetal sex in the ewe. This fetal DNA, named circulating cell free fetal DNA (ccffDNA), has emerged as a valuable source for prenatal fetal sex determination and genetic evaluation. (Asadpour *et al*., 2015). [15]

INVASIVE METHODS

Karyotyping/cytogenetic analysis

Cytogenetic sexing, or karyotyping, was the first method to produce sexed rabbits, calves and sheep. Briefly, the technique involves the biopsy of a small number of cells, which are cultured in a medium with colcemid, a mitosis-arresting agent. The cells are then induced to swell so that the chromosomes disperse. Following fixation and staining with a permanent DNA dye, such as Giemsa, the slides are examined under a microscope. Cells, which are arrested in metaphase, generate a spread of chromosomes that can be identified by their specific banding patterns. The Y -chromosome is easily identified by its small size.

The accuracy of sexing by using this method is nearly always 100%. The difficulty lies in obtaining biopsies that show high quality metaphase spreads of chromosomes. Further the procedure is labour intensive. Approximately, five hours are needed for two experienced cytogeneticists to process 12-15 embryos. Furthermore, success rate rarely exceeds 62 due to poor metaphase chromosomal spreads.

Embryo sexing using V-specific DNA probes

The Y -specific probe technique involves the biopsy of a small number of cells from the embryo, and hybridization of ideal cellular DNA from these cells to a labeled sequence of DNA that is specific to the Y -chromosome and thus the male sex of the embryo. The very small amount of material required for DNA preparation is the most attractive feature of the procedure, although technically an invasive method, the Y -specific probe method does not present the same problems in this regard as does cytogenetic testing.

The generation and characterization of Y -specific DNA probes requires the following steps: isolation of V-chromosomes, isolation of V-specific sequences, determination of sequence copy number and localization of the probe sequence on the V-chromosome.

Recently, several patented Y -specific DNA probes have been made available for sheep (Herr *et al.,* 1990), for cattle (Herr *et al.,* 1990) and for man.

In the human embryo, the zona pellucida is penetrated and a single cell is removed at the 6-10 cell stage, 3 days after *in vitro* fertilization. Its Y-specific DNA is amplified (greatly increased in amount) by the polymerase chain reaction (PCR), using short segments of the 149 base pair V-chromosome specific probe as primers. The amplified DNA is subjected to electrophoresis, stained by ethidium bromide and examined by UV-light. The embryo is judged male if the 149 bp fragment of the Y -specific probe is clearly visible after this amplification. The whole procedure can be completed in five hours, and the results are closely correlated with karyotypic analysis. This biopsy procedure does not seem to affect embryo development adversely, with 37 of the biopsied embryos developing to the blastocyst stage *in vitro, i.e.,* about the same proportion as for unmanipulated embryos.

When applied to domestic animals, Leonard was able to sex *85/150* (*57*) biopsies of 7-8 day old cattle embryos with 95 accuracy, confirmed by karyotyping. achieved better results, sexing 85 *(632/747)* of the embryos biopsied. From the 111 sexed embryos that were thawed and transferred, 37 live calves were born, the sex of which had been predicted with 100 accuracy.

DNA probing is undoubtedly the most accurate method of sexing embryos, but more widespread commercial application is limited by the fact that embryos have to be probed individually, necessitating skillful micromanipulation. It is also an invasive technique, resulting in decreased embryo viability in many reports.

There are some Y-linked gene transcripts which are present only in the early male embryo that might represent useful markers of sex. For example, expression of sex-determining region Y (SRY) has been found as early as the 2- to 4-cell stage in human, murine, and bovine embryos. (Hamilton *et al*., 2012). [16]

PCR based embryo sexing

In the early 1990's the development of the polymerase chain reaction (PCR) opened up new possibilities for embryo sexing (Mara *et al.,* 2004).[17] This technique allows amplification of Y -chromosome- specific repetitive sequences and thus determines the sex of the embryo in a relatively short time and with high reliability (Herr *et al., 1990)*. At present, PCR has become the method of choice in determining the fetal sex in early pregnancies using DNA extracted from maternal plasma.(Cruz *et al.*, 2012). [18] Although not ideal in all aspects (requires removal of cells from the embryo), PCR-sexing is nearly 100 accurate, and the procedure can be carried out in a few hours (Bredbacka *et a1.*, 1995).[19]

Over the last few decades, DNA-based techniques, especially polymerase chain reaction (PCR)-based methods for meat sexing have received particular attention, and have proved to be reliable, sensitive, and fast. (Gokulakrishnan *et al*). [20]. The PCR-based sexing method is generally favored, as it is cost effective, simple and reliable (Malik *et al.*, 2013).[21]. The development of primer directed enzymatic amplification of DNA with thermostable DNA polymerase i.e. PCR and its application to amplify Y-chromosome specific DNA sequences. Sexing of embryo by this method includes 3 steps:

1. Biopsy of embryo (1-4 blastomeres)
2. Amplification of 2 DNA fragments (one species specific and one male specific).
3. Analysis of amplified products and interpretation.

Biopsy of embryo: The embryos for sexing are collected on day 6.5 after 1 st A.1. Only embryos graded as excellent or good from the stage of compact morula to early blastocyst are biopsied, with the help of a micromanipulator.

Amplification: Involves a series of cycle.

1. Template denaturation (94-97^{0}C) for 90 sec.
2. Primer annealing (50-720C) for 90 sec.
3. Extension of the annealed primer at 72^{0}C for 180 sec, by Taq DNA polymerase.

These 3 steps are repeated for 40 cycles. After the last cycle, the samples are incubated at 72^{0}C for 7 minutes. The PCR mixture contains template DNA, 2 sets of primers, 4 deoxyribonucleotides Taq DNA polymerase and buffer. Use of a loop-mediated isothermal amplification (LAMP) technique for bovine embryo sexing has also been reported. (khamlor., *et al* 2015).[22] The feature of LAMP is specific DNA amplification under isothermal conditions. DNA polymerase, with its high strand displacement activity, enables auto-cycling strand displacement DNA synthesis within the range of 60–65 ◦C (zoheir and allam, 2010) [23] Loop-mediated isothermal amplification (LAMP) is a DNA amplification method that can amplify a specific DNA sequence within the range of 60 to 65 C (Hirayama *et al.*, 2013).[24]

Analysis and interpretation

The amplification products are electrophoresed on a 2 agarose gel stained with ethidium bromide and evaluated using ultraviolet light. If only one band of the bovine-specific product is visible, on the gel, the blastomere is considered to derive from a female embryo, whereas the presence of two bands referred to a male embryo.

Embryo sexing by fluorescence in situ hybridization (FISH)

The development of the technique *in situ* hybridisation and fluorescence detection, also called fluorescence *in situ* hybridisation (FISH), have begun to make possible the sensitive detection of specific nucleic acid sequences, or, more simply, identification of individual chromosomes in metaphase and interphase nuclei from many different cell types (Kobayashi *et al.,* 2004).[25] This procedure has been used for the determination of sex and the detection of chromosomal aberrations in human embryos (Griffin *et al.,* 1992;[26] Delhanty *et al.,*1993). When using PCR for embryo sexing, the amplification of non-specific sequences or contamination of samples can be a serious problem (Herr and Reed, 1991). With the FISH technique this problem is negligible; FISH is also able to detect mosaicism and aneuploidy at the same time as determining the embryo's sex (Delhanty *et al., 1993)*.

Bovine embryos are produced by *in vitro* maturation and fertilization (IVMF) and cultured for seven to eight days to develop to the expanded blastocyst stage. The biopsies of the embryos were obtained by cutting th embryos with a microrazor blade with a single downward motion which partly cut the zona pellucida and splitt off a few cells from the trophoblast, to use for sex determination. Blastomeres are fixed individually (Tarkowski, 1996) and thereafter the biopsies are poured into a hypotonic solution of 0.075M potassium chloride with 0.5 bovine serum albumin (BSA), left for 10 minutes and then placed into clean slides coated with poly L-lysine. Approximately 10 µl fixative (acetic acid: methanol, 1 :3) is dropped onto the cells a few times. The specimens are air-dried by continuous blowing for 30 minutes, and the position of the embryos or blastomeres is marked on the back of the slide. Genetic sex determination is based on the fact that numerous polymorphisms are present on two homologous copies of the amelogenin gene on the X and Y chromosomes (Luptakova *et al.*, 2011)[27]

The bovine Y -chromosome-specific DNA sequence used as a probe is termed BC1.2 (Cotinot et a/.,1991).[28] Approximately 50 ng of digoxigenin -labelled probe prepared by PCR is added to 10 µl of a hybridization mixture composed of 50 formamide, 10 dextran sulphate and 2 mg/ml BSA in 2 X SSC (0.3M sodium chloride, 0.03 M sodium citrate, pH 7.0). Ten microlitres of the hybridization mixture is dropped onto the specimens followed by preheating at 720C and denaturing at 720C for 8 minutes on an aluminium block. Immediately after denaturation, the slides are transferred to an incubator maintained at 38.50C and hybridized for 5 minutes. The slides are then washed in 0.5 XSSPE (75 mM sodium chloride, 5 mM monobasic sodium phosphate, 0.5 mM EDTA, pH 7.4) at 720C for five minutes followed by a wash in PN buffer (0.1% sodium phosphate, pH 8.0, supplemented with 0.1% Nonidet-P40) for two minutes at room temperature. The digoxigenin is detected by incubation with 2µl/ml anti- digoxigenin-fluorescin in PN buffer plus 5% non-fat dry milk at 38.50C for 5% minutes. The free anti-digoxigenin -fluorescin is removed by three changes of PN buffer at room temperature. Finally, the preparations are counterstained with propidium iodide (0.3µl/ml). The slides are examined under an epifluorescence microscope with a 4-MWIB (excitation 460 to 490 nm) mirror unit. The nuclei that are male show Y -chromosome specific Signals, whereas the female nuclei do not show any signal.

The most significant problem encountered when using PCR for embryo sexing is contamination, because of the techniques high sensitivity. PCR products from the previous assay pose the greatest risk of assay contamination (Herr and Reed, 1991).[29] In contrast, FISH has the advantage that there is a reduction in the risk of contamination with extraneous cellular material. In addition, cells are actually visualized directly throughout the procedure and can be verified as being of embryonic origin.

Non-invasive methods

X-linked enzymes

In normal mammals, the homogametic sex carries two X-chromosomes, whereas the heterogametic sex possesses only one X-chromosome. embryos theoretically can be distinguished as male or female by measurement of the gene dosage for X-linked enzymes (Bondioli, 2014).[31] To maintain an equivalent number of genes between the sexes, one of the X-chromosomes in the female is inactivated in each cell early in the embryonic life. Although the exact timing of X-inactivation is not known, studies have suggested that there is a brief period between activation of the embryonic genome and X-inactivation in which genes from both X-chromosomes in the female are transcribed. This is reflected in a cellular concentration and activity of certain X-linked enzymes that is twice as high in female as in male embryos. In both cases, X-linked enzyme activity must be compared to autosomal-linked enzyme activity to account for individual variation in embryometabolism.

Based on the hypothesis that the ratio of X-linked enzyme activity to autosomal enzyme activity will be higher in female than in male embryos. studied the activity of glucose- 6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT) and adenine phosphoribosyl transferase (APRT) activities in mice embryos. The study on G6PD showed that 72% (62/86) of female embryos and 57% (54/95) of male embryos were correctly identified as to sex. Both HPRT activity and the ratio of HPRT: APRT activities showed accuracy of sexing as 91% (11/12) for females and 100% (3/3) for males.

In bovine embryos, glucose and glutamine metabolism has been studied by Tiffin *et al.* (1991)[32] and showed greater metabolism of glucose and glutamine in female embryos than male embryos.

The X-linked enzyme activity measurement methods have shown encouraging results, however, have many limitations of collection and examination of small amounts of enzymes.

H-Y (male) antigen

The immunological demonstration of a sex specific antigen provides another non-invasive method of sexing embryos. Studies in this regard have focused on the histocompatibility Y-antigen (or H-Y antigen), which was first identified when females from an inbred strain of mice rejected skin grafts from their male littermates although there was no rejection of female skin grafts in the males. Though the function of this antigen is not known, the gene has recently been mapped to the long arm of the Y chromosome and has been proposed to be one of perhaps, many genes involved in organizing the indifferent gonads into testes.

Two methods exist for detecting H-Y antigen on embryos: a cytotoxicity assay and an immunofluorescent assay. In the cytotoxicity assay, embryos are exposed to dilute H-Y antiserum and complement. Embryos expressing H-Y antigen show a degree of cell lysis, and thus are categorized as male. The immunofluorescent assay system requires antibodies to cell-surfacemolecules specific to male tissues (sometimes referred to - probably incorrectly as the anti-H-Y antigen method. Embryos are incubated for 30-60 minutes with antibodies, and then for an additional 30-60 minutes with an antibody to the first antibody containing fluorescin isothiocyanate (FITC), a fluorescent dye. Embryos are then briefly examined with a fluorescence microscope. Male embryos fluoresce. The advantages of this approach are its speed and lack of need to biopsy embryos. The disadvantages are need for a fluorescence microscope, commercial unavailability of reagents, the subjective nature of determining what is and what is not specific fluorescence and limited accuracy (80 percent). Despite these problems, many people feel that this approach may be developed into an acceptable procedure for routine use.

Hormonal assay for sexing blastocyst

In the light of advances made in fetal endocrinology, suggested that fluid collected from blastocoel could be subjected to hormonal assay for sex diagnosis. However, to be useful for sexing a hormone must be (1) measurable, (2) exist in blastocoelic fluid and (3) differ between sexes unambiguously.

The hormonal assay has been used for predicting fetal sex between 90 and 150 days of gestation in cattle. However, the use of hormonal assays for sexing embryos during transfer is probably remote, but estrogens are produced as early as day 12 (well before gonadal differentiation) in pig embryos. Similarly the pre-attachment horse embryo produces estrogens and androgens as early as day 14, and probably earlier, but it is not known whether steroid production varies with sex nor whether blastocysts large enough to provide an aliquot of fluid for analysis could survive transfer. The method needs more explorations.

Sexing based on cleavage and development

The cells of male embryo have proportionately less amount of DNA as compared to female embryo cells. More amount of DNA means more time needed in its duplication and hence a longer cell cycle. This is expected to effect the cleavage and development rate in male and female embryos. The male embryos are considered to cleave early and develop fast to attain morula and blastocyst stage than female embryos.

Recently, some reports on cleavage and development rate in bovine embryos produced *in vivo* and *in vitro* have appeared, which have shown that

cleavage and development is faster in male embryos than female embryos.

This method of embryo sexing has still many limitations, viz., I*n-vivo*

produced embryos cleavage time cannot be known, besides the difference in developmental rate is very small and needs high skills in separation of fast and slow embryos. DNA probing is undoubtedly the most accurate method of sexing embryos, but more widespread commercial application is limited by the fact that embryos have to be probed individually, necessitating skillful. micromanipulation. It is also an invasive technique, resulting in decreased embryo viability

in many reports.

CONCLUSION AND FUTURE PROBABILITIES

A routine method of embryo sexing must include an accurate sexing procedure, survival of embryos during freezing, transfer and successful development into a calf of desired sex. Sexing methods must also be easy to use and inexpensive to operate.

In view of the above, the evaluation of various methods discussed here suggests that sexing of spermatozoa is the most ideal approach, however, available, technologies are not practicable. Scientists can sort sperm with 90 % accuracy by a process called flow cytometry, a method more commonly used with *in vitro* fertilization; but the process is too slow to provide enough sperm for artificial insemination.

Transplantation of diploid nucleus/blastomere of a sexed embryo into enucleated oocyte can emerge an effective technology for production of sexed clones of farm animals. The cytological methods are very accurate though invasive in nature and will remain applicable at least for testing the results of other effective methods available or emerging in future. The male specific DNA probes are likely to emerge as most preferred methods. The non-invasive methods viz., X-linked enzymes, H-Y antigen, hormonal assay are not of much practical value. The cleavage and development rate have been shown to be faster in male embryos than in female embryos, however, the differences are quite small and need high skills in separation, besides it is possible only with *in vitro* produced embryos. A single copy of male specific target DNA (i.e. single blastomere) is sufficient, thereby enhancing the embryo viability by minimizing the number of blastomeres removed during biopsy. The assay is rapid and accurate at the molecular level to allow the transfer of sexed embryos without the need for cryopreservation. But, the most significant problem encountered when using PCR for embryo sexing is contamination, because of the techniques high sensitivity. The FISH technique, which utilizes a Y-chromosome-specific DNA probe, enables accurate and rapid sex selection of embryos, in order to obtain offspring of the desired sex.

However, techniques that require embryo biopsy still need to be cautious, in case they reduce embryo viability. When successful techniques of embryo transfer and sexing become available, the demand for these techniques is likely to grow considerably. Research to improve sexing and embryo transfer is ongoing, while at the same time, the application of sexing through embryo transfer is being developed.

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