Potential applications of embryo sexing in dairy animals

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ABSTRACT

Manipulating the sex of offspring has great potential for the livestock industry. The ability to determine the sex of embryos prior to transfer to the recipients has commercial application in the embryo transfer technique. This article focuses on the recent advances in the field of embryo sexing and its application in the livestock industry. Six methods, viz. cytological method, X-linked enzymes, differential developmental rates, detection of male specific antigen, Y-specific DNA probes and PCR amplification of Y-chromosome specific gene methods introduced so far for sexing the embryo in farm animals have been discussed along with their merits and demerits. PCR based amplification method has been adjudged the best method with higher accuracy and more efficiency. Several genes such as SRY, ZFY, ZFX, TSPY, AML, etc have recently been used as markers for embryo sexing. Recently, the technique, such as multiplex-nested PCR, Real time PCR, LAMP and FISH are also being used for embryo sexing in different livestock species.

Key words: Embryo Sexing, Y-Specific DNA probe, PCR, Sex Determination.
INTRODUCTION

Genetic improvement for milk production has been one of important goals for enhancing profitability of dairy enterprise. The greatest impact of breed improvement programs has been from infrastructure development for artificial insemination followed by successful embryo transfer programs. Predetermination of sex of offspring in livestock species has various commercial and research applications. The development of embryo transfer procedures coupled with in-vitro fertilization and manipulation of embryo have the potential for embryo sexing in order to control the sex of offspring as well as to accelerate genetic gain.

The desire to manipulate sex is obvious as cows are preferred for milk production and bull/bull calves for beef production. However, a number of methods, such as based on physical difference, H-Y antigen, albumin gradient & percoll, electrophoresis separation and flow cytometry for sexing of semen have been attempted. Jafer and Flint (1996) reported that flow cytometry is most successful method of semen sexing but is limited by the low number of spermatozoa sorted per hour (3.5 x 10^5). On the other hand, sex determination of embryos has been attempted by several methods, which can be broadly divided into invasive methods including cytological analysis of sex-chromatids, Y- specific DNA probe and PCR amplification of Y- chromosome specific gene and non-invasive methods such as quantification of X- linked enzymes, immunological analysis of male specific antigens and differential developmental rates. In invasive technique, the major concern is to minimize the damage to zona pellucida and maximize the viability of embryo. However, in non-invasive technique, the major concern is the stage of X-chromosome inactivation and non-reproducible results.

DIFFERENT METHODS OF EMBRYO SEXING

Different techniques used for embryo sexing have been discussed below:

Cytological Analysis

This is one of the first methods of embryo sexing, in which the sex of embryo was determined by observing the sex chromosomes from metaphase spreads prepared
from trophoblastic biopsies of 6 to 7 day and 12 to 15 days (Betteridge et al. 1981) in bovine embryos. However, the cytogenetic analysis of embryos by chromosome analysis has two major limitations. First, a few cells are in mitosis, even when a metaphase blocker is used (King et al. 1984). Therefore, it is not always possible to detect or identify the degree of mixoploidy (diploidy-haploidy, diploidy-triploidy etc.). Secondly, the occurrence of hypodiploid cells has to be interpreted with caution, as chromosomes can be lost during slide preparation.

Detection of barr body (formed from the inactive X-chromosome) in female cells is not a reliable indicator of sex determination of domestic species because of the granular nature of the cytoplasm which interfere with the visibility of the barr body and some female embryos may be misidentified as males due to the masking of barr body with granular germplasm. Although the accuracy of this method is virtually 100%, the efficiency remains at a dissatisfactory level. However, requirement of a skilled cytogeneticist and the inefficiency of this technique have made it less effective for embryo sexing for the commercial application. This technique is also limited by the number of cells arrested in metaphase from a biopsy.

**X-Linked Enzyme Activities**

Male and female embryos can be distinguished based on measurement of the gene dosage for X-chromosome linked enzymes. The enzymes, such as, hypoxanthine phosphoribosyl transferase (HPRT) and glucose 6-phosphate dehydrogenase (G6PD) activity was measured for sexing of mouse embryos (Monk and Handyside, 1988). The major limitation of this approach is the stage of inactivation of the X-chromosome. It has been observed that the activation of the embryonic genome in bovine embryos occurs between 8-16 cell stages, therefore, if 8-cell bovine embryos were assayed, it is unlikely that a bimodal distribution of enzymatic activity would be observed (Monk and Handyside, 1988). During early development, both X-chromosomes in females are active, and one becomes inactivated during the blastocyst stage in mice. However, the exact timing of X-chromosome inactivation in embryos of domestic animals is not
known, which could lead to diagnosis of female embryos as male because of early X-chromosome inactivation.

**Differential Embryonic Developmental Rates**

Several reports have indicated that the developmental rates of embryos are sex-dependent in cattle. The male embryos develop faster than female embryos. Avery *et al.* (1991) reported that for *in-vitro* fertilized bovine embryos, 95% of the fastest developing embryos were male. A similar result was reported by Bredbacka and Bredbacka (1996), where male embryos cleave faster than female embryos only in the presence of exogenous glucose. The reason for the tendency of males to develop faster is unknown. However, it is unknown that the nuclear transfer embryo derived from male or female diploid nuclei have the same development potential or not. This method is limited by asynchronous ovulation in superovulated animals resulting from variation in the ovulation time of superovulated cows.

**Male-Specific Antigen**

This is a non-invasive antibody-based method that utilizes an immunological approach for embryo sexing. It has been reported that the genes for H-Y antigen located on the Y-chromosome plays an important role in the differentiation of different gonads into testis. The produced antibodies that are targeted towards proteins are found exclusively on male or female cells and this allows for efficient identification of embryos of either sex. Antibody production is performed by injecting bovine sex specific proteins (SSP’s) into an animal. The sex specific proteins are obtained and purified from the cell membrane of adult animal donor tissues (Blecher, 2005). The foreign bovine SSP’s develop an immune response, which produces antibodies specific to the foreign bovine material. Prior to administration in the bovine embryos, the antiserum is tested to determine the sex specificity of the antibodies, which are then tagged with a fluorescent marker for identification purposes (Niemann and Wrenzycki, 2005). Green fluorescent protein (GFP) is most commonly used and can be observed under a fluorescent microscope. Anderson (1987) found an accuracy of 84% using this method in cattle, 85% in goat, 81% in pig and 82% in horse.
However, this method has not shown repeatable results. The drawback of this method was that the sex of bovine embryo can only be detected as early as at 8-celled stage.

**Y-Chromosome Specific DNA Probes**

This approach is based on a molecular biological technique. The pieces of DNA (probes) are made, which bind to DNA on the Y-chromosome but not other chromosomes. Embryos are biopsied, the DNA is extracted from the cells and an enzymatically or radioactively labelled DNA probe is incubated with the extracted embryonal DNA. If Y-chromosomes are present, the probe binds with them. The identification of male-specific bovine chromosomal DNA fragments enables the use of DNA-probe technology in determining the sex of preimplantation embryos from a small embryonic sample. The Y-chromosome specific DNA sequence is identified and accordingly complementary sequence is used as probes. In bovines, 7 to 8 days embryos are collected and 10 to 20 trophoblast cells are removed by biopsy and further processed by *in-situ* hybridization. The accuracy of this method is 95% in bovine (Bondioli *et al.* 1989). However, the accuracy using FISH (Fluorescence *in-situ* hybridization) was 92% (Cenariu *et al.* 2008). The main advantages are that it is highly accurate and a higher percentage of embryos can be sexed since cells need not be in metaphase stage. Major drawback of this method is that the Y- chromosome specific DNA probes for sex determination may be subject to error if the parents have extreme Y-chromosome variants.

**PCR amplification of Y-chromosome specific gene**

Amplification of Y-chromosome specific DNA by means of the PCR technique seems to be the most reliable and practical method of sexing embryos (Watanabe *et al.*, 1992). A small piece of DNA (or genes) which is present exclusively on the Y-chromosome and not on the X- chromosome can be multiplied by polymerase chain reaction (PCR); which provides sensitive, accurate, efficient and rapid sex determination from a small number of cells. Many genes, such as *Amg, ZFX, ZFY, TSPY, G6PD, HPRT* and *SRY* have been used for PCR amplification of Y-chromosome specific gene in different livestock species.
The success rate of this method varies from 90 to 100%. Polymerase chain reaction (PCR) has been standardized by Rao and Totey (1992) for accurate sex determination in sheep and goats, which utilizes a pair of bovine Y-chromosome specific primers and the genomic DNA for recognizing and amplifying the Y-chromosome specific sequences in male goats and sheep.

Shi et al. (2007) studied PCR based method for goat sex determination by amplifying the HMG box of SRY gene and testing its sensitivity in pre-implantation embryos. Goat beta-actin gene together with HMG box of SRY gene was simultaneously amplified as an internal control gene. Hashiba et al. (2000) developed a multiplex PCR assay from a single cell which co-amplifies X (DXZ1) and Y-chromosome (DYZ1) specific repeat sequences. Sathasivam et al. (1995) developed a rapid and reliable method of embryo sexing in pig by amplifying HMG-box of the Y-chromosome in male genomic DNA using PCR. In pig, two sex determination assays targeting the 9-10 bp difference between AMELX and AMELY were developed by Fontanesi et al. (2008).

It is probable that if robust PCR techniques would developed, will allow the analysis of a large number of markers from one biopsy simultaneously, leading to the concept of ‘embryo diagnostics’. Now-a-days, the recent techniques such as multiplex-nested PCR in buffalo (Fu et al. 2007); LAMP (Loop-mediated isothermal amplification) in cattle (Khairy and Ahmed, 2010) and buffalo (Hirayama et al. 2006) have been used for embryo sexing. Khairy and Ahmed (2010) used the novel DNA amplification method, such as LAMP to amplify a target sequence specifically under isothermal conditions and reported 100% accuracy of sex prediction. Zoheir and Allam (2010) developed a new technique by adding ethidium bromide (EB) or 5 μl of CuSO4 at different concentrations to the product of LAMP reaction, which allows detection with the naked eye without the use of electrophoresis or a turbidity meter and the accuracy of this technique is 100%. Although, PCR amplification method is the best method of embryo sexing, the main source of error lies in the extreme sensitivity of PCR, since the assay can amplify and detect a single molecule of DNA, contamination by as little as a single molecule can give a false positive results. Further, due to
invasive technique, it results in disruption of the integrity of the *zona pellucida* which may reduce the viability of the embryo.

**APPLICATION OF EMBRYO SEXING IN LIVESTOCK IMPROVEMENT**

Dairy industries consider embryo sexing as a tool to reduce the number of bulls. It can maximize the number of female animals by transferring only female embryos into the recipients.

The potential increase in the genetic gain and marketability of sexed embryos is used to satisfy both the genetic and the financial objectives simultaneously. Embryo sexing enables producers to reduce the overall cost of embryo transfer by reducing the required number of recipients. Embryo sexing has the potential to control the incidence of genetic disorders such as freemartinism and Complex vertebral malformation (CVM) in farm animals. By utilizing embryo sexing techniques, embryos can be selectively identified and can then be used to obtain the desired sexed offspring. Thus, the beef cattle producers can concentrate on the production of males while dairy producers on the production of female cattle. This allows producers to minimize loss that results from the culling of animals of the undesired sex.

Furthermore, embryo sexing can also be used in the biotechnological sphere for production of transgenic animals and cloning. Embryo sexing can be used synergistically with other artificial reproductive techniques (ART). Hence, further advancement in this technique will prove to be useful if not critical to the future development of beef and dairy industry.

**CONCLUSION**

Various reports on embryo sexing technique demonstrates that PCR amplification of Y-chromosome specific DNA fragments is a highly efficient and accurate approach for embryo sexing. In future, genetic markers associated with quantitative trait loci (QTL) and radioimmunoassay can also be used in embryo sexing. It would also be possible to detect polyploidy and perhaps other abnormal traits in combination with sexing. There are, however, several intrinsic bottlenecks for sexing of embryos. If the embryos of the undesired sex are to be discarded, the process is obviously inefficient because
of the high cost of obtaining embryos. Generally, the procedures are costly clumsy, time consuming, slightly damaging to embryos and costly. Hence, due to various constraints and limitations in the existing embryo sexing techniques, further research is research required to perfect these techniques to obtain the animal of desired sex and this will lead to exploitation of these techniques commercially for higher genetic and financial gains.

REFERENCES
