**Embryo Sexing and it’s future perspective in Livestock Farming**

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**Introduction**

Embryo sexing is a method to determine the gender of bovine embryos before implantation. This is a useful technique in livestock management, especially in dairy production, where female calves are more desirable. The milk production and meat are the result of both female and male cattle that benefits the dairy and beef industries (Sachan et al., 2020). It is more justifiable in country like India where calves are lowering the economy of breeders as well as cow slaughter is also prohibited. Control of the sex ratio by sex prediction of the of pre implanted embryo would be beneficial not only in relation to the aspect of management, production and breeding programmes of livestock but also in diagnosing the genetic disorders at prenatal stage. Pre-implantation sexing of embryos increases the efficiency of embryo transfer, facilitate the transfer of embryos of choice on the basis of their sex (Bredbacka, 2001; Cenariu *et al.* 2008). In recent period artificial insemination or *in vitro* fertilization (IVF) are performed with sex-sorted sperm to get desired sex but it is well expensive (Seidel Jr, 2007) and less efficient compared to conventional, unsorted semen (Trigal *et al*. 2012). The sex prediction of embryos before implantation also helps to prevent freemartins in heterosexual twins. Furthermore, genetic technology with high sensitivity for embryo sexing is applicable to examination to sex chromosomal chimerism in heterosexual twin female calves. Embryo sexing enables the producers to run a fewer recipient female and thus quickly increase the size of their herd. Pre-selection of female has got importance in preserving endangered species. The sexing of embryo is likely to find a widespread application in embryo transfer industry in near future. Importance of pre-determining 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.

**Different techniques of Embryo sexing**

Genetic sex of the zygote, whether it is female or male, is decided with the fertilization of ovum by the spermatozoon having X chromosome or Y chromosome accordingly. Different procedures have been introduced for sexing of embryos in farm animals by means of invasive or non-invasive methods, depending on whether or not a biopsy of embryonic cell is needed (Garcia, 2001). The non-invasive methods are more considerable as integrity of embryo is not damaged i.e. embryos remain intact and viable (Utsumi and Iritani, 1993).

Non-invasive technique can assess the normal growth of embryo but specificity of determining sex of embryo is low (Sharma *et al*. 2017). Though invasive techniques are less considerable due to chances of damage of embryo but more accurate technique to identify the sex of embryo is Y chromosome specific probe. Molecular biology has introduced more rapid and reliable techniques for embryo sexing like polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH).

Methods of embryo sexing can be categorized as:

**I. Invasive methods**

A. Cytological method or Karyotyping

B. Identification of sex chromatin

C. Y chromosome specific DNA probes

D. Polymerase chain reaction (PCR)

E. Loop mediated isothermal amplifications (LAMP)

F. Fluorescence *in situ* hybridization (FISH)

**II. Non–invasive method**

The embryo is not subjected to any harm throughout the procedure

A. Detection of X-linked enzymes

B. Detection H-Y antigens

C. Sexing based on cleavage and development

**I. Invasive methods**

**Cytological method or Karyotyping**

Karyotyping is done using cells at metaphase stage. Some cells are removed from embryo and cultured with colchicines that causes cells to stop dividing at the metaphase stage of mitosis.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% (Seidel, 1999).

**Advantages**:

* Accuracy of the karyotyping method is high
* it requires no sophisticated equipment
* it is inexpensive and easy to perform
* it can identify chromosomal abnormalities before the embryos are transferred (Kitiyanant et al., 2000).

**Disadvantages**

* The procedure is labour intensive. Approximately, five hours are needed for two experienced cytogeneticists to process 12-15 embryos.
* These techniques may cause accidental harm to the survived chromosome was used to distinguish between male and female cells embryo
* Reduce the viability and conception rate of embryos

**B. Identification of sex chromatin**

Identification of sex chromatin depends on the presence of “Barr body”, a dark stained moiety, near to the nucleus in a cell. Barr body formations results from inactivation one of the X chromosome present in female cell not from male cells. Barr and Bertram in 1949 have identified the condensed inactive X chromosome or Barr body in female nucleus in 1949. Demonstration of sex of embryo by evaluating sex chromatin of Rabbit trophoblastic cells was performed by Edward and Gardner (1958).

**Advantages:** This is a simple and rapid technique

**Disadvantages:**

* Barr body may not present in all cells.
* Granular cyctoplasm sometime prevents the detection of Barr body.
* This method is not suitable for cattle, sheep, goat, pig and horse due to coarse nature of the chromatin (Betteridge et al., 1982).

**C. Y chromosome specific DNA probes**

This technique is one of the most accurate method of determining the male sex embryo by presence of Y chromosome. It involves collection of small number of cells the embryo using micro or biopsy blade with proteinases to expose the DNA then hybridized with radioactively labeled Y-chromosome specific probe. By using biotinylated Y-chromosome specific probe, sexing of bovine embryo can be identified within30 minutes (Leonard *et al*., 1987).

**Advantages**

* Highly accurate and a higher percentage of embryos can be sexed.

In this method, from a little quantity of DNA sample fetal sex can be identified

* Fluorescence in situ hybridization (FISH) with a DNA probe for Y-chromosome can be used to distinguish between male and female cells (Cotinot *et al*., 1991)

**Disadvantages**

* It is a quite expensive, complicated and time consuming process.
* Collection of biopsy material from embryo is not accessible all the times. (Vliet *et al*.,1989).

**D. Polymerase chain reaction (PCR)**

At present, it is method of choice for predicting fetal sex using DNA fragment from maternal plasma (Da Cruz *et al.* 2012). Flushed embryos from super ovulated donors can be used for the determination of sex to facilitate the application of embryo transfer to manage sex ratio at farm level. The method of sexing of embryo in bovines by amplifying particular DNA sequences of Y-chromosome using PCR proves an effective tool to influence the sex ratio. Embryo sexing using PCR includes biopsy of embryo (1-4 blastomeres), amplification of DNA fragments (one species specific and one male specific) and interpretation after analysing the amplified products with electrophoresis. the first demonstrated sexing of goat embryo with PCR amplified DNA from blood sample by Aasen and Medrano (1990). Leoni *et al*. (1996) were the first to describe a method for sex determination in goat embryos, using PCR and restriction fragment length polymorphism (RFLP) analysis. PCR sexing procedures based on the amelogenin gene is highly reliable and suitable for sex determination of goats (Chen *et al*., 2007).

**Advantage**

* It is a sensible, accurate and reliable method.
* Less damage ti embryo while collection of sample as very little quantity is needed for PCR.
* It can be used for genotyping and testing of genetic diseases as well.

**Disadvantages**

* It requires high technical skill and time consuming.
* Sometime PCR may give false positive result due to contamination of DNA.

**E. Loop mediated isothermal amplifications (LAMP)**

In PCR, a target DNA sequence is amplified by a temperature change between 50 to 95 0 C, whereas a new isothermal DNA amplification technique has been developed. Loop nediated isothermal amplification LAMP is a DNA amplification method that can amplify a specific DNA sequence within the range of 60 to 650C. Field application of LAMP based embryo sexing has been attempted (Hirayam *et al*., 2004). LAMP can amplify a target sequence within about 15 mins. LAMP based sexing procedure have sufficient sensitivity and accuracy for cattle embryo sexing.

**Advantages**

* Rapid, sensitive method for field application.
* LAMP does not need electrophoresis to detect amplified DND products.
* Less chances of damage of embryonic tissues.

**Disadvantages:** Expensive and high technical knowledge requires operating the technique

**F. Fluorescence *in situ* hybridization (FISH)**

The technique fluorescence *in situ* hybridisation (FISH) can detect specific DNA sequences of individual chromosomes from a cell (Kobayashi *et al.* 2004). This method can be used not only to predict the sex of embryo but also detect the chromosomal mosaicism and aneuploidy in embryos (Griffin *et al.* 1992; Delhanty *et al.* 1993). Unlike to PCR, the risk of contamination of sample is negligible in FISH technique (Sharma *et al.* 2017). By using DNA probe specific to Y chromosome in fluorescence *in situ* hybridization (FISH) male and female embryos can be differentiated (Cotinot *et al.* 1991). Cenariu *et al*. (2011) reported the accuracy of the FISH method of bovine embryo sexing is 86.66%.

**Advantages:**  It is highly sensitive and accurate embryo sexing technique compared to PCA.

**Disadvantage:**  It is complicated, expensive and time consuming method.

**II. Non–invasive method**

**A. Detection of X-linked enzymes**

The female has two X chromosomes whereas the male has only one X chromosomes in somatic cells and hence the enzymes associates with X chromosomes produced in female are almost double than that produced in the males. These enzymes are Glucose-6- phosphate dehydrogenase (G6PD), Hypoxanthine guanine phosphoribosyl transferase (HPRT), Phosphoglycerate kinase, Agalactosidase. These enzymes are measured in embryos. The high concentration of enzyme usually denotes two X chromosomes or female embryo. While low concentration denotes one X chromosomes or male embryo. (Monk and Handyside, 1988). In bovine embryos, glucose and glutamine metabolism has been studied by Tiffin *et al*. (1991) and showed greater metabolism of glucose and glutamine in female embryos than male embryos.

**Advantages**

* Allowing all embryos to be sexed.
* Accuracy is almost 90 percent for female and 100 percent for males sex

**Disadvantage**

* Estimation has to be done for very small quantity enzymes.
* Chances of false diagnosis due to variations.
* This test may toxic to embryo.

**B. Detection H-Y antigens**

The immunological demonstration of a sex specific antigenprovides another non-invasive method of sexing embryos. 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. It has been shown to be expressed by pre-implantation embryos of all mammalian species including the mouse, rat, pig, sheep, goat, cow and horse. The assays for H-Y antigen are approximately 85% accurate in identification of embryonic sex (Anderson, 1987).

**Advantages:** It is a rapid test and no need of biopsy of embryo.

**Disadvantages:** Lengthy process of of embtyo may reduce the conception rate and unavailability of fluorescence microscope.

**C. 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 (Sharma *et al.* 2017).

**Advantages:** Accuracy and sensitivity is very high

**Disadvantages:**

* Need high skills.
* Cleavage time cannot be predicted.
* May decrease the embryo viability.

**Conclusion and future possibilities**

There are various methods of embryo sexing each with advantages and disadvantages. Among all the methods, Polymerase Chain Reaction (PCR), LAMP and FISH are most simple, efficient, highly reliable and accurate procedure for sexing embryos. In a Multiple Ovulation Embryo Transfer (MOET) nucleus breeding scheme, predetermination of sex could either increase the ratio of female to males thereby increasing the accuracy of selection or reduce the number of males produced thereby reducing the cost of MOET. Embryo sexing also helps in conservation of endangered wildlife species and taking proper breeding policy at zoo. It also helps in production transgenic animals and cloning. There are some more studies have been done to differentiate the male and female embryo development based on which more precise method to predict the embryonic sex can be developed. The sexing of embryo might be possible on the basis of difference in hormone profiling of fetus of different sex. In pig and horse, embryonic sexing can be predicted by concentration of estrogens and androgens in blastocoels (Sharma *et al.* 2017). In bovine embryos, there are some sexes specific mechanisms regulating the signalling events of implantation as Larson *et al*. (2001) suggested the higher production of signalling factor like interferon tau in female embryo. Similarly, male embryos have faster development when exposed to higher serum concentrations of glucose *in vitro* (Bredbacka and Bredbacka, 1996; Gutierrez-Adan *et al.* 2001). All these methods are not well established and need more explorations to improve their efficacy and accuracy. Livestock industry needs the techniques of embryo sexing for commercial benefits and as more successful methods will be available, their demand will also be supposed to be increased.

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