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Review Article

# Embryo sexing methods in bovine and its application in animal breed

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**ABSTRACT** The ability to determine the sex of bovine embryos before the transfer is advantageous in livestock management, especially in dairy production, where female calves are preferred in milk industry. The milk production of female and male cattle benefits both the dairy and beef industries. Pre-implantation sexing of embryos also helps with embryo transfer success. There are two approaches for sexing bovine embryos in farm animals: invasive and non-invasive. A non-invasive method of embryo sexing retains the embryo's autonomy and, as a result, is less likely to impair the embryo's ability to move and implant successfully. There are lists of non-invasive embryo sexing such as; Detection of H-Y antigens, X-linked enzymes, and sexing based on embryo cleavage and development. Since it protects the embryo's autonomy, the non-invasive procedure is considered to be the safest.

Invasive methods affect an embryo's integrity and are likely to damage the embryo's chances of successful transformation. There are different types of invasive methods such as polymerase chain reaction, detection of male chromatin Y chromosome-specific DNA probes, Loop-mediated isothermal amplification (LAMP), cytological karyotyping, and immunofluorescence (FISH). The PCR approach is highly sensitive, precise, and effective as compared to invasive methods of farm animal embryonic sexing. Invasive procedures, such as cytological karyotyping, have high accuracy but are impractical in the field due to embryonic effectiveness concerns. This technology can be applicable especially in the dairy and beef industry by producing female and male animals respectively. Enhancing selection accuracy and decreasing the multiple ovulation embryo transfer costs.

**Keywords:** animal breeding, bovine, DNA probes, embryo sexing, PCR

## INTRODUCTION

The ability to determine the gender of bovine embryos before the transfer is useful in livestock management, especially in dairy production, where female calves are favored. 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). Pre-implantation sexing of embryos also assists in the effectiveness of embryo transfers. In farm animals, there are two methods for sexing bovine embryos: invasive and non-invasive. The non-invasive treatment is considered the best since it preserves the embryo's autonomy. The invasive method does not protect the autonomy of an embryo and is likely to affect the embryo's chances of successful transfer. The milk quality and meat of both female and male cattle

benefits from the dairy and beef industries. It is therefore crucial to create animals of the right sex, which can be done by pre-determining the sex of the concept at the time of conception, i.e., sex pre-determination can be economically relevant (Sachan et al., 2020). The effect of determining the sex of pre-implanted embryos is significant for livestock treatment, reproduction, and fetal diagnosis (Wakchaure et al., 2015). Sexing of embryo pre-implantation plays a role in improving the success of embryo transfer efficacy by allowing the transfer of the desired embryo depending on their sex (Cenariu et al., 2008; Sachan et al., 2020). There are two techniques for sexing bovine embryos in agricultural animals: invasive and non-invasive.

Invasive methods like DNA probes (Y-chromosome specific, Polymerase chain reaction (PCR), Loop-mediated isothermal amplification (LAMP) and Flurescenc insitu hybridization (FISH) are some of the invasive methods. There are also non-invasive methods in this case embryo cannot be harmed during the process. From non-invasive techniques like: Detection of X-linked enzymes, H-Y detection antigens, and sexing based on cleavage and development. Therefore, the objective of this review is to discuss different techniques which pre-determine the embryo sex.

## OBJECTIVE

The objective of this seminar was to review the methods of bovine embryo sexing and its applications in animal breeding.

## EMBRYO SEXING METHODS IN BOVINE SPP

### Invasive methods

#### 1) Cytological methods

This approach could be used to determine the gender of such embryo during the mitotic genome's metaphase stage based on the availability or absence of the Y or X chromosome (Wakchaure et al., 2015; Sachan et al., 2020). At this point, individual cells from the embryo are gathered and cultured with colchicine, which inhibits cells from separating during the metaphase stage of mitosis. The cells are then osmotically lysed, and the preparation is set and stained. The metaphase chromosome in females or one Y chromosome in males can be measured microscopically (Wakchaure et al., 2015). Karyotyping has the potential to detect some gross chromosomal abnormality as well as aneuploidies, defects caused by variation in the number of chromosomes among species.

The advantages of karyotyping is less expensive than other techniques, is easy to work with high precision, and does not require the procurement of costly equipment (Kitiyanant et al., 2000; Wakchaure et al., 2015). Besides, this method will sex fewer animals, allowing for fewer metaphasic plates to be prepared for adequate metaphase chromosome dispersion. The disadvantages of this treatment includes the fact that it is time-consuming, labor-intensive, and has a low success rate due to poor metaphasic chromosomal dispersion (Sharma et al., 2017), but it also reduces embryo survivability, viability, and pregnancy risk, and needs well-trained personnel (Wakchaure et al., 2015).

#### 2) Polymerase chain reaction

Sex isolation of home animal is critical for animal breeders to efficiently manipulate their breeding stocks (Tavares et al., 2016). It is now the standard approach for identifying fetal sex using DNA fragments from maternal plasma (da Cruz et al., 2012; Sachan et al., 2020). The sex can be determined using PCR with embryo flushed from super ovulated donors and controlling the sex ratio at the farm level to facilitate embryo transfer. A blastomere biopsy from the pre-implantation embryo is required for sex. This method is used to design the primer and amplify the Y-chromosome-based DNA chain (Lakshmy et al., 2018). The advantages include an approach that is extremely responsive, accurate, dependable, and efficient, and also a consistent pregnancy rate. Considerable many embryos can be sexed using this technique (Malik et al., 2013). Embryo biopsy can be used to perform multiplex genotyping on bovine embryos as well as genetic tests for inherited diseases (Peippo et al., 2007). The disadvantage is the need for trained staff with technical experience and time-consuming skills are required, and there are risks of false positives due to DNA contamination during PCR and electrophoresis processes. This method of sexing an embryo entails three steps. Biopsy of an embryo (1-4 blastomeres), amplification of 2 DNA fragments (one species-specific and one male-specific), and analysis of amplified products and interpretation.

Embryo biopsy: Embryos for sexing are obtained on

day 6.5 after the 1st artificial insemination. Only embryos that have been rated as excellent or strong from compact morula to early blastocyst are biopsied using a micromanipulator. Amplification: involves a series of cycles (template denaturation (94-97℃) for 90 sec., primer annealing temperature 50-72℃ for 90 sec., and extension of the annealed primer at 72℃ for 180 sec, by Taq DNA polymerase).

PCR based sex isolation of bovine embryos produces better outcomes than FISH. The PCR has the invaluable advantage of becoming faster than previous processes. This advantage enabled embryos to be transferred to a female recipient without the need for cryopreservation. However, due to the strict thermal control needed for primer annealing and DNA synthesis, as well as the possibility of false positives due to DNA contamination, PCR is not an easy technology for embryo sexing in the field (Mohammed and Hozab, 2016).

3) Male specific DNA probes (Y-chromosome)

To establish the existence or absence of a certain Y-chromosome DNA sequence that identifies a male embryo, the most accurate methodology for sexing embryos can be utilized (Akiyama et al., 2010; Sachan et al., 2020). The blastocyst biopsy material can be retrieved without harming trophectoderm cells' inner cell masses. Micro sectioning or microblade biopsy is the most often used embryo biopsy technique (Sachan et al., 2020). Proteinases are used to expose the DNA in a very small number of cells from the fetus, which are then hybridized with a radioactively labeled Y-chromosome-specific probe. The existence of the Y-chromosome, and thus the male sex of the chromosome, is indicated by positive hybridization findings. The Y-chromosome-specific DNA probe technique not only needs relatively little material to prepare DNA but also has no harmful effects on the embryo (Sharma et al., 2017).

This procedure is attractive since it needs only a limited number of embryo materials for DNA preparation and detects sex with 100 percent accuracy (Cotinot et al., 1991; Lakshmy et al., 2018). The strengths of this strategy are that fetal sexing can be estimated with just 20 ng of DNA, it is more accurate, and it can sex a greater number of embryos. To differentiate between male and female cells, fluorescent in situ hybridization (FISH) with a Y-chromosome DNA probe is used. This technique has several drawbacks, including the fact that it is complicated, costly, and time-consuming, as well as being limited by the lack of embryonic material for biopsy (Wakchaure et al., 2015).

4) Sex determination based on loop-mediated isothermal amplifications (LAMP)

LAMP is a DNA multiplication method which can duplicate a specific DNA sequence at temperatures as low as 60℃ (Kageyama and Hirayama, 2012). A fastest way of sexing a cow embryo can be employed instead of PCR loop-mediated isothermal amplification. To amplify DNA in isothermal conditions, a DNA polymerase and four simple DNA primers for DNA synthesis, as well as a group of loop primers with the LAMP reaction, can be used (Nagamine et al., 2002; Hirayama et al., 2004; Sachan et al., 2020). Inner and outer primers can be used to create spindle Nucleic acids, which can subsequently be amplified by an auto-cycling process. (Hirayama et al., 2013). LAMP will amplify a target series in around 15 minutes in the best-case scenario. Furthermore, since the target sequence produces a white precipitate of magnesium pyrophosphate (a byproduct of DNA synthesis), LAMP-mediated DNA amplification can be characterized by calculating the turbidity of the reaction solution (Kageyama and Hirayama, 2012). LAMP does not require electrophoresis to detect amplified DNA pieces. These features indicate that LAMP, rather than PCR, would be a safer alternative for in-field DNA analysis (Fig 1).

5) Techniques based on fluorescence in situ hybridization

In a metaphase or interphase cell stage, immunofluorescence method is a tool for analyzing complex DNA sequences from individual chromosomes using fluorescent in situ hybridization (FISH) (Sachan et al., 2020). It is a sensitive method for single-cell chromosome molecular diagnosis that has been used successfully for embryo sexing. Animal breeders are becoming increasingly involved in pre-selection before reproduction, especially in bovine-chromosome sexing of cattle fetuses from biopsied blastomeres using specialized DNA probes that target specific chromosomes (Lee et al., 2004; Singh et al., 2017). This method, in comparison to PCR, has a lower chance of sample contamination (Sharma et al., 2017). In fluorescence in situ hybridization (FISH), male and female embryos may be isolated using a DNA probe specific to the

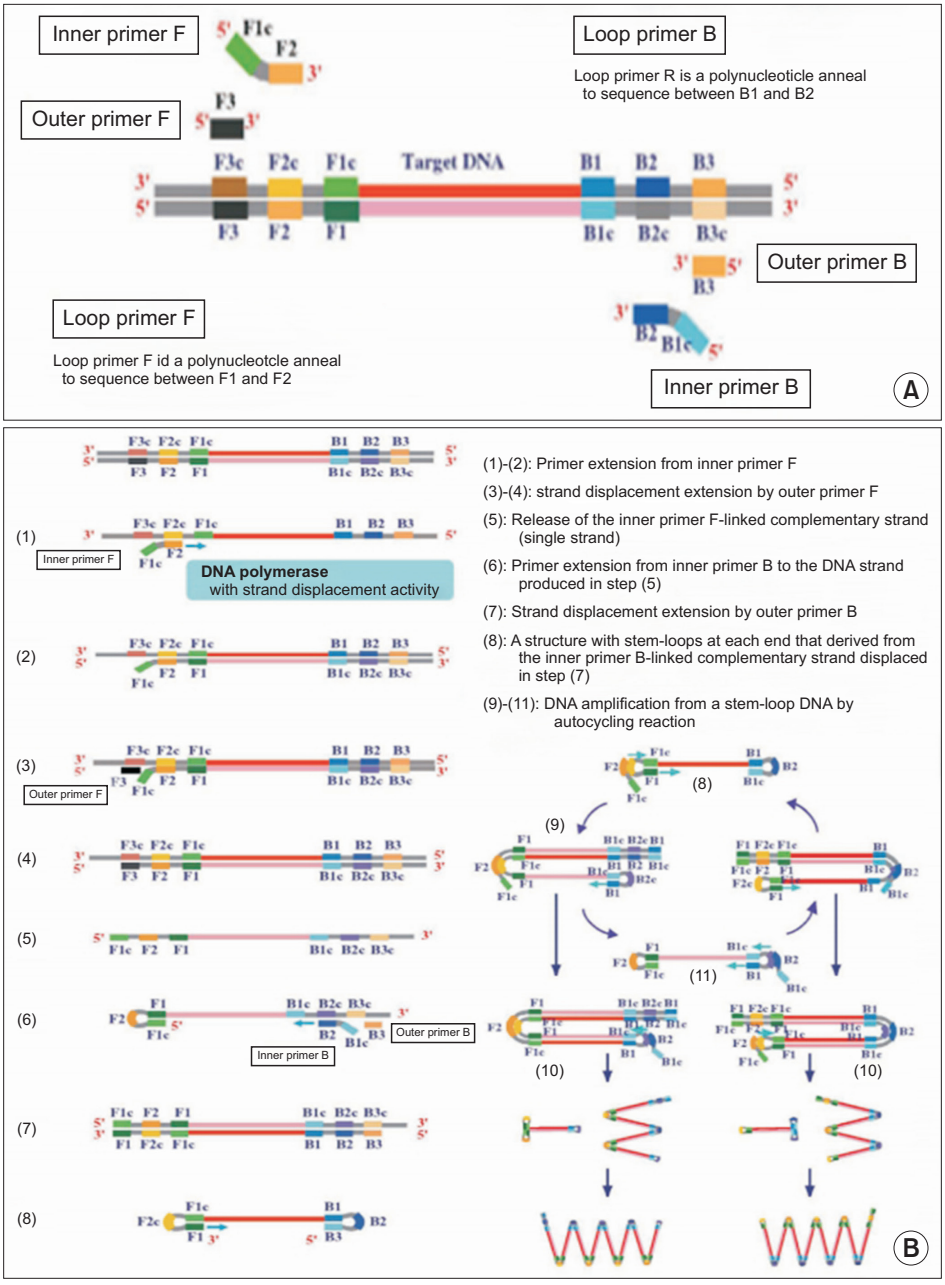


Fig. 1. (A) Loop primer Design (Hirayama et al., 2013). (B) LAMP-based bovine embryo sexing.

Y chromosome (Cotinot et al., 1991; Sachan et al., 2020). When determining the sex of an embryo, FISH can detect mosaicism and aneuploidy (Sharma et al., 2017).This protocol involved the following steps:-blastomere rehydration with diminishing ethanol concentrations; target retrieval with heat and sodium citrate buffer; blastomere digestion with Triton X and the following measures will be included in the fish protocol: blastomere rehydration with declining ethanol concentrations; target retrieval with heat and sodium citrate buffer; -blastomere digestion with Triton X and proteinase K; blastomere fixation with paraformaldehyde; application of in situ frames; application of the hybridization buffer with a 1 percent DNA probe; hybridization at 94℃ for 6 minutes and 37℃ for 16 hours; washing of slides in three PBS baths (Cenariu et al., 2008; Singh et al., 2017). proteinase K (used to digest proteins and removes contamination); blastomere fixation with paraformaldehyde; application of in situ frames; application of the hybridization buffer with a 1% DNA probe; hybridization reaction using the thermocycler using the following

hybridization scheme: 94℃ for 6 minutes and 37℃ for 16 hours; -the washing of slides in 3 baths of PBS (Cenariu et al., 2008; Singh et al., 2017).

6) Sex chromatin identification methods

The inactive x chromosomes, also known as the gender chromatin of female somatic cells, can be the dormant X chromosome. Euchromatin is place where the female chromosome will be wrapped, while the inactive x chromosomes are packaged in heterochromatin. This means that, because both X chromosomes contain the same gene content, the inactive female X chromosome become compact and unavailable to molecules involved in transcription, while the active X chromosome has a larger volume and is more dispersed, or available, allowing transcription to occur (Biogydictionary.net Editors, 2017).

During a mounted cell, a dark staining body termed a Barr body is located near to the nuclear membrane, which is used in the chromatin approach of sex choice. The dormant X chromosome in female somatic cells is known also as Barr body, sometimes known as intercourse chromatin. While one of the X-chromosomes in feminine cells is inactive, it prevents our bodies from taking shape. The x chromosomes are made up of two haplotypes (Wakchaure et al., 2015). Only the cells from a female embryo, not from the male one, are expected to include the Barr body. The existence and identification of Barr bodies are based not only on the cell stage but also on the fixing procedure, i.e., an incorrect cell stage or an unsuccessful fixing and staining technique, resulting in an incorrect diagnosis for embryo sexing (Wakchaure et al., 2015; Sachan et al., 2020). Another drawback to this strategy is that the embryo experiences considerable harm as a result of the need for a vast number of cells.

## Non-invasive method of embryo sexing

A non-invasive technique is favored because it retains the embryo's autonomy and, as a result, is less likely to impair the embryo's ability to move and implant successfully. Non-invasive technologies include x-related enzyme synthesis before X chromosome inactivation and embryo immunoreactions of sex-specific antigen antibodies (Lakshmy et al., 2018). Sexing is preferred because it preserves the embryo's integrity, lowering the likelihood of a failed embryo transfer.

1) X-linked enzymes

In mammals, there are two X-chromosomes for homogametic sex and one for heterogametic sex. In X-linked enzymes, the genic dosage is used to distinguish male and female embryos. To guarantee that the sexes have the same amount of genes, which is the one X-chromosomes in the female cell, is not functioning during the development of each cell. Although the precise timing of X-inactivation remains unknown, experiments have shown that when a brief time between the activation of the embryonic genome and X-inactivation during which the genes in both x chromosomes in the female are transcribed (Sharma et al., 2017).

This shows that in the cell abundance and function of certain X-related enzymes being doubled in both female and male embryos. In both cases, the action of X-linked enzymes must be seen in terms of human differences in embryo metabolism as opposed to autosomal enzymes. For e.g., some enzymes. X-chromosome-related enzymes include glucose-6-dehydrogenase phosphate (G6PD), hypoxanthine phosphoribosyl transferase (HPRT), and phosphoglycerate kinase (Wakchaure et al., 2015; Sachan et al., 2020). Females evolve more quickly than Y-chromosome (Male); this is why males have only one X-chromosome, while females have two. Embryos can be classified depending on their concentration.

2) H-Y antigens detection

Incubated Embryos with antibodies for 30-60 minutes, will be followed by another 30-60 minutes for the first antibody containing a fluorescent dye. H-Y antigen is readily detectable on morulae and is expressed as early as the 8-cell stage. At the blastocyst stage, detection becomes increasingly challenging (Wakchaure et al., 2015). A cytotoxicity assay and an immunofluorescence assay are two methods for detecting H-Y antigens on embryos. Embryos are exposed to H-Y antiserum and complemented in the cytotoxicity assay. Embryos that express the H-Y antigen exhibit cell lysis and are thus labeled as male. The survival rate of embryos in this phase is poor, which is the technique's main disadvantage. Immunofluorescence assay: Embryos are subjected to primary H-Y antibody after 60 seconds, then to a secondary antibody reaction containing fluorescein isothiocyanate (FITC). The embryos are then checked to check if a fluorescent microscope FITC sticker is present (Lakshmy et al., 2018).

Because H-Y antigen is a relatively weak antigen to detect through antigen-antibody reactions, secondary antibodies sometimes show non-specific binding, H-Y antigen may not be limited solely to male embryos, and, finally, subjectivity is involved in the assessment of sex, the accuracy of these two immunological sexing assays may not appear to exceed 90%.

3) Sexing by embryo cleavage and development

Male embryo cells cleave and mature at a faster rate than female embryo cells to reach the morula and blastocyst levels. The DNA content of male embryonic cells is proportionally lower than that of female embryonic cells. More DNA means more time spent duplicating it, resulting in a longer cell cycle. This is supposed to have an impact on the rate of cleavage and growth of male and female embryos. Male embryos are thought to cleave earlier and mature faster to reach the morula and blastocyst stages than female embryos (Sharma et al., 2017; Sachan et al., 2020). However, there are certain disadvantages to using this technique for embryo sexing: The cleavage time of the created embryo cannot be established, the difference in development speed is little, and the separation of rapid and slow embryos necessitates significant skills. While DNA probing is undeniably the most reliable method of sexing embryos, its commercial use is limited since each embryo must be probed individually, necessitating professional micromanipulation. It is also an invasive procedure and has been linked to lower embryo viability in several studies (Sharma et al., 2017).

## APPLICATIONS OF EMBRYO SEXING IN FARM ANIMALS

A pre-implantation of sex during multiple Ovulation of Embryos Transfer (MOET) nucleus breeding techniques could either increase the number of females or males, enhancing selection accuracy, or decrease the number of males born, decreasing MOET costs (Wakchaure et al., 2015). Since the economic advantage of each livestock species differs by sex, sex predetermination is economically essential. It is important for animal control and reproduction, as well as prenatal genetic disease diagnosis (in humans). Farmers are allowed to run fewer recipient females, rapidly increasing the size of their flock, due to embryo sexing. The significance of female preselection in endangered species conservation cannot be overstated. Pre-selection of sex becomes needed in any animal breeding technique by embryo transfer, helping procedures to focus on genetic improvement on their male or female lines through better use of recipient females (Sharma et al., 2017). More heifer progeny from genetically valuable females as a herd supplement for milk production, thus preventing freemartins' multiple births. Knowing the sex of bovine offspring is critical for livestock production and the genomic advancement of cattle. For the genetic regulation of sex-linked diseases in humans. In wildlife management, as a survival technique for an endangered species, as breeding programs in zoos, transgenic animals, and cloning (Maxwell et al., 2004; Wakchaure et al., 2015).

## CONCLUSION

The methods to determine the gender of bovine embryos before a transfer is beneficial in farm animal management, especially in dairy production, where embryo sexing is helpful to promote livestock development productivity. Especially, both the dairy and beef sectors profit from the milk processing of both female and male cattle. It is mainly achieved by two methods: invasive and non-invasive. In contrast, the non-invasive procedure outperforms the invasive method. Since invasively sexed embryos cannot do better in terms of implantation than non-invasively sexed embryos. However, where embryos are sexed using an invasive technique rather than a non-invasive process, the precision of sex determination is higher, or nearly so. As a result, whether embryos are sexed using an invasive or non-invasive technique is primarily determined by the embryo's needs. Generally, bovine embryo sexing allows for the allocation of desirable sex based on the producer's needs to mitigate damage caused by the culling of animals of the undesired sex. These sexing methods have applications far beyond the livestock industry.

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