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Methods of Embryo Sexing in Cattle Breeding: A Review

Review Article

V. Sachan[1*], B. Kumar[2], J. Kumar Agrawal[1], A. Kumar[1] and A. Saxena[1]

[1] Department of Veterinary Gynecology and Obstetrics, College of Veterinary and Animal Science, Deen Dayal Upadhyaya Veterinary Science University, Mathura, Uttar Pradesh, India
[2] Department of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, India

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*Correspondence E-mail: vikassachan@duvasumathura.com

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ABSTRACT

Different livestock sectors as beef industries get benefit from the production of male animals while dairy industries get benefit from the milk production by the female animals. Therefore, it is obvious need to produce the animals of desired sex which can be achieved by predetermining the sex of conceptus at the time of conception i.e. pre determination of sex may be of great economic importance. 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 not only improves efficiency of embryo transfer but also facilitate the transfer of embryos of desired sex. Sex-sorted sperm is a one of the technique fulfilling the requirement but it is well expensive and less efficient. Another concept of getting genetically improved animals of desired sex is embryo sexing. Embryo sexing has great potential to maximize the efficiency of dairy production through controlling the sex ratio of domestic species. There are many methods to determine the sex of embryo categorized as invasive and non-invasive techniques with varying efficiency and merits.

KEY WORDS embryo sexing, fluorescent *in situ* hybridization, karyotyping, loop, PCR.

INTRODUCTION

Different livestock sectors as beef industries get benefit from the production of male animals while dairy industries get benefit from the milk production by the female animals. So, it is obvious need to produce the animals of desired sex which can be achieved by predetermining the sex of conceptus at the time of conception i.e. predetermination of sex may be of great economic importance. 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. Preimplatation 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). Use of sex-sorted sperm is a powerful technique to get desired sex through artificial insemination or *in vitro* fertilization (IVF) but it is well expensive (Seidel Jr, 2007) and less efficient compared to conventional, unsorted semen (Trigal *et al.* 2012).

The use of technique for examining the chromosome in cells from bovine embryo introduced the embryo sexing, a new approach to preselect the sex of conceptus. There are so many other techniques for sexing the embryo. Embryo sexing, if done before transferring the embryos, potentiate the use of embryo transfer technique for production of

genetically improved animals with particular sex at faster rate.

Methods for 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. A lot of work has been done to find out the suitable and easily performing method for embryo sexing with least damage to embryo and high accuracy (Lee *et al.* 2004; Shea, 1998). Several 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).

It may ensure optimum and normal embryonic development. Non-invasive sexing, however, is less accurate as invasive techniques (Sharma *et al*. 2017). High accuracy is found to be with cytogenetic analysis and the use of Y specific probes, but these are invasive methods. 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:

A. Invasive methods

1. Cytological method or Karyotyping
2. Identification of sex chromatin
3. Y chromosome specific DNA probes
4. Polymerase chain reaction (PCR)
5. Loop mediated isothermal amplifications (LAMP)
6. Fluorescence *in situ* hybridization (FISH)

B. Non–invasive method

The embryo is not subjected to any harm throughout the procedure

7. Detection of X-linked enzymes
8. Detection H-Y antigens
9. Sexing based on cleavage and development

Cytological methods or karyotyping

Cytogenetic sexing, or karyotyping is the analysis of genomic framework of a cell. In domestic animals, this technique can be used to identify the sex of the embryo on the basis of presence of the X or Y chromosome in mitotic genome at metaphase stage. Some blastomere cells are taken from 6-8 day old embryo and their division is checked at metaphase stage of mitosis by culturing with mitosis arresting agents like colchicines (Wakchaure *et al.* 2015).

Colchicine binds to tubulin and stop microtubule polymerization thus acts as a spindle poison or mitotic poison. Colcemid (also known as democolcine) is another agent which is closely related to natural alkaloid colchicine but is less toxic. It also depolymerises microtubules and inactivate the spindle formation thus can also be used as mitosis inhibitor (Sharma *et al*. 2017). Cells are subjected to a hypotonic solution in order to lyse them osmotically so that the chromosomes can be dispersed. A permanent staining agent as Giemsa is then used to stain the DNA so that metaphasic chromosomes can be analyzed microscopically for two X chromosome confirming female or one Y chromosome confirming male. Karyotyping also enables one to diagnose any gross abnormality in chromosomes as well aneuploidies, diseases caused by variation in the number of chromosomes species wise. Hare *et al.* (1976) and Wintenberger-Torres and Popescu (1980) described the embryo sexing by karyotyping the trophoblast. Picard *et al.* (1984) has reported to determine the sex of 60% embryos by bisecting and culturing the embryo.

This procedure is less expensive, require no sophisticated instrumentation and can easily be executed with high accuracy (Kitiyanant *et al*. 2000). On other hand lesser embryos can be sexed by this technique because lesser number of metaphasic plates can be prepared with proper dispersion of metaphase chromosomes (Picard *et al*. 1985).

This technique is not only time consuming, labour intensive with poor success rate due to poor metaphasic chromosomal dispersion (Sharma *et al.* 2017) but also reduces the survivability of embryo (Wakchaure *et al.* 2015).

Identification of sex chromatin

Presence of "Barr body", a dark stained moiety, near to the nucleus in a cell during sex chromatin examination can be used to predetermine the sex of the embryo. Inactivated one of the X-chromosome in female cell forms the Barr body i.e. only the cells from female embryo, not from male one, are expected to have the Barr body. Barr and Bertram (1949) have identified the condensed inactive X chromosome or Barr body in female nucleus in 1949. Edwards and Gardner (1967) stained embryonic trophoblastic cells with aceto-orcein and demonstrated the embryo sexing based on presence or absence of Barr body by evaluating sex chromatin in rabbit blastocysts. Although the procedure is quite simple to perform but because of granular nature of chromatin, it is very difficult to observe the Barr body in cells from livestock species as cattle, goat, horse and pig etc (Betteridge *et al*. 1982). Presence and the detection of Barr body depends not only the stage of the cell but also depends on the fixing procedure i.e. improper cell stage or unsuitable fixing and staining procedure may give the false diag-

nosis for embryo sexing (Wakchaure *et al.* 2015). Another limitation of this technique is that because of need of large number of cells, the embryo damage is considerable (White, 1989).

Y-chromosome specific DNA probes

Some Y-linked gene transcripts are present only in the male embryo which are useful sex markers e.g. sex-determining region Y (SRY) (Hamilton *et al.* 2012). The most efficient method for sexing the bovine embryo is to find out the presence or absence of a Y-chromosome specific DNA sequence which indicates a male embryo (Akiyama *et al.* 2010; Cenariu *et al.* 2008).

The presence of these Y-chromosome specific DNA is investigated by using the Y-chromosome specific labeled DNA probes. Several Y-chromosome specific DNA probes for bovines have been reported (Leonard *et al.* 1987; Bondioli *et al.* 1989; Herr *et al.*1990).

The biopsy material for blastocyst should be taken from trophectoderm cells without disturbing inner cell masss. Micro sectioning or microblade biopsy is most popular technique for embryo biopsy. Small biopsy material tends to more loss while manipulations, while large size biopsy material compromises the embryo viability so biopsy material 10-30% of cell mass may be considered compatible for optimum pregnancy rate with highly efficient embryo sexing.

Few numbers of cells are biopsied from embryo and after exposing the DNA with help of protienases, hybridization of this biopsied cell DNA with radiolabeled DNA probe specific to Y-chromosome occurs. If hybridization occurs, it indicates the sex chromosome is of male embryo (Wakchaure *et al.* 2015).

In Y-chromosome specific DNA probe technique, not only the requirement of material to prepare DNA is very less but also embryo is not adversely affected (Sharma *et al.* 2017).

The biotinylated Y-specific probe facilitate the embryo sexing in bovines embryos within 30 hours (Leonard *et al.* 1987). Whether DNA probing is invasive method and it requires skillful micromanipulation of the embryo, it is one of most precise way for embryo sexing (Sharma *et al.* 2017).

Higher percentage of embryos could be sexed with this method than with karyotyping since cells need not be in metaphase.

Polymerase chain reaction (PCR) method

In the early 1990, Kary B. Mullis invented the PCR by which one can make millions of copy of a DNA sample. Use of PCR created new possibilities for embryo sexing (Mara *et al.* 2004).

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 superovulated 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 co-amplification of a Y-chromosome specific gene (Sry) and an autosomal gene (e.g. Aml-X) is done in PCR amplification to yield different fragment sizes of Y-chromosomal and autosomal products. The amelogenin gene (Aml), meant for tooth enamel matrix protein, locates on both X- and Y-chromosomes (AMELX and AMELY) (Kouamo amd Kharche, 2014). Since single pair of primers is needed to amplify the different fragments of the amelogenin genes (Weikard *et al.* 2006) which makes sex prediction easier. On electrophoresis, the presence of Y-chromosomal fragment indicates male and its absence indicates the female sample, while autosomal fragment is present in both the samples. The autosomal gene acts as internal control for the presence of biopsy and suitable conditions of PCR. Some time it is called as duplex PCR as both fragments are co-amplified in the procedure.

The use of PCR is most reliable and simple (Malik *et al.* 2013) method of sexing embryos in bovine (Herr and Reed 1991; Schroder *et al.* 1990; Peura *et al.* 1991; Chen *et al.* 1999), sheep and deer (Pfeiffer and Brening, 2005; Dervishi *et al.* 2008), goats (Weikard *et al.* 2006; Malik *et al.* 2013), pigs (Pomp *et al.* 1995), horses (Peippo *et al.* 1995) and mice (Han *et al.* 1993) and in other related species (Weikard *et al.* 2006).

Whether use of PCR requires technical skill for sexing embryo, this method is nearly 100 % accurate (Kouamo and Kharche, 2014), sensitive, and fast (Gokulakrishnan *et al.* 2013; Ekici *et al.* 2006) as can be carried out within few hours (Bredbacka *et al.* 1995). Good percentage of embryos without disturbing their developmental capacity can be sexed with this technique (Schroder *et al.* 1990; Peura *et al.* 1991; Saiki *et al.* 1989).

Bondioli *et al.* (1989) reported pregnancies more than 40% from frozen-thawed embryos, which has been sexed by PCR before freezing. But at same time PCR has the risk of false positives because of limited amount of DNA in embryo biopsies, cross-species DNA contamination (Aasen and Medrano, 1990), DNA contamination during handling of the DNA products in PCR procedures and electrophoresis (Bredbacka, 1998).

The accuracy of the PCR method of bovine embryo sexing is reported to yield better result (96.4%) in compare to FISH (86.66%) (Cenariu *et al.* 2011).

Loop mediated isothermal amplifications

Embryo sexing based on the basis of specific sequences on Y-chromosome has been demonstrated by amplification through PCR from a small number of blastomeres (Garcia, 2001; Alves *et al.* 2006). But there are a lot of limitations of PCR method in sexing the embryo as discussed above. Hirayama *et al.* (2004) reported a loop-mediated isothermal amplification (LAMP), a simpler method of embryo sexing of a bovine embryo in compare to PCR. In LAMP, DNA amplification is done in isothermal condition using a DNA polymerase and four set of specific DNA primers for DNA synthesis along with a set of primer for accelerating the LAMP reaction called as termed loop primer (Notomi *et al.* 2000; Nagamine *et al.* 2002). Inner primer and outer primers produces a stem-loop DNA structure, and then a large amount of DNA is amplified by the auto-cycling reaction (Hirayama *et al.* 2013). The amplification of target DNA is estimated by measuring the turbidity due to a white precipitate of magnesium pyrophosphate, a by-product of DNA synthesis (Zhang *et al.* 2009). Electrophoresis is a reliable technique for interpretation of result of PCR as well as LAMP but It is time consuming and also needs gel-imaging system and electrophoresis apparatus system so practically difficult to apply for field purpose (Hirayama *et al.* 2004; Zhang *et al.* 2009). On other hand LAMP is rapid and end product is easy to assess with help of turbidity meter.

Use of a LAMP technique for bovine embryo sexing has also been reported (Khamlor *et al.* 2015). Kageyama *et al.* (2004) found a male specific repeated DNA sequence designated S4 in cattle on the basis of which Hirayama *et al.* (2004) developed a bovine embryo sexing kit with LAMP for commercial use. The LAMP-based sexing method is well sensitivity, accurate, quick and easy to perform for cattle embryo sexing at field level (Hirayama *et al.* 2013).

Embryo sexing by 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). This invasive technique is well complicated, expensive and time consuming. Cenariu *et al.* (2011) reported the accuracy of the FISH method of bovine embryo sexing is 86.66%.

Detection of X–linked enzymes

Some enzymes e.g. Glucose-6- phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyl transferase (HPRT), phosphoglycerate kinase are linked or related to X-chromosome (Wakchaure *et al.* 2015). Thier production is more in females in compare to male as male has only one X-chromosome while females has two X-chromosomes. On the basis of presence of their concentration, embryos can be sexed (Monk and Handyside, 1988). The gene production, suppression or inactivation of a large segment of one of the X-chromosome in female occurs at near after blastocyst formation and leads to formation of Barr body. Embryonic genome activation in bovine embryo occurs between the 8 to 16-cell stage (Frei *et al.* 1989) but exact timing of X-chromosome inactivation in domestic animals embryo is not known, it may occur during the blastocyst stage (Chapman, 1985). In the period between activation of the embryonic genome and inactivation of one X-chromosome, male and female embryo can be distinguished by estimating the concentrations of X-linked enzymes. Ratio of X-linked enzyme activity to autosomal enzyme activity is lower in male than female embryo because of variation in embryo metabolism. The activity of autosomal linked enzyme as adenine phosphoribosyl transferase (APRT) is measured, which provides a control for differences in overall enzymatic activities between embryos (Kouamo and Kharche, 2014). In mice, 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 (Sharma *et al.* 2017). In bovine embryos, Tiffin *et al.* (1991) showed the higher activity of glucose and glutamine in female than male one. Although the X-linked enzyme activity measurement has quite fair accuracy but many limitations as difficulty in collecting little amounts of enzymes, longer exposure of embryo to outside, reduced embryo viability and possibilities of false diagnosis due to intermediate values are also encountered (Pratheesh *et al.* 2011).

Embryo viability is reduced, particularly for embryos with very high or very low enzyme activity. Some time results may also be ambiguous due to partial X-chromosome inactivation (Kouamo and Kharche, 2014).

This assay may also be toxic to embryos, which may increase mortality rate (Wakchaure *et al.* 2015).

Detection H-Y antigens

Male specific cell surface antigen is called as histocompatibility-Y or HY antigen. This antigen is found on somatic cells in the heterogametic sex of all species. Detection of HY antigen can be used as method for sexing the embryos.

H-Y antigen on embryos can be detected by a cytotoxicity assay and by an immunofluorescent assay. In cytotoxic assay, polyclonal antisera is developed against HY antigen and in the presence of complement, embryos are incubated with this antisera. Male embryos are killed due to immune reaction while unaffected embryos are female which are available for transfer (Anderson, 1987). The limitation of this assay is that only female individuals are produced as males are destroyed (Kouamo and Kharche, 2014).

On the other hand, the immunological detection of sex specific HY antigen using antibodies is an non-invasive method of sexing embryos. Using immunological techniques (using polyclonal and monoclonal antibodies) the presence of HY antigen has been demonstrated on embryo of 8- cell stage to the blastocyst stage i.e. preimplanted embryo in all mammalian species e.g. bovine (White *et al*. 1987a; Booman *et al*. 1989), porcine (White *et al*. 1985) and ovine (White *et al*. 1987b) etc. At or beyond blastocyst, it is very difficult to detect HY antigen (Wakchaure *et al.* 2015). In this technique, in absence of complement, embryos are incubated with low concentration of HY antibodies and then with fluorescent tagged secondary antibodies which has been raised against HY antibodies. Generally secondary antibodies are labeled with Fluorescein isothiocyanate (FITC) but for increasing intensity Rphycoerythrin (RPE) can also be used (Booman *et al*. 1989).

Fluorescence microscopy examination shows that male embryos fluoresce (H-Y positive) and female embryos non-fluoresce (H-Y negative). Accuracy of the technique is 90% in mice, 85%in sheep and 81% in pig embryos i.e. approximately 85% accuracy in identifying embryonic sex (Anderson, 1987). Although the immunological approach is not accurate as cytotoxic approach (Booman *et al*. 1989), it is comparatively quick, easy to perform, requires no special manipulation skill and also don't need the biopsy of embryos (Bredbacka, 1998).

Indirect fluorescent technique allow the embryo transfer without cryopreservation with apparently less compromised embryo viability (Kouamo and Kharche, 2014).

The disadvantage is that antibodies against HY antigen are not completely sex specific i.e. because of the cross reactions, these antibodies may show false positive result (Wachtel, 1983). Also due to processing of embryo, the conception rate may be affected.

Sexing based on cleavage and development

Cleavage as well as developmental rate is faster in cells of male embryos than in females in order to attain morula and blastocyst stages (Sharma *et al.* 2017). Faster developmental rate of male embryos than females has been reported by many workers while working *in vivo* as well as *in vitro* by using PCR, karyotyping and immunofluorescence (Avery *et al.* 1992; Dominko and First, 1993; Yadav *et al.* 1993; Carvalho *et al.* 1995; Kitiyanant *et al.* 2000).

While this method has been applied up to 7 days old embryo, it may be more informative to determine the sex of embryo as early as the 2-cell stage (Saikhun *et al.* 1997).

The faster growth in male embryos may be due to the faster gene expression caused by Y-chromosomal genes (Pergament *et al.* 1994), effect of H-Y antigen or Y-chromosome growth factors (Burgoyne, 1993; Zwingman *et al*. 1993) or due less amount of DNA in male embryo there is need of lesser time to duplicate i.e. short cell cycle (Sharma *et al.* 2017). Yadav *et al*. (1993) proposed that Y-sperm activates embryonic genome to transcribe growth factors before embryonic transcription which may enhance the rate of cell division in male embryos i.e. faster development rate. Some authors have shown no differences in embryo development rate in the bovine (Holm *et al.* 1998) or porcine (Kaminski *et al.* 1996). Problem in this method of embryo sexing is that it is nearly impossible to access the exact time of cleavage *in vivo* and also it needs great skill to evaluate the development rate difference as it is very meager (Sharma *et al.* 2017).

CONCLUSION

Embryo sexing has great potential to maximize the efficiency of dairy production through controlling the sex ratio of domestic species. It permits the selection of a desired sex based on the requirement of producers in order to minimize the loss because of culling of undesired animals. Embryo sexing can potentiate the multiple ovulation embryo transfer (MOET) technique with the objective of increasing the accuracy of selection and reducing the cost of MOET by increasing the females (Nicholas and Smith, 1983). A good method for sexing the embryo should be least damaging or least affecting the survivability of embryos during freezing or transferring and should lead to the development of a calf of desired sex. Sexing methods must also simple, less ex pensive without compromising the accuracy and applicable at field conditions. The conception rate obtained from unsexed and sexed embryos isreported with no any significant

difference (Karaşahin *et al.* 2014). There are various methods of embryo sexing each with various advantages and disadvantages. Although invasive methods as cytological karyotyping has well accuracy but cannot be applied at field level as embryo survivability is compromised. Detection of X-linked enzymes, H-Y antigen, hormonal assay, cleavage and development rate difference etc are not of much practical value because one or another reasons. Out of all these methods PCR, FISH and LAMP are well efficient, highly reliable and accurate procedures for sexing embryos. 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. Concentration of estrogens and androgens in blastocoels may prove useful in the prediction of embryonic sex in pig and horse (Sharma *et al.* 2017). In bovine embryos, there are some sex 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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