

Immunological semen sexing technology: Present status and future perspective

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Dairy farming in India is facing extreme challenges of high feed costs, shortage of green fodder, low milk productivity of the native breeds, and the burden of raising surplus male calves that have little economic value. One promising solution is the use of sexed semen, which can ensure the birth of mostly female calves from elite high-milk-yielding bulls. This would lower the cost of milk production by avoiding the expenses of rearing unproductive male calves. At present, the only widely used technique for separating male and female sperm is flow cytometry; but it is expensive, time-consuming, and often damages sperm, leading to poor conception rates. As an alternative, researchers are now exploring immunological sex sorting, a simpler and more affordable technique. In this approach, antibodies that recognise differences on the surface of male or female sperm are attached to beads, which selectively trap one sperm type while leaving the other free. Because it is faster, cheaper, and gentler on sperm, immunological sex sorting has the potential to revolutionise cattle breeding and help farmers produce more milk at lower cost.

Keywords: Cattle, FACS based sorting, Immunological sorting

TODAY, India is the world's largest milk producer with 247.8 million tonnes of annual milk production recorded in 2024-25 (Basic Animal Husbandry Statistics, 2025, GOI). Despite this milestone, dairy farmers have to face stiff challenges in making dairy business profitable due to rising feed and fodder costs, shrinking agricultural land, high labour expenses, and competitive milk prices. One promising solution is the use of sexed semen from elite bulls with high milk-yield potential, which ensures the birth of mostly female calves. This practice offers several benefits:

- Reduces the cost and space needed to rear surplus male calves (except those required for breeding),
- Increases the proportion of productive animals in the herd, thus increasing the milk yield and profit, and helps minimise the problem of stray cattle since bovine slaughter is prohibited

in most parts of India.

At present, flow cytometry (FACS) is the only commercially available sperm-sorting method, providing more than 90% accuracy in separating X- and Y-bearing sperm. However, its widespread use is limited by high semen costs, sperm damage caused by prolonged sorting, and lower conception rates with cryopreserved sex-sorted semen. To address these limitations, immunological sex sorting of semen is being explored as a cheaper, faster, and less damaging alternative that could improve the conception rates and make the use of sexed semen more practical for Indian dairy farming.

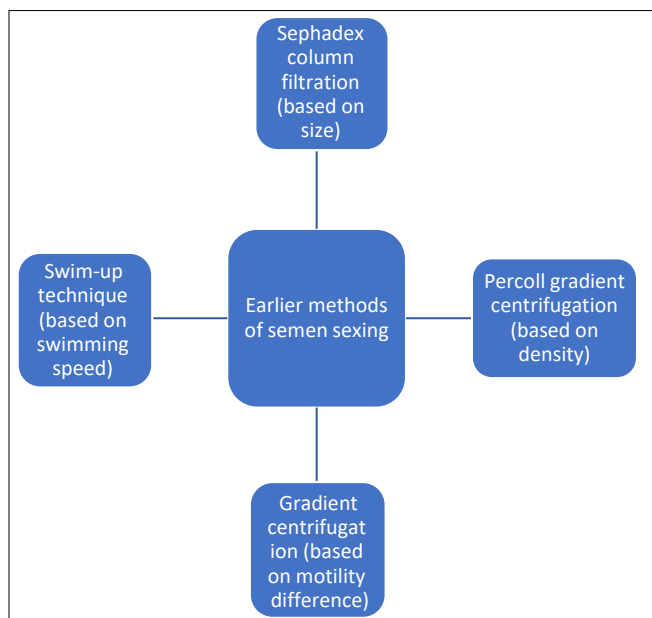
Different methods of semen sexing

Differences in the physical and physiological characteristics of X- and Y-bearing sperm such as size, density, DNA content, surface charge, and motility have inspired the development of several sperm sexing techniques over the

years. Early methods were simple, however, poor reproducibility and limited accuracy prevented their commercial adoption. Even after modifications and optimisation efforts, the accuracy of these approaches rarely exceeded 65–75%. These limitations eventually led to the development of more advanced methods, most notably flow cytometry based sperm sorting (fluorescence-assisted cell sorting, FACS), which remains the only commercially viable technology to date.

Fluorescent assisted cell sorting (FACS)

The flow cytometer-based semen sexing technology was introduced commercially in 2007 by Beltsville Sperm Sexing Technology, XY Inc., which was later acquired by Sexing Technologies (Navasota, TX, USA). The flow cytometer-based sorting of X and Y sperm is based on the difference in DNA content between the X and Y sperm (4% more DNA is present



and causes less damage to sperm is very crucial for enhancing the viability and fertilising ability of sex-sorted sperm and broader applicability of sexed semen by the dairy farmers across the country.

Immunological sorting of X and Y sperm

It is a novel approach based on distinct differences in the surface

sperm revealed higher expression of 15 proteins in X sperm, while two (tubulin alpha 8 and tubulin beta 2B) were highly expressed in Y sperm. These proteins were associated with the structural cytoskeleton of the flagellum, glycolytic enzymes, and calmodulins. However, only the X or Y specific surface proteins have practical relevance in immunological sperm sorting due to the ease of detection and binding by the specific antibodies. Recently, immunological semen sexing uses a polyclonal antibody against surface antigen sex-determining region Y (SRY), which localises specifically on bovine Y sperm. Similarly, a monoclonal antibody against rbSRY (SRY2mab) tagged with a sepharose column was shown to bind specifically to Y sperm. In another recent study, proteome analysis of bovine sperm revealed specific expression of 151 and 88 proteins in X and Y sperm, respectively. Out of these, nine proteins (STX2, CLRN3, CD58, CEACAM20, ADAM21, ABCC12, KIDINS220, RMDN2 and C11H2orf74) in X sperm and three proteins (DPY19L2, PRXL2A, SCAMP1) in Y sperm showed gender-specific expression in more than two samples. In addition, the Hedgehog protein (SHH), SH3D21, TF, and TNF were present only on the Y sperm surface. Prediction of sub-cellular localisation, transmembrane structure, and western blot validation revealed that CLRN3 and SCAMP1 were surface-specific proteins of bovine X and Y sperm, respectively. There is a significant male-biased expression of the sex chromosome gene SCAMP1 in songbirds, and the protein encoded by SCAMP1 co-localises with androgen receptors during development. Therefore, CLRN3 and SCAMP1 could be potential X- and Y-specific surface markers for immunologically sorting X and Y sperm, respectively.

Different sperm separation techniques used in immunological sex-sorting

Gel filtration: First, the antibody against a specific sperm-surface antigen is conjugated with agarose gel beads by using a suitable

in bovine X sperm than Y sperm); and it involves first labelling of the sperm DNA with a cell membrane permeable fluorescent dye (Hoechst 33342) and then forcing these cells to pass through a laser to be detected and formation of charged sperm droplets which are finally separated by applying an electrical field. Although in the last three decades, tremendous advancements have taken place in this technology which permits sorting of X and Y sperm with higher accuracy (>90%), speed and improved viability; however, the high cost of the technology is the main limiting factor preventing widespread application across the country. Besides, decreased fertilisation potential and impaired embryonic development of sex-sorted semen when compared with the unsorted semen are other severe limitations of this technique. The reduction in conception rate (13%) of sex-sorted cattle semen than conventional frozen semen is thought to be due to the reduction in sperm concentration and motility in most commercial doses and the damage to sperm caused during sorting procedures. Sex-sorted sperm also have reduced viability and overall quality after cryopreservation and thawing, reducing pregnancy rates (23%) and increasing indirect costs for the livestock industry. Therefore, the development of an alternative technique that is cheaper and involves less handling of sperm

antigens between the X and Y sperm. Polyclonal antibodies against these surface antigens can specifically bind to either X or Y sperm, which then could be trapped and separated from the other by using suitable means such as magnetic beads or Sephadex gel filtration, etc. The use of any dye or radiation is not required in this method to separate the X and Y sperm avoiding their harmful influence on sperm. In addition, immunological method requires lesser time and exerts lesser stress on sperm cells which in turn minimises damage to sperm and loss in fertility.

Identification of X and Y sperm-specific protein markers

Identification of unique X or Y sperm-specific surface antigen(s) is the pre-requisite for immunological sperm sorting. Several studies employed mass spectrometric characterisation of X and Y sperm to identify the X- and Y-specific protein markers. Scott *et al.* (2018) reported up-regulation of five (FUNDC2, ACACB, NDUFS7, SAMM50, COX2) and three (EFHC1, DNAI2, PDHX) proteins in bovine X and Y sperm, respectively. Notably, the expression of EFHC1 (EF hand-domain-containing protein 1), a microtubule-binding protein involved in the ciliary beating, was deficient in X sperm as compared with Y sperm (X/Y ratio 0.05). Another proteome study of bovine

linker, and then the purified beads are used to specifically capture a specific sperm type either the X or Y; while the other sperm type is washed out using a suitable buffer. However, separation of sperm by this technique requires prolonged exposure of sperm to column matrix which may potentially reduce both sperm motility and fertility.

Magnetic-activated cell sorting (MACS): Magnetic bead-based immunological sorting using antibodies against either X or Y specific surface antigens could be a cheaper and more acceptable technique for sorting of bovine sperm. The MACS technique is routinely practiced in human ART labs to remove apoptotic sperm from an ejaculate by using Annexin V-conjugated magnetic beads. It is relatively simple and fast procedure as compared with the gel filtration. In this technique, first the antibodies against the surface proteins of specific sperm-type are conjugated to supra-magnetic beads. Then, the beads are mixed with semen to

allow binding of the specific sperm type to these beads which are then separated with the help of a magnet.

Potential limitations of immunological semen sexing

One of the major limitations of immunological sex-sorting of sperm is the lack of highly-specific surface antigens that can reliably differentiate X and Y bearing sperm. Besides, antibody cross-reactivity and variation in antigen expression among individuals also affect in developing a standardised protocol reducing the sorting accuracy. Although less damaging and more economical than FACS, current immunological methods still do not achieve the accuracy and efficiency required for large-scale commercial use. Therefore, further research is needed to identify reliable sex-specific surface antigens and improve sperm sorting efficiency.

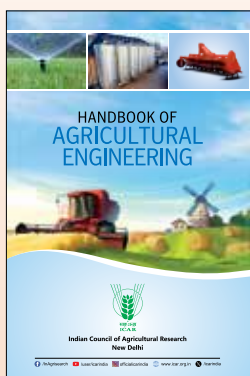
SUMMARY

The use of sexed semen from genetically superior bulls

for artificial insemination has the potential to transform dairy farming, by increasing the proportion of female animals and thus the milk productivity of a dairy herd, and reducing the cost of milk production. Immunological sex-sorting offers a promising alternative to the conventional fluorescent-assisted cell sorting (FACS) technique of X/Y sperm sorting, as it is much cheaper, faster and causes minimal sperm damage. Although several studies have identified multiple potential sperm surface antigens, specific to either X and Y sperm, only a few have been tested in sperm sorting. However, the accuracy and efficiency of these methods remain far behind for commercial application. Therefore, identification of highly-specific X or Y sperm surface antigens is very critical for achieving greater accuracy in immunological sperm sorting.

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