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Immuno-Sexing: A Proteomic Based Approach Towards Next-Generation Biotechnological Advancement in Bovine Reproduction

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Abstract:

Sperm-sexing in bovine is a next generation biotechnological tool to produce desired sexed off-spring. This technological advancement has started gaining popularity since decades of its invention. The only commercial technology apart from the conventional techniques which produce sex-sorted spermatozoa is flowcytometry. This technique has number of constrains, therefore to overcome it biomarker-based sorting technology can be developed where by targeting specifically expressed protein on the surface of X- or Y- chromosome bearing sperm immune based sorting can be established. Proteomic profiling of sperm cells has been studied by many researchers where differentially expressed proteins of either sexed-sperm has been identified. The functional roles of these proteins, along with Gene Ontology (GO), pathway analysis, and protein–protein interaction (PPI) studies, have been thoroughly analysed. Furthermore, finding the epitopic region over the surface region of targeted spermatozoa can be used to raise antibody against it and specific group of spermatozoa either X or Y can be separated from the semen suspension. Accordingly, magnetic bead-based sorting can be employed to optimize this technique and facilitate the development of a novel sperm sexing technology in the future. This concept is having an insightful impact over the breeding strategies of reproduction biotechnology where using an immune based sorting can improve the production of elite milch breed in milk industry. The use of sex sorted semen globally is increasing rapidly and the accuracy of producing female calf by using sexed semen in bovine is 80-90% through artificial insemination (AI). This article aims to explore the potential of proteomics-based sperm sex sorting and highlights how these insights can be leveraged to develop immuno-based sex sorting strategies in the bovine industry.

Keywords: Sperm-sexing, Bovine, Immuno-sexing, Proteomic, specifically expressed proteins (SEPs)

Introduction:

The global adoption of sex-sorted semen is steadily increasing, driven by supportive government initiatives and growing awareness among breeders. Sex-sorted semen refers to

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semen enriched with either X- or Y-bearing sperm, enabling targeted sex selection and its application in artificial insemination according to the requirements of farmers or breeders. In 2024, the global market for sexed semen was estimated at around USD 4.1 billion and is expected to expand at a compound annual growth rate (CAGR) exceeding 11% through 2033, reflecting a strong and growing demand for its application. The only commercial technique through which production and distribution of sexed semen is proceeding is flowcytometry which is based on the difference in DNA content of X- and Y- sperm. This has 90% accuracy of producing sexed semen which is either X or Y- enriched semen straws. However, the use of fluorescent dyes and high-pressure conditions poses significant drawbacks, often leading to sperm membrane damage, reduced viability, impaired motility, and ultimately decreased fertility (Suh et al.,2005). Moreover, the cost of sexed sperm straw is expensive and it is also a highly sophisticated technology where expert handling is needed. Each straw of sorted sperm contains 2million sperm where unsorted straws contain 20 million. Thus, to overcome all these disadvantages of flowcytometry based sexed sperm production researchers are exploring the proteomic profile of X- and Y- chromosome bearing sperm by using LC-MS/MS studies. Several studies conducted worldwide have identified differentially expressed proteins on the sperm membrane between X- and Y-chromosome–bearing sperm (De canio et al.,2014; Shen et al.,2021). These proteins are either highly expressed in X- bearing sperm or Y-bearing sperm. There are several sperm membrane proteins of bovine which has been identified by researchers are viz; FUNDC2, ACACB, TUBA3, HIBADH, PDHX etc (Chen et al.,2012, De Canio et al.,2014, Scott et al.,2018). The concept of immune-sexing arises from the interaction between antigen antibody on the sperm membrane. The targeted protein on the surface of either X- or Y- sperm which is specifically expressed can be analysed to find the epitopic region and synthetic peptide against it can be synthesised. Antibody raising against this peptide can be used as specific antibody against targeted cells. There are several studies where antibody-based sexing has been performed though validation not has been done yet. The H-Y antigen is a male-specific cell surface protein associated with Y-chromosome–bearing sperm in bovines, making it a potential marker for distinguishing Y sperm (Anderson.,1987). Its presence has been explored in immunological approaches for sperm sexing, enabling targeted separation of Y-bearing sperm for breeding applications. Furthermore, magnetic-activated cell sorting (MACS) is an emerging technology in which antibody-coated microbeads are used to selectively isolate target cells, either within a microfluidic device or an Eppendorf tube, while the remaining cells are collected from the mixture. This technique has also been applied by several researchers in sperm sexing to selectively isolate sperm of the desired sex (sringram *et al.*,2022).

Figure: Advantages of sperm sexing in Bovine in a nutshell with pictorial representation.

Flowcytometry Based Sperm Sexing:

In general, there is only one commercially available method used for sex-sorting in bovine, which consists in the individual separation of X- and Y- chromosome- bearing sperm using flow cytometry fluorescence- activated cell sorting (FACS) depending upon the DNA content (Garner et al., 2013; Moore & Hasler, 2017; Seidel, 2014).It is stated that DNA content in X sperm is 3.8% higher compared to Y-sperm in cattle. Although this technique can achieve X-sperm purity exceeding 90%, it is accompanied by several drawbacks, including limited sperm numbers per straw, potential cellular damage, reduced post-insemination conception rates, and high associated costs. Consequently, to overcome these limitations it is important to develop a more economical, less harmful, efficient, and user-friendly sperm sorting approach.

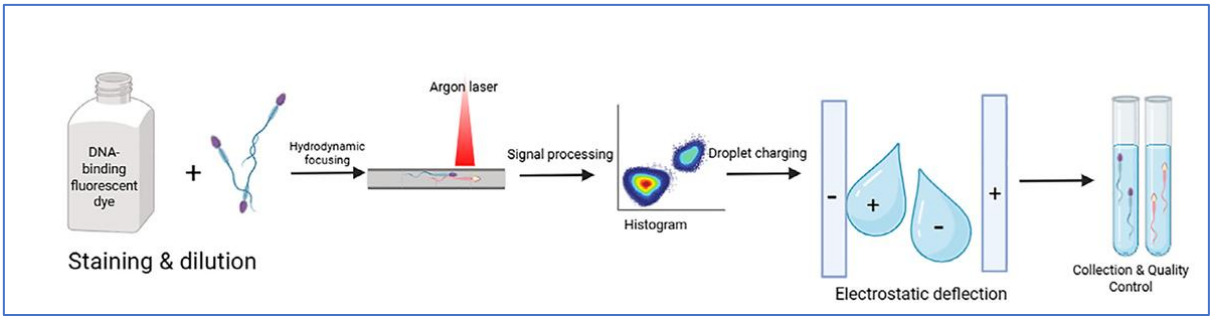

Figure: Flow cytometric sex sorting of sperm based on DNA content (Kinga et al.,2025).

Biochemical Difference Between X and Y- Sperm:

The basic differences between X and Y bearing sperm is in their molecular content as well as structural. The proteomic studies recently done by researchers has also showed that the proteomic profile of X and Y bearing sperm varies. The differences are listed below in the table:

Table 1: The differences between X and Y- bearing sperm given below:

Parameter	X and Y differences	References
DNA	Less DNA in Y sperm	Moruzzi 1979; Pinkel et al., 1982
Size	X sperm larger	Cui and Matthew, 1993
Motility	Y sperm swim faster	Ericsson et al. 1973
Surface charge	X migrate to anode	Kaneko et al. 1984
Sperm surface	H-Y antigen present	Hendriksen et al., 1993
Plasma membrane protein	Differentially expressed protein	Chen et al., 2014; Laxmivandana et al., 2021
F-Body	Long arm on Y chromosome	Barlow and Vosa,1970

Proteomic Insights into X and Y- Chromosome Bearing Bovine Spermatozoa:

The first step of proteomic based sperm sexing arises from the study of label free non-gel based approach called LC-MS/MS. This is basically done to identify differentially expressed proteins or specifically expressed protein on the membrane of X- or Y- chromosome bearing spermatozoa which can act as potential biomarker in immune sexing. Laxmivandana et al., (2021) provided key insights into bovine sperm membrane biology by identifying 13 differentially expressed proteins on the plasma membrane of X- and Y-bearing sperm. Likewise; Shen et al.,2021 has studied plasma membrane proteome of bovine X- and Y-sperm where CLRN3 was stated as X-specific and SCAMP1 was stated as Y-specific. Similarly, Sharma and colleagues (2022) studied the whole sperm proteome and reported differential expression, with around three proteins upregulated in Y-bearing sperm and 27 in X-bearing sperm.

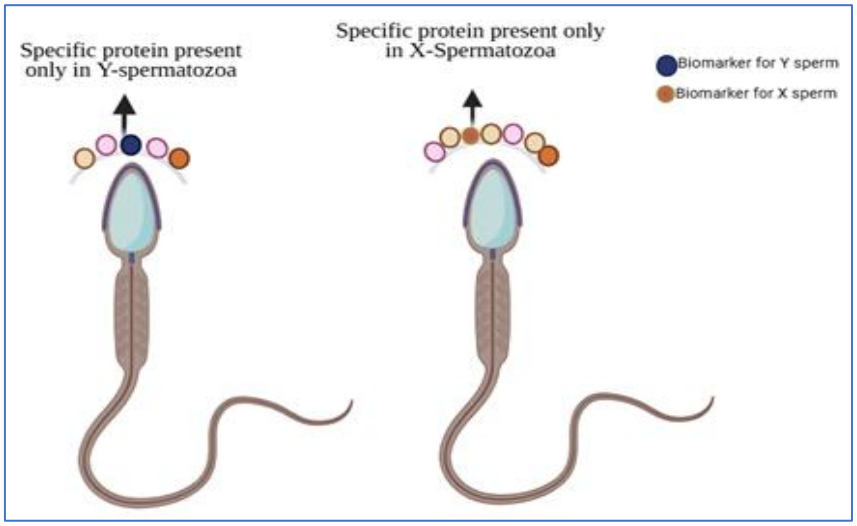

Figure: Specifically expressed protein on the surface of X- and Y- bearing sperm (Created by BioRender.com)

Immuno Sexing:

Immunological approaches to sperm sexing are depends on the presence of distinct surface proteins expressed by X- and Y-bearing spermatozoa. The isolation of such proteins offers the possibility

of generating specific antibodies to selectively identify and separate sperm carrying either chromosome type. Among the targeted markers, the male-specific H-Y antigen has received considerable attention as a potential indicator of Y-bearing sperm; however, inconsistent findings have limited its practical application in sperm sexing.

Furthermore, studies have reported a set of differentially expressed proteins between X- and Y-bearing spermatozoa, which are believed to contribute to their functional differences. Chen and co-workers identified 14 proteins that show differential expression between X- and Y-bearing spermatozoa, potentially contributing to their distinct functional characteristics. These proteins are associated with key biological processes such as energy metabolism, stress response, cytoskeletal organization, enzymatic activity, sperm–oocyte interaction, and early embryonic development, thereby influencing the distinct roles of each sperm type during fertilization. Further, few sexing kits are also available based to antigen-antibody reaction for bovine like WholeMom kit (Nuri Science Inc.), Emlab Genetics kit. But due to the number of limitations like low fertility, high cost and lack of availability it is difficult to rely on.

Magnetic-activated cell sorting is increasingly being directed toward immuno-sorting, thereby facilitating the development of innovative immuno-based sperm sexing technique. By using scFv antibody coated with magnetic microbeads sorting of X- and Y- bearing spermatozoa done under magnetic field (Sringram et al., 2022).

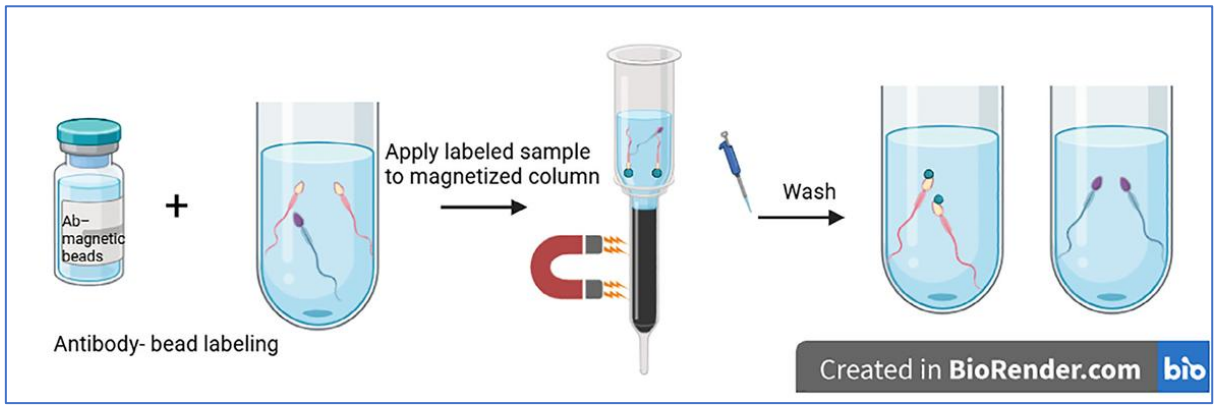

Figure: Magnetic-activated cell sorting (MACS) based sperm sexing (Kinga et al.,2025).

Future Research Directions of Sperm Sexing:

The concept of immuno-based sperm sexing is both novel and scientifically robust. Identifying antigenic regions on selectively expressed membrane proteins represents a significant advancement, as targeting these markers can enable the efficient separation of sperm of the desired sex. Moreover, this approach holds promise as a cost-effective, less complex, and labour-efficient alternative to existing technologies. A highly fertile population of sorted sperm can be obtained, ensuring improved efficiency and reliability for artificial insemination doses. Further research is required to identify specific surface proteins on X- and Y-bearing bovine spermatozoa, which will be essential for developing a novel sperm sexing technique.

Research Impact on This Topic:

Despite extensive investigations into the proteomic profile of spermatozoa, the application of antibody-based sorting and the precise identification of antigenic regions on selectively expressed proteins remain largely unexplored. This gap is particularly evident in indigenous bovine breeds, where proteomic studies on sorted sperm are limited. Addressing this, our laboratory has undertaken LC–MS/MS-based proteomic analysis, with ongoing efforts focused on identifying proteins that are differentially and specifically expressed between X- and Y-bearing spermatozoa.

This research establishes a critical foundation for the discovery of potential biomarkers and the subsequent development of targeted antibodies, thereby paving the way for a novel, efficient, and immunologically driven sperm sexing strategy.

Conclusion:

The sperm plasma membrane serves as a strategic target for developing next-generation sperm selection techniques. Exploiting the differential protein composition between X- and Y-bearing sperm can enable the design of efficient, non-invasive, and economical sexing approaches. Identification of uniquely expressed membrane proteins would allow the generation of highly specific antibodies, facilitating selective separation with minimal cellular damage and sperm loss.

The concept of immuno-based sperm sexing should be promoted more widely at the global level to accelerate research efforts, enabling the development of antibody-based techniques for sperm sexing in the near future. A cost-effective and farmer-friendly sperm sexing technique will not only enhance production efficiency but also significantly contribute to the generation of elite bulls.

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