B-4530
[1-10]

REVIEW ARTICLE Indian Journal of Animal Research

Spermatozoa Sorting Techniques for the Sex Pre-selection: A Review

S.J. Naidu, A. Arangasamy[1], S. Selvaraju, B.K. Binsila,
J.P. Ravindra, I.J. Reddy, R. Bhatta

10.18805/IJAR.B-4530

ABSTRACT

Furtherance of sex pre-selection techniques is extremely beneficial for the farm productivity and economic growth of the country. Several techniques such as flow cytometry, swim-up, percoll gradient centrifugation, lumisort and immunogenic spermatozoa sexing developed so far are reviewed with their principles, advantages, disadvantages and possible ways for developing a highly reliable and efficient technique to achieve success in obtaining offspring of the desired sex. Out of all these techniques available till now, sorting X- and Y- spermatozoa using fluorescence-activated cell sorter (FACS) is the most successful and commercially available technique, which employs sorting of spermatozoa based on DNA content. Despite its effectiveness, there are disadvantages concerning cost, sperm damage, trained technical person, low conception rate, *etc*. An alternative approach that might have potential significance could be the identification of sex-specific membrane marker proteins for the immunological method of spermatozoa sorting.

Key words: Flow cytometry, Immunological methods, Sex ratio, Spermatozoa sorting, X- and Y- spermatozoa.

There has been significant progress in the understanding of reproductive biology and more particularly the biology of sex determination; this knowledge has given an impetus for taking forward the basic research to a translational mode for benefiting mankind in terms of improving economic and nutritional status. Production of offspring of desired sex to suit the farm productivity is highly economical for the growth of the country. Although India is the highest milk producer, certain percent of the country's demand for the milk is still unmet. The present production being 187.7 million tons (Annual report 2019-20, Department of Animal Husbandry, Dairying and Fisheries, Government of India). India's requirement for milk is likely to ascend up to 200-210 million tons close to 2021-22. Therefore the advancement of spermatozoa techniques along with the implementation of these techniques in the field level will assist in wisely organizing the dairy or beef farms.

Spermatozoa sorting technique helps to produce offspring of the desired sex, genetically superior cows and elite bulls. Several approaches have been employed to sort X- and Y- spermatozoa based on the physical differences in spermatozoa. For example, motility differences between X- and Y- spermatozoa have been exploited using the swim-up technique (Han *et al.* 1993) and sedimentation velocity has been used for the percoll density gradient centrifugation (Iizuka *et al.,* 1987). The presence of sex-specific spermatozoa membrane protein (Bennett and Boyse, 1973) is also used to separate X- and Y- spermatozoa. Discontinuous albumin gradient centrifugation has been used to sort spermatozoa based on the sedimentation rate (Wang *et al.,* 1994). In recent years, Raman spectroscopy based on assessing Raman peaks associated with the DNA content of the head-neck region of the spermatozoa has been developed (De Luca *et al.,* 2014). Flow cytometry is found to be a highly reliable and commercially translated technique so far (Johnson and Welch, 1999). Recently, laser-based system based on the differences in DNA content that selectively kills undesired sex of spermatozoa (LumiSort) has been developed as a potential high-speed sorting technique ("Lumisort - Microbix Biosystems" 2019). However, separation of the spermatozoa based on protein-based techniques is yet to be established for commercial application.

In this review, several techniques developed and tried so far are reviewed to update the knowledge on the techniques employed in the field of sex-sorting and sex-skewing considering their principles, advantages and disadvantages. This review also foresees possible ways for developing a highly reliable and efficient technique to effectively skew offspring sex ratio in farm animals.

Animal Physiology Division, Reproductive Physiology Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru-560 030, Karnataka, India.
[1]ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru-560 030, Karnataka, India.

Corresponding Author: A. Arangasamy, ICAR-National Institute of Animal Nutrition and Physiology, Bengaluru-560 030, Karnataka, India. Email: arangasamyars@gmail.com

How to cite this article: Naidu, S.J., Arangasamy, A., Selvaraju, S., Binsila, B.K., Ravindra, J.P., Reddy, I.J. and Bhatta, R. (2021). Spermatozoa Sorting Techniques for the Sex pre-selection: A Review. Indian Journal of Animal Research. DOI: 10.18805/IJAR.B-4530.

Submitted: 18-05-2021 **Accepted:** 02-08-2021 **Online:** 16-09-2021

Modified swim-up technique

The swim-up technique has been developed to separate or enrich the motile spermatozoa from the immotile for AI (Artificial Insemination). The standard swim-up technique employed a 4ml tube filled with 1.5 ml sperm-TALP (Tyrode's albumin lactate pyruvate) medium and 0.5ml of semen sample being deposited under the media. This technique was employed to enrich highly motile spermatozoa for IVF (*In vitro* fertilization). A swim-up procedure used before IVF influenced the sex ratio in humans where an optimal percentage of Y- spermatozoa could be obtained in about 30 to 45 minutes, although this method requires proper validation (Check *et al.,* 1989; Claassens *et al.,* 1989). This technique was modified further to separate the X- and Y- spermatozoa in domestic animals.

The modified swim-up technique consists of allowing spermatozoa to swim-up through a vertical glass pipette of certain height filled with sperm-TALP (Azizeddin *et al.* 2014; Asma-ul-Husna *et al.,* 2017). After incubation for about 10-45 minutes, the supernatant rich with Y- spermatozoa are collected. Since the Y-spermatozoa swims faster, the supernatant will be rich with Y-spermatozoa while the bottom portion will be rich with X-spermatozoa (Azizeddin *et al.* 2014). The modified swim-up method was efficient in sorting Nili-Ravi buffalo spermatozoa (Asma-ul-Husna *et al.* 2017). The same was observed in the case of bovine semen obtained from the upper portion preferentially produced male embryos (62%) and the results were confirmed through FISH and *in vitro* fertilization (Azizeddin *et al.,* 2014). This technique, as validated through real-time PCR also has shown a 4-5 fold increase in X- spermatozoa in X-chromosome bearing fractions and 4 fold increase in Y-spermatozoa in Y-chromosome bearing fractions (Asma-ul-Husna *et al.,* 2017). To conclude, the modified swim-up technique being a simple one found to have low spermatozoa damage and high spermatozoa recovery rate. However, it has to be performed in large scale and the conception rates and sex ratio have to be validated through field trials.

Percoll density gradient centrifugation technique

Percoll density gradient centrifugation employs the sedimentation density differences between the X- and Y-spermatozoa populations for their separation. A 12-step gradient ranging from 30% to 80% of discontinuous percoll density gradient centrifugation was found to greatly enrich human X- spermatozoa cells. About 94% of purified X-spermatozoa were obtained from the bottom fraction of the gradient (Iizuka *et al.,* 1987). Six healthy female infants were born without any abnormalities from the spermatozoa obtained from the bottom fractions of the eighth layer (35-84%) discontinues percoll gradient (Iizuka *et al.* 1987). In this trial, the validation done by the quinacrine mustard staining method may be unreliable since it stains only 25-50% of the diploid male cells containing the Y- chromosome (Pearson *et al.,* 1970). Another group reported higher false positive and false negative rates as there were chromosomal variants or non-existing bands or low fluorescence (Thomsen and Niebuhr, 1986). The validation by employing southern blot also showed no difference in the X- and Y- ratio obtained after percoll gradient centrifugation (van Kooij and van Oost, 1992).

Later, multiple groups have tested the efficiency of percoll density gradient method (Wang *et al.,* 1994; Lin *et al.,* 1998; Kobayashi *et al.,* 2004; Wolf *et al.,* 2008; Promthep *et al.,* 2016). The twelve-step percoll density gradient was prepared from a 25-80% gradient layer. The difference was scored for the neat semen and 80% fraction by double-labeled FISH and chromosome-specific probes (Wang *et al.* 1994; Lin *et al.,* 1998). Significant enrichment of X- to Y-spermatozoa in the 80% fraction was obtained, but the enrichment is not sufficient for sex selection (Wang *et al.* 1994; Lin *et al.,* 1998; Lucio *et al.,* 2012). Whereas no difference in the ratio of X- and Y- spermatozoa, although slight enrichment of bovine Y- spermatozoa was obtained from the top layer of discontinuous percoll gradient after washing with modified Brackett and Oliphant's medium (Kobayashi *et al.* 2004). Slight enrichment of X- spermatozoa of bovine was obtained through continuous percoll density gradient centrifugation (Wolf *et al.,* 2008). In a recent study, about 60-75% of the X- spermatozoa were enriched in a 65-70% percoll gradient layer (Promthep *et al.,* 2016). Although the method has been reported to enrich a population of spermatozoa, there is an inconsistent opinion about the technique protocol. Thus, proper validation of the results coupled with enhancement in the yield of spermatozoa is required.

Flow cytometry technique

Among all techniques, fluorescence-activated cell sorter (FACS) is considered the most successful technique at present. Sex pre-selection of spermatozoa using flow cytometric spermatozoa separation is based on the difference in DNA content between the X- and Y- spermatozoa. The DNA content (%) of X- and Y- spermatozoa nuclei differs in various species ranging from 2.8 in human to 7.5 in chinchilla. The DNA content (%) in animals such as rabbit, boar, stallion, dog and ram are 3.0, 3.6, 3.7, 3.9 and 4.2, respectively (Johnson and Welch, 1999). Likewise, the DNA content differences (%) in X and Y-spermatozoa nuclei of various breeds of cattle such as Jersey, Angus, Hereford, Holstein and Brahman are 4.22, 4.07, 3.98, 4.01 and 3.70, respectively (Garner *et al.,* 1983).

To date, the sexed semen is used for cattle (Garner and Seidel, 2008; DeJarnette *et al.,* 2011), rams (De Graaf *et al.,* 2007), horses (Clulow *et al.,* 2008; Buchanan *et al.,* 2020), water buffalo (Lu *et al.,* 2010), elk (Schenk and DeGrofft, 2003), boars (Johnson *et al.* 2000), Western Lowland Gorilla (O'Brien *et al.,* 2005), White-Tail deer (Kjelland *et al.,* 2011), cats (Pope *et al.,* 2009); bottlenose dolphins (O'Brien *et al.,* 2009); Pigs (Johnson 1991, Martinez *et al.,* 2001; Rath *et al.,* 2003), goats (Bathgate *et al.,* 2013) and dogs (Wei *et al.,* 2017) have resulted in the birth of desired sex.

Table 1: Spermatozoa sorting techniques developed based on the physical differences between X- and Y- spermatozoa.

Techniques	Principle	Advantages	Disadvantages	References
Modified swim up	Motility	Low spermatozoa damage and high spermatozoa recovery rate.	Conception rates and sex ratio has to be further validated through field trials. Not commercialized yet.	(Asma-ul-Husna *et al.,* 2017; Azizeddin *et al.,* 2014; Check *et al.,*1989; Claassens *et al.,* 1989)
Percoll density gradient centrifugation	Density	Fair increase in spermatozoa count	Proper validation coupled with enhancement in the yield of spermatozoa is required.	(Kobayashi *et al.,* 2004; Lin *et al.,* 1998; Promthep *et al.,* 2016; Wang, *et al.,* 1994; Wolf *et al.,* 2008)
Flow cytometry	DNA content	More than 90% purity of cells, 90% of desired sex of offspring is obtained, commercially available	Spermatozoa damage, low fertility rates, sorting rate, high cost and specialized machine with skilled personnel. sorting facility need to be linked with bull semen bank, as sorting of the fresh semen is a pre-requisite for the semen cryopreservation	(Johnson and Welch 1999)
Lumisort	DNA content	Efficient, higher sorting rates	Reports on the conception-rates of the laser sexed-semen are not available	("CNW | Microbix Announces Proof-of-Concept Demonstration for LumiSortTM - its Livestock Semen Sexing Technology" 2018; "Lumisort – Microbix Biosystems" 2019.)
Immuno-sexing approach	Protein	Overcomes sperm damage, easy and cost effective	Reliable method but is still at the experminental level	(Chen *et al.,* 2014, 2012; Chowdhury et al. 2019; De Canio et al. 2014; Domínguez et al. 2018; Han *et al.,* 2018; Hashimotoa *et al.,* 2013; Li *et al.,* 2011; Scott *et al.,* 2018; Umehara *et al.,* 2019; Yang *et al.,* 2014)

Working principle of flow cytometry

Initially, stained spermatozoa with a membrane-permeable nucleic acid-specific fluorophore, Hoechst 33342 are pumped in a stream, one at a time as a droplet in front of a laser beam at a specific wavelength coming from a photomultiplier. Spermatozoa samples are broken into 70,000 to 80,000 droplets per second. As bovine X-spermatozoa have 4% more DNA, more dye binds to X- than Y- spermatozoa, to give off 4% more fluorescence. This fluorescence is quickly measured by using PMT (Photo multiplying tubes). PMT converts the fluorescence into an electric pulse. About 85-90% purity is obtained while separating the spermatozoa through a specially designed nozzle, HiSON (Johnson and Welch, 1999). If the droplet contains Y-spermatozoa, a negative charge is imparted and if the droplet contains X-spermatozoa, a positive charge is imparted and if the droplet contains no or multiple spermatozoa, no charge will be imparted (Fig 2). Thus, these droplets can be collected in three different tubes (Seidel, 2003).

With this technique, the spermatozoa sorting rate of bovine species is said to be at 8000 spermatozoa/sec with an input of 40,000 spermatozoa. In this speed, approximately, 28 million spermatozoa/hour can be sorted and if 2 million cells/straw is used for AI, 300 straws/day can be produced (Sharpe and Evans, 2009; Garner *et al.,* 2013).

Using low dose and conventional AI, the production of progeny in several species has been reported (Buchanan *et al.,* 2000). The improvement in the sorting efficiency has led to the commercialization of the equipment for the production of offspring of animals such as cattle (Maxwell *et al.,* 2004). Sexing Technologies (ST) acquired all the technology rights to facilitate the bull studs to access the sexed semen of consistent quality at sensible costs. In 2013, developments in the existing technology along with the optimization of the extenders and media, redesign of the equipment, *etc*. resulted in improvements in the semen quality and a new-product label "4M sexedULTRA™" was launched officially. 4M SexedULTRA™ provided 4 million spermatozoa cell/straw, unlike the previous technology which provided 2.1 million spermatozoa/straw. This resulted in a comparable conception rate with the conventional semen. In 2017, ST genetics launched 4M Sexed ULTRA™ and is expected to become the new industry standard for sexed semen (Thomas *et al.,* 2017; González-Marín *et al.,* 2018; Vishwanath and Moreno, 2018; Dell'Eva *et al.,* 2019). IVF using sorted semen showed high purity, repeatability and comparable rates of cleavage and blastocyst formation were observed between conventional and sorted semen (Puglisi *et al.,* 2006; Xu *et al.,* 2009; Carvalho *et al.,* 2010; López *et al.,* 2013). However, reduction in the blastocyst development and yield in the case of sorted semen was observed (Mikkola and Taponen, 2017). Nevertheless, overall calving rates were not different from sorted and conventional spermatozoa (Rasmussen *et al.,* 2013). The use of sorted semen has bought about 90% gender bias. The developmental rates, pregnancy rates and cryoresistance of embryos produced were comparable between conventional and sorted semen (Trigal *et al.* 2012). The conception rates of Holstein heifers and cows were analyzed using sorted semen. Compared with that of the conventional semen, the conception rates for cows and heifers were only 70% and 83% good (Norman *et al.* 2010). However, increasing the count of sorted spermatozoa further improves the conception rate (Garner and Seidel, 2008; DeJarnette *et al.,* 2010, 2011).

FACS is the commercialized technique, provides more than 90% purity and accuracy. However, sex sorting using flow cytometry has serious limitations, which include spermatozoa damage and low fertility rates apart from slow sorting rate, high cost and specialized machine with skilled personnel. Besides these, the sorting facility needs to be linked with the bull semen bank, as sorting of the fresh semen is a pre-requisite for the semen cryopreservation (Seidel, 2003, 2007). The spermatozoa are exposed to various kinds of stressors during the sorting process such as staining of the gametes using fluorescent stains, laser exposure, high dilution, elevated pressure and several changes in the media composition (Garner, 2006). Although spermatozoa viability of the sexed semen is affected by these stressors resulting in low conception rates, offsprings have no detectable abnormalities compared to conventional semen (Seidel, 2003; Healy *et al.,* 2013).

Raman spectroscopy technique

Raman spectroscopy works on the principle of the inelastic scattering process (De Luca *et al.,* 2014). The energy is transferred in either way; from the incident photon to the molecule or from molecule to scattered photon and therefore, there is a difference in the energy level between the Raman scattered photon and incident photon. This property allows it to depict the structure and properties of the molecule from their stretching and bending vibrational transitions (De Luca *et al.,* 2014). Raman micro-spectroscopy was primarily employed in the study of fixed amembranous human spermatozoa (Huser *et al.,* 2009). Protein- and DNA- related differences in the Raman spectra of spermatozoa chromatin in normal and abnormal spermatozoa nuclei were observed (Huser *et al.,* 2009). Raman spectroscopy is suitable for *in vivo* studies as the laser power and excitation wavelengths used are not harmful to the biological sample (De Luca *et al.,* 2014). The study targeted the nucleus in the neck-region of X- and Y-spermatozoa which show the major biochemical differences. Major variations were observed in the DNA content and proteins in the nuclear neck-region between X- and Y- chromosome bearing spermatozoa. Variations are mainly because of the differences in the quantity of DNA between X- and Y- spermatozoa. Increased intensity in the peaks in the X- spermatozoa indicates high DNA content compared to Y-spermatozoa. Raman spectroscopy is a label-free, convenient and non-invasive method; however, the data

Table 2: Differentially expressed proteins identified through proteomic approaches so far. The identified proteins are arranged in the table according to their biological functions. The differential proteins present on the spermatozoa membranes can be employed for sorting of X- and Y- spermatozoa through Immunological approaches.

Protein	X- spermatozoa	Molecular function	References
L-asparaginase	↑	Amino acid catabolism	(De Canio *et al.*, 2014)
Calmodulin	↑	Regulatory protein	(De Canio *et al.*, 2014)
A Chain A, episelection: Novel Ki~nanomolar inhibitors of serine proteases selected by binding or chemistry on an enzyme surface	↑	Inhibitor of serine proteases	(Chen *et al.*, 2012)
Histone demethylase UTY	↓	Demethylase	(Chen *et al.*, 2014)
DPH3 homolog	↑	Protein synthesis	(Chen *et al.*, 2014)
Toll-like receptor 7 and 8 (TLR7/8)	↑	Immunity	(Umehara *et al.*, 2019)
Sex-determining region Y (SRY)	↓	Male phenotype	(Hashimotoa *et al.*, 2013)
FUN14 domain-containing protein 2	↑	Mitochondrial outer membrane	(Scott *et al.*, 2018)
Sorting and assembly machinery component 50 homolog	↑		(Scott *et al.*, 2018)
EF-hand domain-containing protein 1	↓	Fertilization	(Scott *et al.*, 2018)
Seminal plasma protein PDC 109	↑		(De Canio *et al.*, 2014)
Sperm acrosome membrane associated protein 1	↑		(De Canio *et al.*, 2014)
Outer dense fiber protein 2	↑	Structural protein	(De Canio *et al.*, 2014)
Tubulin beta 4A	↑		(De Canio *et al.*, 2014)
Dynein intermediate chain 2, axonemal	↓		(Scott *et al.*, 2018)
Tubulin alpha-3 chain (TUBA3)	↑		(Chen *et al.*, 2014; De Canio *et al.*, 2014)
Outer dense fiber protein 1	↑		(De Canio *et al.*, 2014)
A kinase anchor protein 3	↑		(De Canio *et al.*, 2014)
Tubulin beta 4B	↑		(Chen *et al.*, 2012; De Canio *et al.*, 2014)
Outer dense fiber protein 3	↑		(De Canio *et al.*, 2014)
Tubulin alpha 8	↓		(De Canio *et al.*, 2014)
Chain A, the structure of crystalline profilin-beta-Actin	↓		(Chen *et al.*, 2012)
Tubulin beta 2B	↓		(De Canio *et al.*, 2014)
F-actin-capping protein subunit beta (CAPZB)	↓		(Chen *et al.*, 2012)
Glyceraldehyde 3 phosphate dehydrogenase	↑	Energy metabolism	(De Canio *et al.*, 2014)
L-lactate dehydrogenase A	↑		(De Canio *et al.*, 2014)
Iron-sulfur cluster assembly enzyme ISCU, mitochondrial	↑		(Chen *et al.*, 2014)
Glyceraldehyde 3 phosphate dehydrogenasetestis specific	↑		(De Canio *et al.*, 2014)
Triosephosphate isomerise	↑		(De Canio *et al.*, 2014)
Chain A, crystal structure of bovine heart mitochondrial	↓		(Chen *et al.*, 2012)
Bc1 with Jg144 inhibitor			(Chen *et al.*, 2012)
ATP synthase subunit beta, mitochondrial (ATP5B)	↓		(Chen *et al.*, 2012)
3-hydroxyisobutyrate dehydrogenase (HIBADH)	↑		(Chen *et al.*, 2012)
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (IDH3A)	↑		(Chen *et al.*, 2012)
Cytochrome b–c1 complex subunit 1, mitochondrial (UQCRC1)	↑		(Chen *et al.*, 2012)
Isocitrate dehydrogenase 3 (NAD+) alpha (IDH3A)	↑		(Chen *et al.*, 2012)
Cytochrome b	↑		(Chen *et al.*, 2014)
NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	↑		(Scott *et al.*, 2018)
Pyruvate dehydrogenase protein X component	↓		(Scott *et al.*, 2018)
Cytochrome c oxidase subunit 2	↑		(Scott *et al.*, 2018)
Oxidase heme a, cytochrome	↑		(Chen *et al.*, 2012)
Acetyl-CoA carboxylase, type beta	↑		(Scott *et al.*, 2018)
Glutathione-S-transferase, mu 3 (brain) (GSTM3)	↓	Cellular defense and stress	(Chen *et al.*, 2012)
PREDICTED: similar to neutral sphingomyelinase (N-SMase) activation associated factor (NSMAF)	↑		(Chen *et al.*, 2012)

on final application of the technique for the sorting process is not available.

Lumisort technique

Lumisort is a next-generation laser-based technology developed by a Canadian Company, Microbix Biosystems. Lumisort technology was initiated in the year 2005. The main focus of the technology is to obtain good quality sexed spermatozoa. The mechanism is quite similar to that of flow cytometry which allows the spermatozoa cells to move in a fluid steam one at a time. Based on the laser detection a particular population of spermatozoa is eliminated. The technique kills one population of cells and the desired spermatozoa cell population are undisturbed, hence minimal damage to the desired cells. The technique can be useful to sort all kinds of non-symmetrical and non-spherical cells such as spermatozoa cells, unlike conventional flow cytometry. Reports on the conception-rates of the laser sexed-semen are not available; it is expected to be identical to that of conventional semen. The yield is expected to be 250% higher compared to the current market technology like flow cytometry with sorting speed of 100,000 cells/sec ("CNW | Microbix Announces Proof-of-Concept Demonstration for LumiSort™ - its Livestock Semen Sexing Technology" 2018; "Lumisort - Microbix Biosystems" 2019). Lumisort enables one to obtain a good yield of sexed semen with good quality, rapidness and fertility and it overcomes the drawback of existing spermatozoa sorting technology in terms of damage, sorting speed and yield. However, the quality of the spermatozoa, conception rate and abnormalities related to the development of the fetus has to be further examined.

Immuno-sexing approach

Studies on the identification of spermatozoa protein markers to sort X- and Y- spermatozoa through immunological spermatozoa sexing are being carried out extensively by many researchers. The drawbacks in the above mentioned different techniques considering the efficiency, cost, effectiveness, time, spermatozoa damage, *etc*. necessitate the development of an alternate method with a convenient, non-invasive and inexpensive approach. Immunological spermatozoa sexing can be one such convenient method. Though the presence of sex-specific proteins and their usefulness in sorting spermatozoa has been described for the past four decades since the 1980s (Ali, 1986; Bradley, 1989), commercial application of this technology has not picked up the momentum yet. Sex-specific antigens are explored to identify the differences in protein profile between X- and Y- chromosome bearing spermatozoa. Detectable serological H-Y antigen (male-specific) on half of the population of mammalian spermatozoa could be used as a potential target for immuno-sexing (Bradley, 1989). A preliminary study raised the sex-specific antibodies against sex-specific proteins on the bovine spermatozoa membrane and set an initial step for sexing experiments through an immunological approach (Blecher *et al.,* 1999). Another elegant study, employing 2-Dimensional gel electrophoresis identified 42 significant spots, which were differentially expressed between the X- and Y- spermatozoa of the bull. High-throughput proteomic analysis of these proteins spots revealed that these proteins were closely associated with the cytoskeleton of flagella, stress maintenance, energy metabolism and the activity of serine proteases (Chen *et al.,* 2012). Recently, suppressive subtractive hybridization coupled with cDNA microarray analysis of bovine spermatozoa revealed 27 and 4 up-regulated genes in X- and Y- spermatozoa, respectively (Chen *et al.,* 2014). Such differential expression of genes may induce differences in protein content as well which becomes the basis for immunological spermatozoa sexing (Table 2).

Nano ultra-performance liquid chromatography-tandem mass spectrometry was employed for a comparative study of pooled sex samples of bovine semen. The study identified 17 differentially expressed proteins among which 2 proteins were up-regulated in Y- spermatozoa such as tubulin alpha 8, tubulin beta 2B and 15 proteins in X-spermatozoa such as seminal plasma protein PDC 109, glyceraldehyde 3 phosphate dehydrogenase, outer dense fibre protein 1 *etc*. (De Canio *et al.,* 2014). These differentially expressed proteins were suggested to be associated with the cytoskeleton of flagella and some were glycolytic enzymes (De Canio *et al.,* 2014). Added to it, protein profiling by SWATH-Mass Spectrometry analysis identified eight proteins which were differentially expressed between X- and Y-

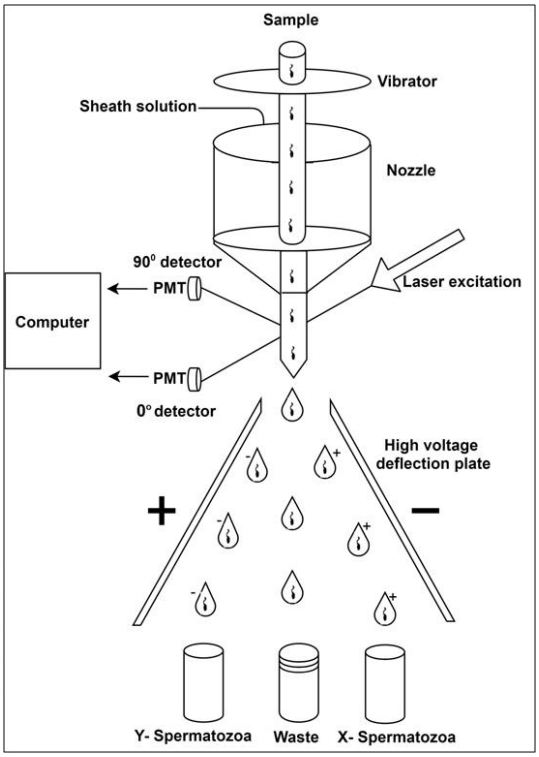

Fig 1: Schematic representation of Flow cytometry (Referred from Naniwa *et al.,* 2019).

bearing spermatozoa. EF-hand domain-containing protein-1 was found to be abundant in X- spermatozoa and FUN14, acetyl-CoA carboxylase type beta, cytochrome C oxidase subunit 2 in Y- spermatozoa. Many of these were mitochondrial proteins involved in energy production (Scott *et al.,* 2018).

In further studies, sex-specific antibodies against X-spermatozoa of bovine were produced and were used to immuno-precipitate proteins in unsorted or sorted X- or Y-bovine spermatozoa. Three major sex-specific proteins (30 kDa) in X- spermatozoa were identified by 2- dimensional electrophoresis. Also, these X- specific antibodies were used to sort X- spermatozoa and used for ICSI (Intracytoplasmic Sperm Injection). About 74.3% were found to be female embryos although more promising validation was needed (Yang *et al.,* 2014). Recently, a monoclonal antibody (Wholemom˜) raised against the bull spermatozoa surface epitope was employed for the production of sex preselected embryos (Chowdhury *et al.,* 2019). Such immunogenic spermatozoa sexing methods are non-invasive and also can be efficient and economical for the livestock sector as sophisticated equipment is not needed. Another advantage of immuno-sexing of spermatozoa is that it may provide less stress as compared to flow cytometric sorting.

Sex-determining region Y (SRY), is identified to be specific to Y- spermatozoa become a key protein for the separation of the X- and Y- spermatozoa (Li *et al.,* 2011; Han *et al.,* 2018). In a study conducted in mice, researchers have conjugated the Cy3-SRY antibody-containing a magnetic bead to pull down the Y- spermatozoa. Higher purity of Y- spermatozoa was obtained than X- spermatozoa (Hashimotoa *et al.,* 2013). Magnetic nanoparticle-based separation based on the interaction between the magnetic nanoparticle and zeta potential of spermatozoa employed in donkeys resulted in 90% purity of X- spermatozoa (Domínguez *et al.,* 2018). Magnetic nanoparticle-based sorting of spermatozoa is a simple, easy and fast. However, higher purity can be obtained by improving the efficiency of antibody tagging.

Recently, a simple method employed to separate X- and Y- spermatozoa yielded 90% male and 81% female litters in mice. The authors focused on X- spermatozoa specific receptor, toll-like receptor 7 and 8 (TLR7/8). Treating mice spermatozoa with TLR7/8 receptor-specific ligand yielded a large proportion of fast swimming Y- spermatozoa and slow swimming X- spermatozoa. The slow movement of X- spermatozoa was linked to the blockage of the TLR7/8 receptor linked with the production of ATP (Umehara *et al.* 2019). Further, the application of the technique in larger mammals has to be ensured.

Challenges in the field of immunogenic spermatozoa sexing are, it requires identification of spermatozoa surface protein because internal protein may not be effective for sorting. It can be overcome by employing advanced mass spectrometers it is possible to identify the surface proteins with great precision. The identified membrane protein it can be effectively integrated with the immunogenic methods such as magnetic-activated cell sorting (MACS), microfluidics, aptamer ion-exchange chromatography, affinity chromatography etc., Such potential techniques will be efficient in sorting X- and Y- spermatozoa in the future.

CONCLUSION

Obtaining the offspring of the desired sex is every farmer's goal to suit the farm productivity and improve the economy. Overall, significant efforts have been made to come up with techniques to produce the offspring of the desired sex. So far, flow cytometry is the only reliable technique to obtain the desired sex which is validated in variety of species. Although, lumisort seems to be having higher efficiency and efficacy to sort X- and Y-spermatozoa, the sex ratio and conception rates has to be confirmed through field trials at a large scale. The techniques including modified swim-up, percoll gradient centrifugation and Raman spectroscopy is still at the experimental level and further improvement is needed. Immunological sexing is a reliable approach; however, it has to be translated to the field level. Hence more research is needed in sorting of X- and Y- spermatozoa and development of newer techniques with a minimum input cost as well as field verification of conception rate in larger scale and cross checking of sex ratio at parturition is warranted.

Conflict of interest

The authors declare no conflicts of interest.

ACKNOWLEDGEMENT

This research did not receive any specific funding

REFERENCES

Ali, J.I. (1986). Seperation of bovine X- from Y-chromosome bearing spermatozoa using monoclonal H-Y antibodies. ETD collection for University of Nebraska, Lincoln. https://digitalcommons.unl.edu/dissertations/AAI8624577.

Annual report (2019-20). Department of Animal Husbandry and Dairying Ministry of Fisheries, Animal Husbandry and Dairying Government of India.

Asma-ul-Husna, Awan, M.A., Mehmood, A., Sultana, T., Shahzad, Q., Ansari, M.S., Rakha, B.A., Saqlan Naqvi, S.M., Akhter, S. (2017). Sperm sexing in Nili-Ravi buffalo through modified swim up: Validation using SYBR ® green real-time PCR. Animal Reproduction Science. 182: 69-76.

Azizeddin, A., Ashkar. F., King, W., Revay. T. (2014). Enrichment of Y-Chromosome-Bearing Bull Spermatozoa by Swim-up Through a Column. Reproduction in Domestic Animals. 49: e1-e4. https://doi.org/10.1111/rda.12252.

Bathgate. R., Mace. N., Heasman. K., Evans, G., Maxwell, W.M.C., de Graaf, S.P. (2013). Birth of kids after artificial insemination with sex-sorted, frozen-thawed goat spermatozoa. Reproduction in Domestic Animals. 48: 893-898.

Bennett. D., Boyse, E.A. (1973). Sex Ratio in Progeny of Mice Inseminated with Sperm treated with H-Y Antiserum. Nature. 246: 308-309.

Blecher, S.R., Howie, R., Li, S., Detmar. J., Blahut, L. (1999). A new approach to immunological sexing of sperm. Theriogenology. 52: 1309-1321. https://doi.org/10.1016/S0093-691X (99) 00219-8.

Bradley, M.P. (1989). Immunological Sexing of Mammalian Semen: Current Status and Future Options. Journal of Dairy Science. 72: 3372-3380.

Buchanan, B.R., Seidel, G.E., McCue, P.M., Schenk, J.L., Herickhoff, L.A., Squires, E.L. (2000). Insemination of mares with low numbers of either unsexed or sexed spermatozoa. Theriogenology. 5: 1333-44.

Carvalho, J.O., Sartori, R., Machado, G.M., Mourão, G.B., Dode, M.A.N. (2010). Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in *in vitro* embryo production. Theriogenolog. 74: 1521-1530. https://doi.org/10.1016/j.theriogenology.2010.06.030.

Check, J.H., Shanis, B.S., Cooper, S.O., Bollendorf, A. (1989). Male sex preselection: Swim-up technique and insemination of women after ovulation induction. Achives of Andrology. 23: 165-166.

Chen, X., Yue, Y., He, Y., Zhu, H., Hao, H., Zhao, X., Qin, T., Wang, D. (2014). Identification and characterization of genes differentially expressed in X and Y sperm using suppression subtractive hybridization and cDNA microarray. Molecular Reproduction and Development. 81: 908-17. https://doi.org/10.1002/mrd.22386.

Chen, X., Zhu, H., Wu, C., Han, W., Hao, H., Zhao, X., Du, W., Q in, T., Liu, Y., Wang, D. (2012). Identification of differentially expressed proteins between bull X and Y spermatozoa. Journal of Proteomics. 77: 59-67.

Chowdhury, M.M.R., Lianguang, X., Kong, R., Park, B.Y., Mesalam, A., Joo, M.D., Afrin, F., Jin, J.I., Lim, H.T., Kon, I.K. (2019). *In vitro* production of sex preselected cattle embryos using a monoclonal antibody raised against bull sperm epitopes. Animal Reproduction Science. 205: 156-64. https://doi.org/10.1016/J.ANIREPROSCI.2018.11.006.

Claassens, O.E., Stander, F.S., Kruger, T.F., Menkveld, R., Lombard, C.J. (1989). Does the wash-up and swim-up method of semen preparation play a role in sex selection? Archives of Andrology. 23: 23-26.

Clulow, J.R., Buss, H., Sieme, H., Rodger, J.A., Cawdell-Smith, A.J., Evans, G., Rath, D., Morris, L.H.A., Maxwell, W.M.C. (2008). Field fertility of sex-sorted and non-sorted frozen-thawed stallion spermatozoa. Animal Reproduction Science. 108: 287-297. https://doi.org/10.1016/j.anireprosci. 2007. 08.015.

De Canio, M., Soggiu, A., Piras, C., Bonizzi, L., Galli, A., Urbani. A., Roncada, P. (2014). Differential protein profile in sexed bovine semen: shotgun proteomics investigation. Molecular BioSystems. 10: 1264-1271.

De Graaf, S., Evans, G., Maxwell, W., Downing, J., O'Brien, J. (2007). Successful Low Dose Insemination of Flow Cytometrically Sorted Ram Spermatozoa in Sheep. Reproduction in Domestic Animals. 42: 648-653. https://doi.org/10.1111/j.1439-0531.2007.00837.

De Luca, A.C., Managó, S., Ferrara, M.A., Rendina, I., Sirleto, L., Puglisi, R., Balduzzi, D., Galli, A., Ferraro, P., Coppola, G. (2014). Non-invasive sex assessment in bovine semen by Raman spectroscopy. Laser Physics Letters. 11: 055604.

DeJarnette, J.M., Leach, M.A., Nebel, R.L., Marshall, C.E., McCleary, C.R., Moreno, J.F. (2011). Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: Is comparable fertility of sex-sorted and conventional semen plausible? Journal of Dairy Science. 94: 3477-3483. https://doi.org/10.3168/jds.2011-4214.

DeJarnette, J.M., McCleary, C.R., Leach, M.A., Moreno, J.F., Nebel, R.L. and Marshall, C.E. (2010). Effects of 2.1 and 3.5×106 sex-sorted sperm dosages on conception rates of Holstein cows and heifers. Journal of Dairy Science. 93: 4079-4085.

Dell'Eva, G., Bolognini, D., Iacono, E. and Merlo, B. (2019). Superovulation protocols for dairy cows bred with SexedULTRA™ sex sorted semen. Reproduction in Domestics Animals. 54: 756-761. https://doi.org/10.1111/rda.13421.

Domínguez, E., Moreno-Irusta, A., Castex, H.R., Bragulat, A.F., Ugaz, C., *et al.* (2018). Sperm Sexing Mediated by Magnetic Nanoparticles in Donkeys, a Preliminary *In Vitro* Study. Journal of Equine Veterinary Science. 65: 123-127. https://doi.org/10.1016/j.jevs. 2018. 04.005.

Garner, D.L. (2006). Flow cytometric sexing of mammalian sperm. Theriogenology. 65: 943-957.

Garner, D.L., Seidel, G.E. (2008) History of commercializing sexed semen for cattle. Theriogenology. 69: 886-895. https://doi.org/10.1016/j.theriogenology.2008.01.006.

Garner, D.L., Evans, K.M., Seidel, G.E. (2013). Sex-Sorting Sperm Using Flow Cytometry/Cell Sorting. Methods in Molecular Biology. 279-295.

Garner, D.L., Gledhill, B.L., Pinkel, D., Lake, S., Stephenson, D., *et al.* (1983). Quantification of the X- and Y-Chromosome -Bearing Spermatozoa of Domestic Animals by Flow Cytometry 1. Biology of Reproduction. 28: 312-321.

González-Marín, C., Góngora, C.E., Gilligan, T.B., Evans, K.M., Moreno, J.F., Vishwanath, R. (2018). *In vitro* sperm quality and DNA integrity of SexedULTRA™ sex-sorted sperm compared to non-sorted bovine sperm. Theriogenology. 114: 40-45.

Han, C.M., Chen, R., Li, T., Chen, XL., Zheng, Y.F., Ma, M.T., Gao, Q.H. (2018). The bovine sex-determining region Y (Sry) gene and its mRNA transcript are present in Y sperm but not X sperm of bulls. Animal Biology. 68: 321-322. https://doi.org/10.1163/15707563-17000105.

Han, T.L., Flaherty, S.P., Ford, J.H., Matthews, C.D. (1993). Detection of X- and Y-bearing human spermatozoa after motile sperm isolation by swim-up. Fertility and Sterility. 60: 1046-1051. https://doi.org/10.1016/S0015-0282 (16) 56408-5.

Hashimotoa, H., Eto, T., Suemizu, H., Ito, M. (2013). Application of a new convenience gender sorting method for mouse spermatozoa to mouse reproductive engineering technology. Journal of Veterinary Medical Science. 75: 231-235. https://doi.org/10.1292/jvms.12-0303.

Healy, A., House, J., Thomson, P. (2013). Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. Journal of Dairy Science. 96: 1905-1914.

Huser, T., Orme, C.A., Hollars, C.W., Corzett, M.H., Balhorn, R. (2009). Raman spectroscopy of DNA packaging in individual human sperm cells distinguishes normal from abnormal cells. Journal of Biophotonics. 2: 322-332. https://doi.org/10.1002/jbio.200910012.

Iizuka, R., Kaneko, S., Aoki, R., Kobayashi, T. (1987). Sexing of human sperm by discontinuous Percoll density gradient and its clinical application. Human Reproduction, (Oxford, England) 2: 573-75. https://doi.org/10.1093/oxford journals.humrep.a136591.

Johnson, L., Guthrie, H.D., Fiser, P., Maxwell, W.M.C., Welch, G.R., Garrett, W.M. (2000). Cryopreservation of flow cytometrically sorted boar sperm: effects on in vivo embryo developmen. Journal of Animal Science. 78.

Johnson, L.A. (1991). Sex Preselection in Swine: Altered Sex Ratios in Offspring following Surgical Insemination of Flow Sorted X and Y Bearing Sperm. Reproduction in Domestic Animals. 26: 309-314.

Johnson, L.A., Welch, G.R. (1999). Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology. 52: 1323-1341.

Kjelland, M.E., González-Marín, C., Gosálvez, J., López-Fernández, C., Lenz, R.W., Evans, K.M., Moreno, J.F. (2011). DNA fragmentation kinetics and postthaw motility of flow cytometric-sorted white-tailed deer sperm. Journal of Animal Science. 89: 3996-4006.

Kobayashi, J., Oguro, H., Uchida, H., Kohsaka, T., Sasada, H., Sato, E. (2004). Assessment of bovine X- and Y-bearing spermatozoa in fractions by discontinuous percoll gradients with Rapid Fluorescence In Situ Hybridization. Journal of Reproduction and Development. 50: 463-469.

Li Chunjin., Sun,.Y, Yi, K., Li, Chengjiao., Zhu, X., Chen, L., Zhou, X. (2011). Detection of the SRY Transcript and Protein in Bovine Ejaculated Spermatozoa. Asian-Australasian Journal of Animal Sciences. 24: 1358-1364.

Lin, S., Lee, R.K.K., Tsai, Y.J., Hwu, Y.M., Lin, M.H. (1998). Separating X-bearing human spermatozoa through a discontinuous percoll density gradient proved to be inefficient by double-label fluorescent in situ hybridization. Journal of Assisted Reproduction and Genetics. 15: 565-569.

López, S.R., de Souza, J.C., González, J.Z., De Ondiz Sánchez, A., Romero-Aguirregomezcorta, J., de Carvalho, R.R., Rath, D. (2013). Use of sex-sorted and unsorted frozen/thawed sperm and in vitro fertilization events in bovine oocytes derived from ultrasound-guided aspiration. Revista Brasileira de Zootecnia. 42: 721-727.

Lu, Y., Zhang, M., Lu, S., Xu, D., Huang, W., Meng, B., Xu, H., Lu, K. (2010). Sex-preselected buffalo (*Bubalus bubalis*) calves derived from artificial insemination with sexed sperm. Animal Reproduction Science. 119: 169-171.

Lucio, A., Resende, M., Dernowseck-Meirelles, J.A., Perini, A.P., Oliveira, L., *et al.* (2012). Assessment of swim-up and discontinuous density gradient in sperm sex preselection for bovine embryo production. Arquivo Brasileiro de Medicina Veterinária e Zootecnia. 64: 525-532.

Lumisort™ technology. In: Microbix, 2019 https://microbix.com/pages/lumisort-old. Accessed 3 Jan 2020.

Martinez, E., Vazquez, J.M., Roca, J., Lucas, X., Gil, M., Parrrilla, I., Vazquez, J., Day, B. (2001). Successful non-surgical deep intrauterine insemination with small numbers of spermatozoa in sows. Reproduction. 122: 289-296.

Maxwell, W.M., Evans, G., Hollinshead, F., Bathgate, R., de Graaf S., *et al.* (2004). Integration of sperm sexing technology into the ART toolbox. Animal Reproduction Science. 82: 79-95.

Mikkola, M., Taponen, J. (2017). Quality and developmental rate of embryos produced with sex-sorted and conventional semen from superovulated dairy cattle. Theriogenology. 87: 135-140.

Naniwa, Y., Sakamoto, Y., Toda, S. and Uchiyama, K. (2019). Bovine sperm sex selection technology in Japan. Reproductive Medicine and Biology. 18: 17-26.

Norman, H.D., Hutchison, J.L., Miller, R.H. (2010). Use of sexed semen and its effect on conception rate, calf sex, dystocia and stillbirth of Holsteins in the United States. Journal of Dairy Science. 93: 3880-3890.

O'Brien, J.K., Steinman, K.J., Robeck, T.R. (2009). Application of sperm sorting and associated reproductive technology for wildlife management and conservation. Theriogenology. 71: 98-107. https://doi.org/10.1016/j.theriogenology.2008.09.052.

O'Brien, J.K., Stojanov, T., Crichton, E.G., Evans, K.M., Leigh, D., Maxwell, W.M.C., Evans, G., Loskutoff, N.M. (2005). Flow cytometric sorting of fresh and frozen-thawed spermatozoa in the western lowland gorilla (Gorilla gorilla gorilla). American Journal of Primatology. 66: 297-315.

Pearson, P., Bobrow, M., Vosa (1970). Technique for identifying Y chromosomes in human interphase nuclei. Nature. 226: 78-80.

Pope, C.E., Crichton, E.G., Gómez, M.C., Dumas, C., Dresser, B.L. (2009). Birth of domestic cat kittens of predetermined sex after transfer of embryos produced by *in vitro* fertilization of oocytes with flow-sorted sperm. Theriogenology. 71: 864-871.

Promthep, K., Satitmanwiwat, S., Kitiyanant, N., Tantiwattanakul, P., Jirajaroenrat, K., Sitthigripong, R., Singhapol, C.S. (2016). Practical use of percoll density gradient centrifugation on sperm sex determination in commercial dairy farm in Thailand. Indian Journal of Animal Research. 50: 310-313.

Puglisi, R., Vanni, R., Galli, A., Balduzzi, D., Parati, K., Bongioni, G., Crotti, G., Duchi, R., Galli, C., Lazzari, G., Aleandri, R. (2006). *In vitro* fertilisation with frozen-thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. Reproduction. 132: 519-526. https://doi.org/10.1530/rep.1.01173.

Rasmussen, S., Block, J., Seidel, G.E., Brink, Z., McSweeney, K., Farin, P.W., Bonilla, L., Hansen, P.J. (2013). Pregnancy rates of lactating cows after transfer of *in vitro* produced embryos using X-sorted sperm. Theriogenology. 79: 453-461. https://doi.org/10.1016/j.theriogenology. 2012.10.017.

Rath, D., Ruiz, S., Sieg, B. (2003). Birth of female piglets following intrauterine insemination of a sow using flow cytometrically sexed boar semen. Veterinary Record. 152: 400-401. https://doi.org/10.1136/vr.152.13.400.

Schenk, J., DeGrofft, D, (2003). Insemination of cow elk with sexed frozen semen. Theriogenology. 59: 514.

Scott, C., de Souza, F.F., Aristizabal, V.H.V., Hethrington, L., Krisp, C., Molloy. M., Baker, M.A., Dell'Aqua, J.A. (2018). Proteomic profile of sex-sorted bull sperm evaluated by SWATH-MS analysis. Animal Reproduction Science. 198: 121-128.

Seidel, G.E. (2003). Sexing mammalian sperm-intertwining of commerce, technology and biology. Animal Reproduction Science. 79: 145-156.

Seidel, G.E. (2007). Overview of sexing sperm. Theriogenology. 68: 443-446.

Sharpe, J., Evans, K. (2009). Advances in flow cytometry for sperm sexing. Theriogenology. 71: 4-10.

Thomas, J.M., Locke, J.W.C., Vishwanath, R., Hall, J.B., Ellersieck, M.R., Smith, M.F. and Patterson, D.J. (2017). Effective use of SexedULTRA™ sex-sorted semen for timed artificial insemination of beef heifers. Theriogenology. 98: 88-93.

Thomsen, J.L., Niebuhr, E. (1986). The frequency of false-positive and false-negative results in the detection of Y-chromosomes in interphase nuclei. Human Genetics. 73: 27-30. https://doi.org/10.1007/BF00292659.

Trigal, B., Gómez, E., Caamaño, J.N., Muñoz, M., Moreno, J., Carrocera, S., Martín, D., Diez, C. (2012). *In vitro* and *in vivo* quality of bovine embryos in vitro produced with sex-sorted sperm. Theriogenolog. 78: 1465-1475.

Umehara, T., Tsujita, N., Shimada, M. (2019). Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. PLOS Biolology. 17: e3000398.

van Kooij, R.J., van Oost, B.A. (1992). Determination of sex ratio of spermatozoa with a deoxyribonucleic acid-probe and quinacrine staining: a comparison. Fertility and Sterility. 58: 384-386.

Vishwanath, R., Moreno, J.F. (2018). Review: Semen sexing - current state of the art with emphasis on bovine species. Animal. 12: s85-s96.

Wang, H.X., Flaherty, S.P., Swann, N.J. and Matthews, C.D. (1994). Assessment of the separation of X-and Y-bearing sperm on albumin gradients using double-label fluorescence in situ hybridization. Fertility and Sterility. 61: 720-726.

Wei, Y.F., Chen, F.L., Tang, S.S., Mao, A.G., Li, L.G., Cheng, L.G., Chen, C., Li, F.X., Wang, B., Xu, T., Zhang, Y.J., Li, J., Wan, J.S. (2017). Birth of puppies of predetermined sex after artificial insemination with a low number of sex-sorted, frozen-thawed spermatozoa in field conditions. Animal Science Journal. 88: 1232-1238.

Wolf, C.A., Brass, K.E., Rubin, M.I.B, Pozzobon, S.E., Mozzaquatro, F.D., De La Corte, F.D. (2008). The effect of sperm selection by Percoll or swim-up on the sex ratio of *in vitro* produced bovine embryos. Animal Reproduction. 5: 110-115.

Xu, J., Chaubal, S.A., Du, F. (2009). Optimizing IVF with sexed sperm in cattle. Theriogenology. 71: 39-47. https://doi.org/10.1016/j.theriogenology.2008.09.012.

Yang, W.C., Sang, L., Xiaon,Y., Zhang, H.L., Tang, K.Q., Yang, L.G. (2014). Tentative identification of sex-specific antibodies and their application for screening bovine sperm proteins for sex-specificity. Molecular Biology Reports. 41: 217-223.