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Sex sorting technology: A brief review

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Abstract
The most useful tool in assisted reproduction is called "sperm sorting," which divides sperm cells into two groups based on which chromosomes are X (female) or Y (male). This process permits the selection of "Healthy" sperm and the purpose of particular traits, like sex. Dr. Pinkel also developed the first flow cytometer. The sperm sorting technology known as the Beltsville sperm sexing technology was patented by the USDA in April of 1991. Advanced cell sorting devices called flow cytometers employ a laser to stimulate fluorescent dye that attaches to spermatozoa's DNA. Limitation of sex sorting technology includes the cost of equipment and maintenance etc. The area of sexed semen is to increase pregnancy rates. The usage of sexed semen for the generation of female calves is beneficial because the demand for milk production is rising everyday. This technology is a good technique which is largely required at the field level for the production of desired offspring and for sexed semen to be adopted nationally, farmers must receive financial assistance and education.

Keywords: Sperm-sorting, flow cytometer, Beltsville, laser

1. Introduction
For many years, animal scientists and livestock owners have dreamed of having a calf of their desired sex. In the case of cows, pre-conception selection for a particular sex is typically economically justified. Due to the mechanisation of agriculture, male cows are raised by livestock owners less frequently, and when they do, the animals are either slaughtered—which is illegal in the majority of Indian states—or left to starve to death. So, we can use sex-sorted semen technology to protect ourselves from this unusual predicament of having "unwanted" male calf born (Gaur *et al.*, 2020) [12]. The most useful tool in assisted reproduction is sperm sorting, which divides sperm cells into two groups with X (female) and Y (male) chromosomes, respectively. This process allows for the selection of "healthy" sperm and the determination of particular traits, such as sex (Garner *et al.*, 1983) [11]. It is possible to distinguish between X- and Y-chromosome-bearing sperm in cattle rapidly because an X-chromosome-bearing sperm has 3.8% more DNA than a Y-chromosome-bearing sperm (Holden and Butler, 2018) [17]. Employing sex-sorted semen raises the percentage of female dairy offspring from artificial insemination, which speeds up herd growth. This also makes it possible to profitably sell surplus breeding females, which encourages increased use of beef semen to raise the value of an excess calves sold for beef production and boost profitability (Murphy *et al.*, 2016) [32]. Many bovine field studies that reported around 90% of female progeny had been attained by separating X- and Y-bearing sperm (Norman *et al.*, 2010) [33].
For conventional semen straws, liquid semen has a distinct advantage over frozen semen because the reduced sperm concentration per straw (approx. 3-5 × 10^6 vs 15–20 × 10^6 sperm) allows for approximately 3 to 5 times more semen straws to be produced per ejaculate. Flow cytometry is a well-established technique that has been used in cattle for commercial purposes to sort sperm cells (Rath *et al.*, 2013) [39]. The genetic level of the herd increases when sexed semen is used on genetically superior animals (Ettema *et al.*, 2011) [9]. Regarding sperm cell quantity and quality, semen that has been sorted by sex poses special difficulties for use in fixed-time artificial insemination.

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Sex-sorted sperm are seen to be an excellent tool for increasing production efficiency and optimising reproductive management in farm animals, which in turn ensures an adequate supply of food for the expanding human population. This is especially true for *in vitro* fertilisation and artificial insemination (Cottle *et al.*, 2018 and Diers *et al.*, 2020) [2, 7]. Since sperm sexing is still an emerging technology, improvements in the product such as increased fertility, more sexing accuracy, more flexibility in delivery systems, and lower costs continue to be created (Sedial, 2014) [45]. The conception rate from inseminations using frozen sex-sorted semen is usually between 70% and 80% of that from conventional semen at the regular dose rate due to a suboptimal dose rate and damage done to sperm during the sorting process (Xu, 2014) [58]. However, It is not possible to completely compensate for the decreased conception rate with frozen sex-sorted semen by raising the sperm dose rate.

2. History

There was no reliable way to identify the sex of sperm cells before the 1980s. At the Lawrence Livermore National Laboratory, Dr. Daniel Pinkel created the first sperm sorting technology. Additionally, Dr. Pinkel created the first flow cytometer. Researchers from Oklahoma State University and Lawrence Livermore National Laboratory showed that flow cytometry could distinguish between X and Y sperm based on changes in DNA content (Loggan, 2019) [27]. The labelling procedure did not prevent the dye from killing the sperm cells, despite the enhanced sorting procedure. A group of researchers led by Johnson, Flook, Look, and Pinkel figured out the solution when they found that the sperm cells were not killed by the fluorescent dye Hoechst 33342, which is a bisbenzimidazole. The USDA patented the Beltsville sperm sexing technology in April 1991. It is a method of sorting sperm. The machine's inventor was identified as Dr. Lawrence Johnson. The seminal study on semen sexing was published by Dr. Larry Johnson of the US Department of Agriculture (USDA). Using analytical flow cytometry, the first attempts were made to separate sperm containing X and Y. A significant advancement in sperm sexing was documented in 1989 when live progeny was produced from sex-sorted live rabbit spermatozoa following oviductal insemination (Johnson *et al.*, 1989) [25]. Sperm sexing using flow cytometry is a patent process, and the patent currently belongs to M/s X–Y–INC Colorado (USA) (Sharma and Sharma, 2016) [46]. According to Dr. Duane Garner's research, flow cytometry can reliably detect variations in the DNA content of X and Y sperm cells in 4 different mammal species: Sheep, pigs, cattle, and rabbits. A presexed calf and lamb with fresh sorted semen A.I. were born in 1997, while a presexed calf with MOET was born in 2004. Prior to that point, non-frozen sex-sorted semen was utilised to create progeny. The problem was resolved in 1999 when the first effective cryopreservation of the sex-sorted semen was achieved using the egg-yolk-tris buffer media (Schenk *et al.*, 1999) [41].

There was a decrease in the difference in conception rates between conventional and sex-sorted semen. Sexed ULTRA^{TM} 4M semen was introduced by Sexing Technologies (Navasota, Texas) in April 2017. In contrast to the typical dose of 2 x 10^6 sexed sperm cells obtained using the XY technique, the latest product contains 4 x 10^6 living sex-sorted sperm cells (Lenz *et al.*, 2017) [26s]. The technology for sexing bovine semen has improved, leading to the creation of Sexed Ultra^{tm}. The Gensis III sorting technique is the approach now employed by Sexing Technologies. According to the manufacturer, the new technique harms sperm cells less severely (Thomas *et al.*, 2017) [52]. In India the First laboratory has been set up in BAIF, Pune (On September 2018) and under Rastriya Gokul Mission (RGM) First laboratory has been set up in Rishikesh, Uttarakhand (On March 2019) and the 2nd laboratory has been set up in Bhopal, Madhya Pradesh (On March 2021).

Table 1: Functional changes between X and Y sperm

Parameters	Difference measurable
DNA content	Less in Y sperm
Size	X sperm is larger
Fluorescence property	X chromosome is more fluorescence
Motility	Y sperm faster
Surface charge	X sperm migrate towards cathode
Cell surface antigen	H-Y antigen on Y sperm

Johnson, 1995, [22]

3. Methods of sex sorting technique

3.1 Albumin gradient/Gradient swim-down method

The foundation of this technique lies in the variations in the capacity of X and Y bearing spermatozoa to descend in a gradient solution. Spermatozoa having Y chromosomes swim faster downward than spermatozoa bearing X chromosomes because they are smaller and more motile (Moruzzi, 1979) [31]. Ericsson *et al.* (1973) [8] reported the first successful separation of X and Y containing spermatozoa with an albumin gradient. It has been reported that the success rate for this procedure is approximately 75%. Only human spermatozoa could be successfully separated using this procedure; those of other mammals were not. As such, it was never used as a traditional sperm sexing procedure.

3.2 Percoll density gradient

A Percoll column is covered with layers of semen, and spermatozoa are permitted to pass through the column. Because X-bearing spermatozoa have a high sedimentation density, they settle at the bottom of the column whereas Y-bearing spermatozoa remains near the top. The method's success rate varied between 86% and 94% (Van Kooij and Van Oost, 1992) [54]. According to Iwasaki (1988), the method was not successful in isolating spermatozoa containing X or Y. Other research revealed that in 80% of the percoll gradient, the ratio of X to Y sperm was nearly equal, which contradicted the findings of several experts and colleagues (Wang *et al.*, 1994) [56]. The approach was shown to be inefficient for sperm sexing because of these kinds of variations in the results.

3.3 Swim up procedure

Sarkar *et al.* (1984) [40] observation served as the foundation for this methodology. Because they are smaller than X-bearing spermatozoa, Y-bearing spermatozoa are said to swim more quickly. According to reports, this method's success rate was 81%.

3.4 Free flow electrophoresis

The X spermatozoa surface has a negative charge, whereas the Y spermatozoa surface has a positive charge. X and Y spermatozoa have been separated using the variations in surface charges based on the electric field of separation (Mohri *et al.*, 1986) [30]. The sperm motility is greatly decreased after electrophoresis, which limits the procedure's current application. This method's drawback was the sperm's

corresponding decrease in motility following electrophoresis.

3.5 Identification of H-Y antigen

Using the H-Y antigen, attempts have been made to use immunological techniques to render the Y sperm inactive. Because the membranes of both X and Y spermatozoa contain the H-Y antigen, sorting sperm is challenging (Hendriksen *et al.*, 1993) [16]. Large-scale sperm sorting can use this sorting technique. According to Hoppe and Koo (1984) [18], spermatozoa carrying X and Y most likely have a similar surface antigen. Research indicates that, in normal conditions, the X and Y spermatozoa's membranes contain the HY antigen. Therefore, using the HY antigen to sex the spermatozoa is ineffective.

3.6 Sperm sexing based on the volumetric differences

In order to show a difference in sperm head volume depending on the DNA content among X and Y chromosome bearing spermatozoa, this method employs interference microscopy image analysis of spermatozoa. This idea has led to the development of a technique for classifying live spermatozoa utilising interference microscopy optics and a flow cytometer. It has been reported that this method's success rate is less than 80% (Van Munster, 2002) [55]. Since the purity of the spermatozoa from either sex did not surpass 80%, this approach suffered from poor quality assurance, and regrettably, attempts to enhance it were not effective.

3.7 Centrifugal counter current distribution

It was discovered that there was only a 0.0007 g/cm^3 density difference between X and Y that carried bovine spermatozoa, hence this trait was likewise unsuitable for use as a sex sperm characteristic. Using an aqueous two-phase system with centrifugal counter current distribution, Ollero *et al.* (2000) [34] sought to sex ram spermatozoa. It has been reported that this approach has a 75% success rate.

3.8 Immunological sorting of semen

Blecher *et al.* (1999) [1] conducted research in this area. To increase antibodies to sperm membrane proteins, male and female rabbits were immunised by subcutaneously injecting sperm preparations with Freund's incomplete adjuvant. It was discovered that only the "anti-X" antisera caused spermatozoa to agglutinate, while the "anti-Y" antisera did not cause any spermatozoa to agglutinate.

3.9 Flow-cytometric sorting of semen

The most dependable and reproducible technique for producing sex-preselected animals is to sex X and Y chromosome containing sperm based on the difference in DNA content. Because the sperm exhibited random orientation in the flow-cytometer fluid stream, the previous investigations were unable to detect any differences in the DNA quantity between X and Y carrying sperm (Johnson and Welch, 1999) [24]. Pinkel *et al.* (1982) [36] reported the first sperm flow sorting to separate X from Y-bearing spermatozoa. Advanced cell sorting devices called flow cytometers employ a laser to stimulate fluorescent dye that attaches to spermatozoa's DNA. The main concepts for sperm sexing with flow cytometry are the DNA percentage and DNA specific dye. Hoechst 33342, a dye that binds to DNA and is permeable to undamaged sperm membranes, is applied to the spermatozoa in this sorting technique. The sperm cells that have been dyed exhibit a blue glow, with the X-chromosome sperm displaying a stronger tone compared to the Y-chromosome sperm cell (Garner and Seidel, 2008) [10]. The fluorescent dye is quenched by the red dye, revealing only viable sperm cells under illumination.

3.9.1 Procedure

- Spermatozoa that have been stained are moved to an area where each one is subjected to a UV laser beam with a wavelength of 351-364 nm. The intense blue fluorescence that results is then observed and examined.
- Spermatozoa that have been stained are moved to an area where each one is subjected to a UV laser beam with a wavelength of 351-364 nm. The intense blue fluorescence that results is then observed and examined.
- X chromosome-bearing spermatozoa require more dye than Y sperm because of their higher DNA concentration. Before sorting, the living and dead sperm need to be identified.
- A crystal vibrator aligns the stained semen in a single-file stream and divides the stream into droplets holding a single sperm with its head facing the lasers, making it possible to identify the sperm cell (Garner and Seidel, 2008) [10].
- X-bearing sperm are among the 20–30% brightest sperm cells that illuminate. Y-bearing sperm cells make up the 20–30% of sperm cells that glow less brightly. It is impossible to determine with accuracy which sperm cells are left.
- Opposite electric charges are applied to droplets containing recognised sperm cells. Through an electromagnetic field, the sperm drops descend. Brass plates attract cells, which allows sperm to flow into various collection containers.
- The difference in the amount of DNA between spermatozoa containing X and Y in domestic animals ranges from 3 to 4.5% (Johnson, 2000) [23]. There have been reports of an 85–95 percent success rate with this procedure.
- The damage done to sperm by the applied electric charge, UV laser, dye, sorting speed, and pressure is one of the main disadvantages of this technology (Silva *et al.*, 2006) [47].
- The most widely used technique for separating spermatozoa specific to a given sex is flow cytometry-based, and so far, no other technique has consistently shown to be successful in producing progeny of the expected sex (Prakash *et al.*, 2014) [37].

3.9.2 Factors impacting the flow cytometer's sorting efficiency

- Sperm head and fluid orientation in the nozzle.
- Presenting sperm at an appropriate angle of 45^0 degrees to the excitation source.
- 20 to 40 percent of live sperm are undetectable and go straight into the waste tube, even with the proper orientation of the cell and fluid.
- Optical techniques.
- Speed of computer processor.

4. Effect on Conception Rate by Using Sex-Sorted Semen

One significant drawback of sex-sorted semen is that its conception rates are lower than those of conventional semen. Reports on the difference between sex-sorted and conventional semen conception rates range from 60% to 90% (Healy *et al.*, 2013) [15]. According to Hutchinson *et al.* (2013) [20], fresh, non-frozen sexed semen was associated with higher

pregnancy rates. Pregnancy rates for fresh-sexed and frozen-thawed-sexed semen were 94% and 75%, respectively. Similar findings were observed in a New Zealand investigation, where 90–95% of traditional frozen-thawed semen had conception rates. The stress of the sorting process is the reason for the lower conception rates in sexed semen. Stress factors for sperm cells include the following: Light from the laser used to illuminate the DNA; pressure from the collection process; dilution of the semen sample; dying the sperm cells with a DNA binding agent (Hoechst 33342); mechanical forces such as sending the sperm cells via the flow cytometer at 60 miles per hour at 40 pounds per square inch and finally, centrifugation to purify the sample.

The quantity of sperm cells per straw also has an impact on conception rates in sex-sorted semen. A single straw of sexed semen typically contains about 2×10^6 sperm cells. Standard doses of conventional straws contain roughly 15-20 $\times 10^6$ sperm cells (Healy *et al.*, 2013) [15]. The reason for the reduced quantity of sperm cells in sexed straws can be attributed to several factors, including the high expense of the tools and knowledge needed for sorting, the duration required to produce a dosage of sexed semen, and the fluctuations in the viability of bulls' semen during the sorting process. When the amount of sperm per dose was the same for both, the pregnancy rate for sexed sperm was sometimes between 60 and 80 percent of that of unsorted control sperm. Following AI, China reported a conception rate of 69.7% for sexed sperm and 66.5% for unsexed sperm (Lu *et al.*, 2010) [28]. DeJarnette *et al.* (2010) [5] discovered that there was no increase in conception rates when the number of sexed semen cells was increased from 2.1 x 10^6 to 3.5 x 10^6. The conception rates of both dosages of sex-sorted semen were almost 75% of those of conventional semen. Pregnancy rates increased just little (5-7%) when semen dosages being doubled or tripled (4 x 10^6 or 6 x 10^6). When sexed cryopreserved semen was placed into uterine bodies, heifers with 7–20 times more sperm per dosage had a higher pregnancy rate (Seidel *et al.*, 1999) [41].

5. Defects in sperm because of sorting process

Spermatozoa defects are caused by several factors such as dye, sorting speed, pressure, laser light, electrical charge, deviation, and changes in the medium.

5.1 Defects caused by dye

Chromatin DE condenses when DNA-specific dye Hoechst 33342 is added. In sexed sperms, dye-mediated disruptions of the heat shock protein HSP70 and capacitation-like alterations in the sperm membranes have also been documented (Spinaci *et al.*, 2006) [48].

5.2 Sorting speed and pressure

When sorting at a fast speed (55–60 mph) and high pressure (40–60 psi), sperm are more susceptible to DNA damage (Suh *et al.*, 2005) [49].

5.3 Defect caused by U-V laser

The known effect of UV radiation on DNA integrity is negative. Because the chromatin integrity of rabbit sperm was destroyed by laser power of 200 MW or more, their capacity to fertilise was negatively impacted more so than that of sperm subjected to 125 MW (Silva and Gadella, 2006) [47].

5.4 Defect due to electrical charging and deviation

The midpiece and tail sperm membranes depolarize as a result of electrical charge and electrical deviation. Additionally, the electrical forces' production of relative oxygen species decreased sperm mitochondrial function (Rath and Johnson, 2008) [38]. Stressors arising from the sorting process have the potential to cause DNA damage, which could make spermatozoa with normal DNA less viable for embryogenesis (Tesarik *et al.*, 2004) [50].

5.2 Defect due to changes in the medium

Sperm fertilising capacity is reduced during sorting due to changes in pH and osmolarity (Harrison and Gadella, 2005) [14]. Any change in sperm physiology, such as adjustments to motility, membrane stability, or acrosome homeostasis, directly affects the sperm's ability to fertilise, while changed DNA quality influences the quality of the embryo and results in syngamy following gamete fertilisation. De Graaf *et al.* (2008) [59] revealed changes in membrane proteinase as a result of sorting and freezing. Spermatozoa have a shorter lifespan as a result of the sorting processes. A shorter life span results in decreased motility, which lowers spermatozoa fertility through sex sorting (Peippo *et al.*, 2009) [35].

6. Actions Taken to Minimise Spermatozoa Defects during Sex Sorting

- With no discernible drop in sorting efficiency in bull and stallion, the sorted spermatozoa quality was enhanced by reducing the sorting pressure from the normal 50 psi to 40 psi. It has been demonstrated that sperm membrane and DNA defects can be lessened by using a solid-state laser or UV laser with argon.
- Seminal plasma (10% v/v) works as an inhibitor of capacitation. It maintains the pH as alkalinity of spermatozoa in the female reproductive canal, which has been demonstrated to improve the viability, motility, and decrease capacitation-like alterations in boar and ram spermatozoa.
- The sperm viability and motility were enhanced and their fertility was maintained by adding protamine before the sorting process and adding bovine sheath fluid (197 mM tris, 55.4 mM citric acid, and 47.5 mM fructose) to the extender (Gosalvez *et al.*, 2011) [13].
- Before sperm sexing, gradient centrifugation increased sorting rates and resolution. One way to lessen the shortcomings caused by dye in sorted spermatozoa is to use impermeable or permeable dyes at low concentrations.

7. Application of sex sorting technology

7.1 Herd expansion

These studies showed that in seasonal, pasture-based dairy herds, the use of sexed semen, either fresh or frozen, can enable faster, more profitable herd development.

7.2 Improved biosecurity

Utilising sexed semen to produce more replacement heifers may be a benefit for herd expansion and also for improved biosecurity (Weigel, 2004) [57]. The ability to produce all replacements on-farm removes the need to purchase inventory from other sources, which could expose you to disease.

7.3 Reduced dystocia

Dystocia reduces productivity, fertility, cow and calf mortality, culling, veterinary and management expenses, and other factors that affect the profitability of dairy herds. Heifer dystocia cases have decreased due to the use of sexed semen.

After fertilising spermatozoa and sorting the resulting flow, offspring from seven mammalian species, mostly cattle, have been created; nevertheless, no observable defects have resulted from this process. Dystocia expenses in first-calving heifers may be 20% lower when female sexed semen is used (Seidel, 2003) [42].

7.4 Increased rate of genetic gain
By using sexed semen to identify just the best-ranking cows to breed replacements from, dairy herds can increase the rate of genetic gain. The availability of sexed semen from the greatest genetic merit sires is a prerequisite for obtaining better genetic gain using sexed semen. Usually, there is a bigger market for traditional semen straws than there is for the best genetic merit sires.

7.5 Beef production from the dairy herd
By breeding the remaining herd with semen from beef sires, this gives dairy farmers the chance to raise profits from their sales of calves for meat production (McCullock *et al.*, 2013) [29].

7.6 Shortening gestation length
A cow that calves late in the spring will have a longer dry period (higher cost) and a shorter lactation (lower income) than a cow that calves at the beginning of the calving period in late winter/early spring. Lactation length is one of the major factors influencing the profit generated per individual cow in seasonal calving systems. Depending on geographical variations in the price of milk and beef, there may be a relative economic benefit to either shortening the gestation period or increasing the beef yield from the dairy herd.

8. Limitation of sex sorting technology
- The equipment's high cost and exclusive technology.
- Higher the cost of maintenance.
- Because sorting methods waste a lot of sperm, sires with the highest genetic quality are usually left unsexed.
- Manpower with the necessary skills is needed to supervise and operate machinery.
- Slow process, meaning that fewer spermatozoa are sorted every hour because sperm are sexing one at a time instead of several at once. This results in fewer sperm being identified for their sex or in the production of fewer straws (7–10 doses/hour) (Seidel, 2007) [43].
- Only 30% of sperm are sexable, and only 15% of those are responsible for producing female children, meaning that roughly half of sperm samples are unsexable and wasted. This increases the cost of sexed semen relative to unsexed semen.
- Since fresh sperm are most effective for sexing, sorters must to be positioned close to the bull's stations.
- A greater fraction of capacitated sperm cells are produced during the sex-sorting process. The length of time that sperm cells which have undergone sex sorting can remain fertile may be restricted by this and other cell-sorting-related stressors.
- Lower rates of conception. Conception rates can range from roughly 60 to 90% of conventional semen, according to numerous research (Healy *et al.*, 2013) [15].
- Dairy cattle continue to exhibit rising rates of inbreeding. Because there are so few bulls with sex-sorted semen available, using sex-sorted semen going forward will only increase the percentage of inbreeding (De Vries *et al.*, 2008) [10].

9. Future Prospects
The goal of sexed semen in the future is to increase the rate of conception. The use of sexed semen to produce female calves is beneficial because the demand for milk production is growing daily. The female reproductive system can more readily polarise sperm by adding certain compounds to semen or in extenders, enabling sperm of the desired sex to fertilise the egg. In the future, new methods will be developed to reduce sperm loss and shorten the sorting time, since post-thaw fertility in sexed semen is lower (47%) than in conventional semen (54%). More study is required to increase the effectiveness of sperm sorting and to conduct extensive field trials to increase the pregnancy rates of low-dose, sexed sperm.

10. Conclusion
Sex sorting technology is a good technique which is largely required at the field level for the production of desired offspring. The older techniques need to be upgraded so, that the loss of sperm cells can be minimized, the shelf life of sexed straws can be increased, sexed straws should be easily available and the cost of the straw/dose can be reduced. To adopt sexed semen nationwide, Indian farmers must have financial assistance and educational opportunities.

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