REVIEW ARTICLE

Technologies for Separation of 'X' and 'Y' Spermatozoa in Bovines: An Overview

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Abstract

Several approaches to sperm sexing such as Albumin gradient, Identification of H-Y antigen, Free-flow electrophoresis, Detection of sex-specific proteins, Centrifugal counter current distribution, Volumetric differences, Percoll density gradient, Flow-cytometry, Laser ablation, etc were put forward by the scientists. However, the alteration in DNA content between X- and Y-sperm remains the key and only discriminating feature for sperm sorting based on which only Flow cytometry and Laser ablation techniques are being used commercially. Flow cytometry is based on precise staining of the DNA of sperm with the nucleic acid-specific dye, Hoechst 33342, to differentiate between the subpopulations of X- and Y-sperm. The fluorescently stained sperm are then sex-sorted using a specialized highspeed sorter. Laser ablation (LA) is a system and method for sorting a mixture of stained particles in a fluid flow path. It includes an electromagnetic radiation source for exciting fluorescence emissions from the stained particles, a photodetector for detecting the fluorescence emissions, a processor for classifying the stained particles, and a photo-damaging laser for damaging selected particles in the flow path.

Key words: Flow cytometry, Laser ablation, Semen sexing, Sex-specific proteins

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Introduction

Sexed semen is an emerging reproductive biotechnology which is designed to alter the sex ratio of the progenies towards a desired gender. It was found that the efficiency of this technology is around 90 %, that is, out of all the progenies 90 % will be females (DeJarnette *et al*., 2009; Sharma *et al*., 2017). The differences present between X and Y spermatozoa form the foundation of the sexed semen technology. Till date, the difference in DNA content between X- and Y-sperm remains the primary and only determining feature to separate them (Garner, 2009). Even though various alternatives to this have been researched and worked upon, they mostly lack in accuracy, repeatability, and practical application (Seidel, 2012). The difference in DNA content amongst the farm species ranges between 3.6% in swine to about 4.4% in deer. On average, there is also a difference in size between X- and Y-sperm which is about 4% in cattle with some subtle alterations between the breeds (Garner *et al*., 2013). On the basis of these differences, there are various methods that have been devised for sorting spermatozoa such as Albumin gradient, Identification of H-Y antigen, Free-flow electrophoresis, Detection of sex-specific proteins, Centrifugal counter current distribution, Volumetric differences, Percoll density gradient, Raman spectroscopy, Flow cytometry, Laser ablation etc. Out of these only flow cytometry and laser ablation are being used commercially with around 90% efficiency.

Differences between X and Y Spermatozoa

The presence of sex chromosomes was reported by Guyer (1910). In 1925, without any reference to DNA, Lush endeavoured one of the first significant attempts to preselect sex. That study showed no skewing of the sex ratio in rabbits and pigs based on centrifugation (Lush, 1925). After this numerous groups started their investigation on sex preselection and plentiful of the work in this area was presented in a symposium held at Penn State University in 1970 (Kiddy and Hafs, 1971). With the invention and use of flow cytometry in the late 1960's, measuring the DNA content in different cells for various purposes became a point of interest, specifically for cancer analysis (Kamentsky *et al*., 1967). Another facet of that initial work was that several

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weapons systems were being developed or used at that time and the DNA could have been a subtle indicator of mutagenic events associated with them.

The use of flow cytometry for measuring DNA in sperm to analyse the changes that might occur with genetic damage was reported by Gledhill *et al.* (1976). It was observed that differential fluorescence was caused due to asymmetric shape and when it was joined with random orientation then the known difference in DNA content between X- and Y-bearing sperm was masked by the differential fluorescence and therefore the difference could not be measured. It was observed that fluid streams in flow cytometer could be altered from cylindrical to flat and when this was applied to sperm, it became possible to analyse asymmetrical-shaped sperm for its DNA content with the help of a flow cytometer / analyzer (Fulwyler, 1965; Dean *et al.*, 1978).

The attention to the use of DNA as a potential marker for sex preselection was highlighted by Moruzzi (1979) with the publication of data that showed that there is a variety of differences between X and Y chromosome bearing sperm for several species (Table 1). These differences in DNA content were measured by the differences in chromosome length. It was reported that there is on average a difference of 6.6% in DNA content among several species.

Table 1: Differences between X and Y spermatozoa

Parameter	Difference	References
F body	Long arm on Y chromosome	Barlow *et al.,* 1970
Motility	Y sperm is faster	Ericsson *et al.,* 1973
DNA content	Less in Y spermatozoa	Moruzzi *et al.,* 1979; Pinkel *et al.,* 1982
Surface charge	X sperm has negative charge	Kaneko *et al.,* 1984
Size	X spermatozoa is larger	Cui and Mathews, 1993
Cell surface antigen	H-Y antigen is present on Y spermatozoa	Hendricksen, 1993
Sperm surface	Specific protein	-

An experimental laser-based flow cytometer with high-resolution was built (Penfold *et al.*, 1998). It was orthogonally configured so that it could orient the sperm. It was used to measure a difference in DNA content in fixed mouse sperm nuclei which turned out to be 3.2%. A simpler analytical but non-sorting flow cytometer which was based on mercury-lamp excitation was used to measure a small difference in DNA in nuclei of livestock sperm and it was observed that these differences ranged from 3.6 to 4.0% (Garner *et al.*, 1983) (Table 2). In different breeds of cattle and buffaloes, the difference in the DNA content was found to be 3.98% in Holstein Friesian, 4.24% in Jersey, 4.05% in Angus, 4.03% in Hereford, 3.73% in Brahman, 3.59% in Murrah and 3.55% in Nili-Ravi (Figure 1). Another conference was held which served to bring the work on sex-preselection research up to date and also demonstrated a broad interest in the area and an update in the potential technologies available to separate X and Y sperm (Amann and Siedel, 1982).

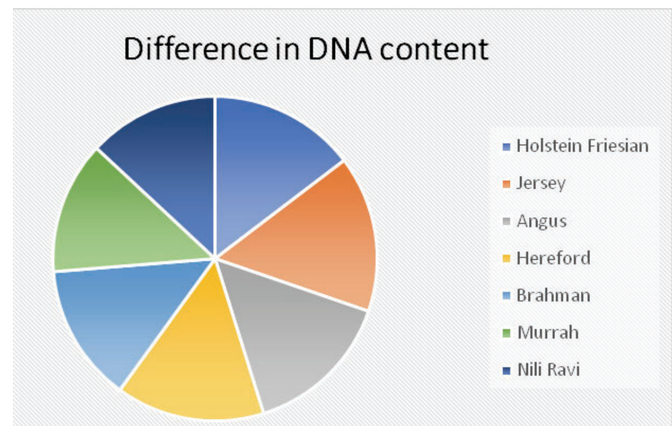

Fig. 1: Differences in the DNA content between X- and Y- bearing spermatozoa among different breeds of cattle and buffaloes (Prakash *et al.,* 2014; Kumar *et al.,* 2017)

This knowledge in the field of sperm sexing that was gained over a period of 71 years (1910- 1981) donated vastly to the developmental successes of the most recent years in which the only effective marker for separating viable X and Y sperm successfully was found out to be the difference in DNA content (Johnson *et al.*, 1989).

Table 2: DNA content differences between X and Y sperm of various species (Garner and Siedel, 2003)

Species	DNA content differences (%)	References
Australian Possum (*Pseudocheirus peregrinus*)	2.3	Johnson, 1995
Human (*Homo sapiens*)	2.8, 2.9, 3.0	Johnson *et al*., 1993; Johnson, 1992; 1995; Fugger, 1999
Dromedary Camel (*Camelus dromedarius*)	3.3	Johnson, 2000
Asian Elephant (*Elephas maximus*)	3.4	Johnson, 2000
Mouse (*Mus musculus*)	3.6, 4.9	Pinkel *et al*., 1982
Swine (*Sus scrofa*)	3.7, 3.5, 3.6	Garner *et al*., 1983; Johnson, 1992; 1995
Horse (*Equus caballus*)	3.7, 4.1	Johnson, 1992; 1995
Cattle (*Bos taurus*)	3.9, 3.8	Gledhill *et al*., 1982; Garner *et al*., 1983; Johnson, 1995
Dog (*Canis familiaris*)	3.9	Johnson, 1995; 2000
Rabbit (*Oryctolagus cuniculus*)	3.9, 4.2	Garner *et al*., 1983; Johnson *et al*., 1989; Johnson 1992; 1995
Sheep (*Ovis aries*)	4.1, 4.2	Garner *et al*.,1983; Johnson, 1992; 1995
Baboon (*Papio hamadryas*)	4.2	O'Brien *et al*., 2001
Chinchilla (*Chinchilla langier*)	7.5	Johnson, 1992; 1995

Albumin Gradient

The fundamental idea behind the Gradient Swim-Down technique is the difference between X and Y-bearing spermatozoa's capacity for swimming down in a gradient solution. This technique relies on the spermatozoa's organic motility (Singh *et al.,* 2019). The discontinuous bovine serum albumin medium, which gets gradually less concentrated from top to bottom, is first created. The medium is then topped with the semen sample, which is then given an hour of incubation at 37°C. During migration, the majority of the sperm with mobility descend into the gradient. Due to their smaller size, Y chromosome-bearing spermatozoa have strong motility and travel more quickly downhill than X chromosome-bearing spermatozoa. As a result, a higher percentage of X or Y spermatozoa can be seen at different albumin gradients when a segment of semen is separated from that gradient. The X chromosome is bigger than the Y chromosome, according to Ericsson *et al.* (1973), who was also the first to describe successfully sorting X and Y spermatozoa utilizing an albumin gradient.

According to Maxwell *et al.* (1984), the approach proved successful in increasing the quantity of motile spermatozoa and eliminating irregular forms. They also noted that there was not much of a variation in the proportion of X- to Y-bearing spermatozoa. Later, when Beal *et al.* (1984) attempted to distinguish X and Y spermatozoa in bull sperm employing an albumin gradient, they could not get sufficient results. In a different investigation, rabbit spermatozoa were divided using an albumin gradient in 1985. The sex ratio did not change significantly, but it was seen that the spermatozoa at the bottom of the column moved more quickly (Zavos, 1985). Despite the fact that the success rate with this approach is said to be about 75%, the efficiency of this specific method is questioned due to the inconsistent outcomes (Beernik *et al.,* 1993; Kumar *et al.,* 2017; Singh *et al.,* 2019).

Identification of H-Y Antigen

In 1955, Eichwald and Silmser provided the first description of the H-Y antigen, a male-specific antigen. The first study about antibodies against the H-Y antigen was reported in 1971 (Goldberg *et al.,* 1971). Sperm was routinely tested for anti-H-Y antibody titers in the initial stages of H-Y serology. It was hypothesized that the H-Y antigen may only be present in sperm that carry Y chromosomes since the majority of anti-H-Y antibodies only bind a small portion of the sperm (Hendrikson *et al.,* 1993). This procedure uses a technique called affinity chromatography or magnetic bead separation to sort sperm, with the possibility of using a particular antibody towards the surface protein of spermatozoa harbouring the Y chromosome (H-Y antigen) (Kumar *et al.,* 2017; Singh *et al.,* 2019).

The initial description of H-Y antigen was made by Eichwald *et al.* in 1955. In order to distinguish Y sperm from X sperm, several immunological inactivation techniques were used to target the H-Y antigen in the Y sperm in 1971 (Goldberg *et al.,* 1971). However, Watchel (1983) confirmed that under normal conditions, H-Y antigen is in fact found in the cell membranes of both X and Y spermatozoa. Similarly, Hoppe and Koo (1984) asserted that spermatozoa carrying X- and Y-chromosomes most likely shared the same surface antigen. However, Zavos *et al.* (1983) discovered a considerable change in the progeny's sex ratio when they used semen that had been beforehand treated using H-Y antisera with its complement. The H-Y antigen may be beneficial as a way to select just the spermatozoa containing the X gene, according to some additional studies from the trials conducted on rabbit semen (Zavos, 1985). In light of their experimental findings, Hendrikssen *et al.* (1993) concluded that Watchel's 1983 assertion on the existence of preferential expression was unsupported. When semen samples were tagged positively to the H-Y antigen, Ali *et al.* (1990) noticed that there was a preponderance of Y-bearing spermatozoa; these results did not agree with those of Hoppe and Koo in 1984. Human semen tests including certain surface antigens were carried out in 1998, although the results were inconclusive (Sills *et al.,* 1998). Hoppe and Koo's (1984) assertion that H-Y antigen can be found on the outermost layer of both Y and X chromosome-bearing sperm and that, thus, sexing the spermatozoa based on HY antigen was shown to be ineffective and was backed by Prasad *et al.* (1984).

Sperm Sorting by Swim-up Procedure

Scientists exploit the difference in swimming speed between sperm cells with smaller Y chromosomes and those with large X chromosomes to sort sperm (Van *et al.,* 1998). According to studies (Check *et al.,* 1989; Kumar *et al.,* 2017; Singh *et al.,* 2019), this approach has an 81% success rate. A modified swim-up method has been effective for sperm sexing in Nili-Ravi buffaloes using a real-time PCR. The fact that the sperm quality is not diminished during separating and freeze-thawing is one of the technique's potential benefits. Real-time PCR using SYBR green has been used to confirm the sperm sexing achieved by this method (Husna *et al.,* 2017; Yadav *et al.,* 2018).

Human X spermatozoa travel slower- that is, they have lower angular velocity - than Y-spermatozoa in the flow stream, according to an experiment done by Sarkar and his colleagues on human sperms in 1984. Additionally, they discovered that both cells move in a manner that is generally comparable during the stationary period (Sarkar *et al.,* 1984). According to reports, 81 % of male calves were produced when sperm sorted using this method was utilized for AI (Check *et al.,* 1989). The measurement of the spermatozoa explains the fundamentals of this method. Numerous specialists exploited the difference in sperm size for sperm sorting since the Y spermatozoa travels more quickly than the X spermatozoa due to its smaller size (Van *et al.,* 1998; Ollero *et al.,* 2000). A rate of success of 81% has been reported for

the swim-up method of sperm sorting (Check *et al.,* 1989; Kumar *et al.,* 2017). In their research, Alminana *et al.* (2014) discovered results that were in conflict with earlier findings and showed that the motility of X- and Y-spermatozoa had not significantly changed. However, they also discovered that the X-sperm moves to almost a straight course with a significant drop in angular velocity while in a flowing stream. When the swim-up approach was used on the sperm of Nili-Ravi, it was successful (Yadav *et al.,* 2017). However, this approach only recovers 10% of the entire number of sperm, which is a significant downside.

Free Flow Electrophoresis

Electric charge variations on the surface of sperm containing X and Y chromosomes form the basis of this method (Yadav *et al.,* 2018). The spermatozoa with the X chromosome have a negative surface charge, whereas the spermatozoa with the Y chromosome have a positive surface charge. This difference in surface charges is exploited in electric field separation for sperm sorting (Kumar *et al.,* 2017).

Sex Specific Proteins

Proteins that are exclusive to one sex can be used to establish an immunological technique for sperm separation. Different proteins are expressed differently in the protein profiles of X- and Y-sperm. These proteins may have diverse effects on phenotypic, sperm functions, oocyte-sperm contact, and zygote formation (Li *et al.,* 2016; Kumar *et al.,* 2017). As molecular identifiers, these differently expressed proteins can distinguish between X- and Y-sperm. In the near future, this technique could lead to a revolution in sperm sorting (Singh *et al.,* 2019).

It has been noted that spermatozoa have different surface charges. The foundation for the electric field separation used in sperm sorting is that spermatozoa with the X chromosome have a negative charge, whereas those with the Y chromosome have a positive charge (Kaneko *et al.,* 1984; Mohri *et al.,* 1986; Kumar *et al.,* 2017).

A birth rate of 50.4 % of female calves was observed when utilizing semen rich in X-bearing spermatozoa that were separated using this method and utilized for AI on 1185 animals. The outcomes weren't what were expected. After going through electrophoresis, sperm cells lost some of their ability to move (Sharma *et al.,* 2016). Electrophoresis-based sperm sorting was unable to distinguish between any discernible sex ratios (Foote, 1985). The X chromosome's plasma membrane contains sialic acid, which gives it a negative charge (Bradley, 1988). According to Prasad's (2010) findings, the anode contains a substantially higher concentration of glycoproteins that contain neuraminic acid on its membrane surface, that is why X chromosomes travel there.

Centrifugal Counter Current Distribution

A chromatography technique called counter-current distribution (CCD) uses an upper phase that is mobile and a lower phase that is stationary. One system and two stages are used to split the cell sample in a methodical manner. It comes into contact with brand-new opposing phases. This approach requires a CCD camera. The device includes 60 chambers that are arranged in a circle and enable transfers between the mobile and stationary phases. By retaining the lighter phases in the upper half of each chamber and the denser phases at the bottom, the CCD may be obtained during centrifugation. A cyclic multistep shift of 60 upper-over 60 bottom-batch stages makes up the whole operation. Each transfer involves thoroughly mixing each stage at unit gravity and then centrifuging them at 1000g to separate them. The top phases are moved to the following chambers during rotation once the phases have been separated. After the deceleration, the next one can be executed. This approach might not be as effective for animals where there is less variation in the DNA content of spermatozoa carrying X- and Y-chromosomes (Johnson, 1995). According to many sources (Ollero *et al.,* 2000; Yadav *et al.,* 2018; Singh *et al.,* 2019), this approach has a claimed success rate of 75%.

For this approach, a counter-current distribution (CCD) device that was created using Akerlund's idea from 1984 is necessary. In this technique, there are two phases: an upper mobile phase, and a lower stationary phase. The sperm cell sample is then separated into 2 phases that come into touch with fresh opposite phases using 1 system in a methodical manner. According to physical factors such as size, shape, mass, specific gravity, and the density differential among the cell and the suspending medium, each sperm has a unique sedimentation velocity. Since the two sperm pools differ physically, it is thought that the two will be separated accordingly**.** Meistrich (1982) asserted that the method is ineffective for sperm sorting since there is a very little (0.0007 g/cm3) difference in concentration between X- and Y-sperm. No noticeable results were observed when Foote (1985) copied this method. The first reputable attempt to separate X and Y sperm groups from bovine semen using CCD was undertaken by Cartwright *et al.* in 1993. Ollero *et al.* (2000) found that the Y spermatozoa-enriched population was around 75% on the right part of the focal peak with high viability, whereas the X sperm-enriched population was about 63% and was mostly on the left side with non-viable cells. In order to improve the selection process using counter-current distribution, Bhattacharya (1977) postulated that the injection of an appropriate microampere current would drive spermatozoa that carry the Y and X alleles to move closer to the anode and toward the cathode, respectively. This approach has been shown to have a 75% rate of success and 57% viability.

Volumetric Differences

This technique uses interference microscopy analysis of images of spermatozoa to show a variation in sperm head volume dependent on the amount of DNA between spermatozoa harbouring the X and Y chromosomes (Yadav *et*

al., 2018). Interference microscopy in visible light may be used to measure the volume differences between sperm heads carrying X and Y chromosomes. These volume variations may be measured using DIC microscopy, or differential interference contrast. The measurements may be performed with visible light of 550 nm without the need for staining (Van *et al.,* 1998; Singh *et al.,* 2019).

Van Munster *et al*. (1999a) employed interference microscopy and subsequent image processing to show that there is a difference in sperm head volume that correlates with the variances in the amount of DNA between X and Y-bearing bovine spermatozoa. Unfortunately, based on theoretical considerations, the expected quality of the amounts of sorted sperms that have been differentiated using volumetric dimensions could not exceed 80% of either sex (Van Munster *et al.,* 1999b). A fresh technique has been created for sorting live spermatozoa that is based on interference microscopy optics alongside a flow cytometer (Van Munster, 2002).

Percoll Density Gradient Method

This technique involves layering semen on a top of a percoll column and allowing spermatozoa to pass through the column. This method is based on the variations in sedimentation density between spermatozoa containing X and Y (Yadav *et al.,* 2018). During sperm sorting, the Y chromosome-bearing spermatozoa maintain a high percentage at the top of the column, whereas the X chromosome-bearing spermatozoa have a greater sedimentation density and sink at the bottom. According to studies by Singh *et al*. in 2019 and Van *et al*. in 1992, the success rate ranges from 86% to 94%.

Semen is permitted to be put on top of the percoll column using this technique. Because they are larger than Y chromosome-bearing spermatozoa, X chromosome-bearing spermatozoa have a higher sedimentation density. During sperm sorting, it sinks to the bottom of the column while the spermatozoa harboring Y chromosomes remain disproportionately abundant at the top. According to reports from Van *et al*. in 1992, the success rate ranges from 86% to 94%. It was initially recommended the therapeutic application of intermittent percoll density gradient for X-chromosome-carrying sperm enrichment of the semen. Because there is more DNA present and the sperm head is larger, X chromosome-bearing sperm have a 0.006% greater density that may be detected. This is the fundamental idea behind the Percoll density gradient method, which was used by Curry *et al*. in 2009 as a method for separating X- and Y-sperm. Using a 12-layer percoll density gradient, Wang *et al*. in 1994 achieved a modest enrichment of semen. Nevertheless, it was insufficient to have a major impact on the sex ratio at birth. Again in 1996, Flaherty and Matthews attempted sperm sorting using this approach and obtained a modest enrichment of X-bearing sperm; nevertheless, the procedure was not sufficient to successfully change the sex ratio at birth (Flaherty and Matthews, 1996). In a different research, a 7-layer (70%-10%) percoll gradient of density was employed, and it was shown to yield a 20.48% enrichment in Sahiwal semen and 29.9% enrichment in crossbred sperm. Further the findings were supported by Raman spectroscopy, which did not influence the semen's quality (Chaudhary, 2018).

Raman Spectroscopy

Raman spectroscopy is a label-free technique that uses inelastic light scattering to detect vibrations in chemical bonds in molecules (Jonas *et al.,* 2010; Dochow *et al.,* 2011). Without the need for external labelling, chemical information collected from Raman spectra provides the crucial details about a cell, indicating the presence of nucleic acids, proteins, carbohydrates, and lipids. In order to distinguish between distinct cell kinds and states, it gives precise chemical information that includes cellular molecular arrangement, structure, and physiological status (Mazilu *et al.,* 2010; Ashok *et al.,* 2011; Rusciano *et al.,* 2011). For *in vivo* or *in situ* analyses of aqueous materials, such as biological samples, Raman spectroscopy is more appropriate. Additionally, it does not need a label or marker, like fluorescence, for example, making it possible to analyze the materials *in situ* quickly and non-intrusively. Bovine X and Y sperm cells are selectively and sensitively distinguished using a technique based on Raman spectroscopy. In X-bearing or Y-bearing sperm, Raman spectroscopy enables the study of numerous sex-associated membrane protein components. More specifically, the spectra of Y-sperm are more intense than those of X-bearing sperm. This could be because the membrane of X-bearing sperm lacks the HY antigen, but it is present on Y-bearing sperm (Yadav *et al.,* 2018).

Quinacrine Mustard Staining

Certain chromosomal areas exhibit extremely high fluorescence when stained with quinacrine mustard (Caspersson *et al.,* 1968). Previously, this technique was used to confirm the enrichment of either X- or Y-sperm, with the sperm that is thought to contain the Y chromosome displaying a fluorescent spot or F body and the sperm that is thought to contain the X chromosome remaining unstained (Barlow *et al.,* 1970). Pearson *et al.* (1971) carried out quinacrine staining in various species, including humans, in a different investigation. They claimed that only humans and African great apes exhibit the Y-chromosome's quinacrine fluorescence, which is not a feature shared by other mammalian Y-chromosomes.

Numerous investigations have shown that this procedure can yield confusing and misleading findings involving human sperm (Thomsen *et al.,* 1986; Flaherty and Mathews., 1996). Quinacrine also produces false positives and false negatives in interphase cells. As a result, according to Yadav *et al.* (2018), it is not an appropriate approach for sorting sperm in the majority of mammalian species.

Polymerase Chain Reaction and Fluorescence *in situ* Hybridization

In comparison to single label FISH, the use of double FISH with X- and Y-chromosome-specific markers has allowed for a more precise assessment (Han *et al.,* 1993). The development of PCR and FISH has increased the likelihood of precisely identifying X- and Y-sperm, opening up the possibility of evaluating the purity of various sperm sexing techniques. Single and double-label FISH may be used to directly see the sex chromosomes in each sperm. By using specialized probes combined with fluorescent molecules for the X- and Y-sperm, FISH precisely determines the sex chromosome of each individual sperm (Flaherty and Mathews., 1996).

The main advantage of FISH in comparison to single cell PCR analysis and flow cytometry reanalysis is that it is incredibly qualitative and quantitative (Parrilla *et al.,* 2003). It takes a lot of individual sperm to analyze for single sperm PCR to accurately determine the sex ratio. Quantitative real-time PCR, however, makes determining the sex ratio of semen easier and more accurate (Parati *et al.,* 2006). The sex and sex ratios of the sperm in a semen sample may be determined using specific DNA sequences on X- and Y-sperm (Colley *et al.,* 2008). Specific sequences of the X-chromosome-located bovine proteolipid protein gene and the Y-chromosome-located SRY gene were utilized in an experiment to assess the sex ratio in bovine semen.

Tan *et al.* (2015) employed SYBR® Green qPCR to detect the sex of bovine sperm using two sets of primers for the zinc finger protein X (ZFX), particular sequences for the X-chromosome, and SRY, unique sequences for the Y chromosome. ZFX and SRY genes are both present in the bovine genome as single copies and are typically employed as DNA markers to identify gender. To distinguish between X- and Y-sperm, PCR is a very sensitive and specific method. However, its application to cell populations is only partially useful in evaluating sex selection strategies.

However, the main issue with FISH is the quantity of DNA condensation in sperm, which makes it difficult to retrieve certain hybridization sites. To allow the entry of DNA markers to the sperm chromatin during FISH, the sperm nuclei must be decondensed (Wyrobek *et al.,* 1993). Additionally, it requires a lot of labour to use it for screening a lot of individual sperm (Flaherty and Mathews., 1996).

Raman Micro-spectroscopy

De Luca *et al.* (2014) analyzed the Raman spectra of X- and Y-sperm from three bulls and found that the differences between X- and Y-sperm may be distinguished by a spectroscopic signature, which is a combination of the sperm’s spectral constituents (DNA, protein, lipids, etc.). The main metabolic variations between X- and Y-sperm are shown by the nucleus.

The peak at 726, 785, and 1581/cm (given to nucleic acids and DNA backbone) are differences between X- and Y-sperm, and a strong peak at 1095/cm is indicative of the PO2 backbone. When X-sperm was compared to Y-sperm, the strength of the peaks increased, indicating a higher total DNA concentration (Manag *et al.,* 2014; De Luca *et al*., 2014).

The peaks in the spectra of the acrosomal vesicle that correspond to proteins, Amide I (1600-1680/cm), Amide III (approximately 1200-1300/cm), and lipids (C-H vibration at 1480/cm), all have higher intensities. The three nuclear regions - the neck, middle, and acrosomal regions - have stable relative intensities and peak locations. DNA content and sex membrane proteins were found to be the primary variations in Raman peaks. According to De Luca *et al.* (2014), Raman spectroscopy is a good candidate for the creation of a highly effective and non-invasive sperm sexing method.

Sperm Labelling using Gold Nano-Particles (AuNPs)

This method relies on a qualitative detection of sperm variations associated with sex. The X- and Y-chromosome DNA sequences of haploid spermatozoa differ, which is a well-known characteristic of these cells. The present state of research using Y-chromosome-specific sequences in morphologically and physiologically intact sperm is described in Yadav *et al.*’s 2018 study. There are three key considerations in this.

1. Functionalized AuNPs penetrate the sperm membranes.
2. Non-invasive triplex binding that couples a particular DNA probing with the whole DNA double strand.
3. Identification of the sperm population’s sex-specific signal pattern.

Laser Ablation Technology

In order to remove material from the irradiated zone, a laser beam is aimed on a sample surface using the laser ablation technique. It contains a brand-new instrument design that breaks through technological barriers to sorting speed and product viability. Both the laser ablation technology analyze each individual sperm cell to determine whether it is “female” (bearing an X chromosome) or “male” (bearing a Y chromosome) by moving sperm cells through a laser-based detection zone in a fluid stream at high speed. After the sex of the cells is identified by the equipment, the unwanted sex cells are selectively eliminated from the process of preparing using a powerful laser that harms them and renders them infertile. The destructive pressure, velocity, or electric field effects that are the foundation of traditional cell sorting technologies do not affect cells of the target sex as they pass through unharmed.

Sperm cells are labelled with a DNA-binding fluorescent dye prior to sexing; this dye produces light when activated by a laser. Female cells with X chromosomes have more DNA than male cells with Y chromosomes, making them brighter. Although slight, the distinction between cells that contain X and Y chromosomes may be made with adequate accuracy. Even though sperm cells have a distinct form and randomly oriented as they travel through the sensing zone of the

instrument, laser ablation technology employs a specific optical system developed to identify sex with great precision. A conception rate of 78% of that of conventional semen has been observed using this technology (Perry *et al.,* 2020).

Flow Cytometry

A flow cytometer is a device that analyzes the physical characteristics and multi-color fluorescence of cellular particles moving in a stream of fluid. It is made up of four basic systems: software handling, fluidics, optics, and electronics. The cell suspension moves through a tubular system and is illuminated in certain locations by a laser (or mercury arc lamp in some earlier devices). Results are produced by digitizing and computer-handling the emissions from the cells that were observed as a result of this illumination.

The Hoechst 33342 dye, which diffuses across an unbroken cell membrane and specifically binds to the A/T base pairs within the minor groove, is the key component of the procedure. H33342's fluorescence emission and absorption spectra range in wavelength from 350 to 460 nm. The precise amount of DNA present in the sperm cell may be fixed using this shift as a marker (Seidel and Garner, 2002; Garner, 2009). Two fluorescence detectors that evaluate the strength of the signal from the H33342 when activated by a laser are used in the flow cytometric method to determine the DNA difference between the two types of sperm cells. The sperm are streamed in a single line by the jet in the air flow cytometer, which produces a terminal droplet and a distinguishing droplet charge to differentiate the sperm populations. The two distinct populations can be twisted into opposing streams for collecting tanks to the charged plates at the discharge point. Fluorescence histograms on the flow cytometer are used to distinguish between the sorted populations, and the software also allows for the gating out of dead and moribund sperm. The harvesting of very enriched populations of X- and/or Y-sperm is made possible by the relative gating of particular populations (Vishwanath *et al.,* 2018).

Conception Rate of Semen Produced by Flow Cytometry

The fertility of sex-sorted sperm in cattle has always been lower than that of conventional sperm, and dealing with compensable factors, such as greater sperm numbers or a greater number of sperm with improved morphological features, that typically would boost the fertility of a sub-fertile sire, did not produce better results with sexed sperm (DeJarnette *et al.,* 2011). The sex-sorting procedure involves 20 additional sub-processes, including an extended holding period before staining, exposure to a laser beam to promote fluorescence, separation into X- and Y-sperm, and exposure to an electrical field for forming a relatively pure population into an appropriate vessel, all of which contribute to the decline in fertility (Seidel and Garner, 2002). The many metabolic changes that sperm go through throughout this sex-sorting process have been attributed as the causes of the lower fertility of sex-sorted semen. The sperm cells undergo some rather drastic changes in their surroundings throughout the sex-sorting process, which adds to their stress. This is necessary because the physiology of the sperm must be changed in order to allow the H33342 stain to enter and remain inside the cell until it fluoresces (Johnson, 2000; Seidel, 2012; de Graaf *et al.,* 2014). A sperm cell that has already undergone significant stress must now endure the cryopreservation process.

The conception rates for traditional semen and sexed semen were found to be 48-76% and 39-57%, respectively (Borcherson *et al.,* 2009; DeJarnette *et al.,* 2009), with mean conception rates of 56% and 39%, respectively, in the USA (Norman *et al.,* 2010); 60.3% and 52%, respectively, in Denmark (Borcherson *et al.,* 2009); 35% and 24.9% in India (http://naas.org.in, 2020). According to Sharma *et al.* (2017), conventional and sexed semen have conception rates in Uttarakhand of 49.32% and 40.0%, respectively. At concentrations of 2.1 million, the comparative fertility of sex-sorted semen is almost 70% of that of conventional semen (DeJarnette *et al.,* 2011; Vishwanath *et al.,* 2014). In buffaloes, Ingawale *et al*. (2022) recorded conception rate of 38.63 % and 45.00% with sex-sorted and conventional semen, respectively, under field conditions and the female birth was 88.23% and 55.55% with two types of semen.

Conclusion

Semen sexing technology can reduce or eliminate surplus male calves by using sperm sorted based on sex. This method generates offspring of the chosen sex using an X or Y chromosome. However, current technology has poor process speed and physical restrictions, leading to reduced fertility in sex-sorted semen. A more effective, affordable, practical, and less intrusive sperm sorting method is needed. Using current technology we can also identify protein differences between separated populations of X- and Y-spermatozoa, which could be used for immunological methods in sperm sorting.

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