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Original Research Article

Sperm Sexing and its Application in Livestock Sector

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A B S T R A C T

Livestock farmers always have a wish for producing young ones of desired sex, to meet the increasing demand of meat & milk which ultimately leads to economical benefit to farmers. Sexual pre-selection plays an important economic role in animal production industries. In past, various techniques have been used to separate the X and Y sperm which were based on principle of difference in mass, size, swimming pattern, immunological structure and surface charges etc. Some of them showed the encouraging results but lacking scientific validation and some remained yet to be established. But at present only one, flow cytometery, is effective. This process consistently results in semen sorted with 90% of the desired sex. However, while the accuracy is great, the speed and yield of the process is slow and low. In addition, the equipment is extremely expensive and specially trained technicians are needed to assure sorting accuracy. Despite these limitations, production of sexed semen usually followed by cryopreservation for AI is being used commercially for cattle production.

Keywords

Sperm sexing, livestock sector, farmer, economical benefit

Introduction

The demand for livestock products like meat, milk and dairy products is increasing day by day globally; to meet this demand, utilization of modern technologies to promote sustainable production of animals assumes paramount importance. Pre-sexed sperm or embryo mediated livestock production along with other genomic, proteomic and phenomics technologies offers a promising breeding strategy to meet the increased demand for food production (Rath *et al.*, 2013). Among this, sperm sexing or sexed semen is one of the newest reproductive technologies available to livestock sector.

Separation of X and Y-sperm for pre-selection of the desired sex is economically important in livestock production, which allows the livestock sector to produce the optimal proportion of males and females. This is really needed because,

Female cattle are required for the dairy industry while males are preferred in the beef cattle industry

Controlling the sex ratio entails direct returns in the livestock sector, allowing improved management of food production, animal welfare improvement, faster genetic

selection, and a decrease of environmental impact.

Benefits of Sex-Sorted Semen

Determination of sex at the earliest stage can reduce the management cost thorough selective management of superior bulls or cows

Calves of desired sex can be produced. 90:10 female to male ratio or vice-versa can be ensured.

Dystokia can be reduced by preventing production of male calves.

It lowers the cost of progeny testing programs and embryo transfer and enhances the value of genetic markers.

Fewer quantity of sexed sperm is used in genetically superior dairy females for heifer replacement

Sperm sexing techniques

Many different techniques for sorting Y and X bearing sperm were tried over 20 to 30 years. All this techniques are mostly based on many theoretical differences in X and Y sperm, like in mammals, the X-sperm contain more DNA than the Y-sperm. The degree of differences varies from species to species and amounts to approximately 2.9% in human sperm (Johnson *et al.,* 1993), 3.8% in cattle (Johnson and Welch, 1999) and as much as about 7.5% in chinchilla (Johnson, 1992). In addition to DNA content, other differences include the size (X-sperm > Y-sperm) (Cui, 1997), surface charges on sperm (Y-sperm has a positive charge and X-sperm has a negative charge) (Kiddy and Hafs, 1971) and cell surface antigens (Hoppe and Koo, 1984). Furthermore, in a study with bull sperm, Penfold *et al.,* (1998) reported that Y-sperm does not swim faster than X-sperm. However, it may be distinguished from X-sperm on the basis of linearity and straightness of path.

Quinacrine mustard staining

Quinacrine mustard staining produces very intense fluorescence to certain regions of chromosome (Caspersson, 1968). Previously, quinacrine staining was used to verify X- or Y-sperm enrichment, in which the putative Y-chromosome bearing sperm exhibit a fluorescent spot or F body, and the putative X-chromosome bearing sperm remain unstained (Barlow and Voss, 1970). However, quinacrine produces false positive and false negative results in interphase cells and several studies have shown that this technique can produce misleading and imprecise results with human sperm (Flaherty and Matthews, 1996). Furthermore, quinacrine fluorescence is not universal property of all mammalian Y-chromosome. Therefore, it is considered an inappropriate approach for selection of sperm for most mammalian species.

Raman micro-spectroscopy

De Luca *et al.,* (2014) reported discrimination of X- and Y-bovine sperm can be done based on Raman spectroscopy. The spectral component in the sperm (DNA, protein, lipids, etc.), can be used to identify the differences between X- and Y-sperm. The nucleus reveals the main biochemical differences between X- and Y-sperm. Raman peak positions and relative intensities are consistent in the three nuclear regions, viz., acrosomal, middle, and neck regions in Y-sperm. The main variations of Raman peaks were observed due to DNA content together with the sex membrane (De Luca *et al.,* 2014). Hence, De Luca *et al.,* (2014) advocated that Raman spectroscopy

is a promising candidate for the development of a highly efficient and non-invasive technique for sperm sexing.

Centrifugal Counter Current Distribution based on Density Characteristic

Meistrich (1982) found the difference in density between X-bearing bovine spermatozoa and Y-bearing bovine spermatozoa to be only 0.0007 g/cm^3, hence this feature was not suitable to be exploited as a characteristic to sex sperm. Ollero *et al.,* (2000) have attempted to sex ram spermatozoa by centrifugal counter current distribution using an aqueous two-phase system.

Albumin Gradient

Successful separation of X and Y-bearing spermatozoa using an albumin gradient was first reported by Ericsson *et al.,* (1973). Moruzzi (1979) reported that Y chromosome is smaller than X chromosome. Maxwell *et al.,* (1984) recorded that though the method was effective in increasing the proportion of spermatozoa with motility and elimination of abnormal forms, there was no much difference in the ratio of X- Y-bearing spermatozoa.

Sperm Sorting based on Volumetric Differences

Van Munster *et al.,* (1999) used interference microscopy and subsequent image analysis to demonstrate a difference in sperm head volume that matched differences in DNA content between X and Y-bearing bovine spermatozoa.

Swimming Patterns under Laminar Flow

This method was based on Y-bearing spermatozoa swim differently and more quickly than X-bearing spermatozoa in a column of flowing media. The feasibility of this technique is questionable as only 10 % of the total number of spermatozoa placed in the system could be recovered (Sarkar *et al.,* 1984).

Percoll Density Gradient

Semen is layered on top of a percoll column and spermatozoa are allowed to penetrate the column. This technique was not effective in separation of X or Y-bearing spermatozoa (Iwasaki, 1988).

Free flow electrophoresis

It is based on the possibility that the electric charge on the surface of X-bearing spermatozoa differs from that of Y-bearing spermatozoa, uses an electric field to separate spermatozoa into the two major classes (Kaneko *et al.,* 1984). However, inseminations with semen separated by this technique yielded disappointing results.

Counter Current Galvanic Separation

Application of a suitable micro-ampere current attracts Y-bearing spermatozoa to the anode and X-bearing spermatozoa to the cathode (Bhattacharya, 1977). However, it could not succeed in producing any significant alteration of sex ratio the same technique (Foote, 1985).

Immunological Sexing of Semen

Immunization of male and female rabbits by injecting sperm preparations with Freund's incomplete adjuvant subcutaneously was done to raise antibodies to sperm membrane proteins. The anti-sperm antisera obtained from the female rabbit were putative "anti-Y" and those obtained from male rabbit were "anti-X" antisera. Sperm doses after

suitable treatment were mixed with either of these antisera and incubated for 60 min at 38.5C and 5% CO2. It was found that only the "anti-X" antisera resulted in agglutination of spermatozoa whereas the "anti-Y" antisera failed to show any agglutination in the spermatozoa. The agglutinated sperm population was separated from the free-swimming sperm by glass wool filtration and the free-swimming sperm population (potentially Y-bearing spermatozoa) was isolated. This method has to be validated by further experiments and another constraint is that the method was successful in isolating Y-bearing spermatozoa only and attempts to isolate X-bearing spermatozoa by agglutinating Y-bearing spermatozoa was not successful. (Blecher *et al.,* 1999).

Polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH)

With the introduction of PCR and FISH, it has now become possible to accurately identify the X- and Y-sperm and this has opened the way for assessment of sorting purity of different sperm sexing methods (Flaherty and Matthews, 1996). Specific DNA sequences on X- and Y-sperm have been reported which can be used to identify the sex of individual sperm and sex ratios of sperm in semen sample (Wang *et al.,* 2011). Accurate determination of the sex ratio using single sperm PCR necessitates analysis of a large number of individual sperm. However, sex ratio of semen can be determined more simply and accurately by quantitative real-time PCR (qPCR) (Parati *et al.,* 2006). However, its application to populations of cells is of limited use in the assessment of sex selection methods. Moreover, it is labor intensive to be used for screening large number of individual sperm (Flaherty and Matthews, 1996). Single and double label FISH can be used for the direct visualization of sex chromosomes in individual sperm. FISH precisely identifies the sex chromosome of individual sperm using specific probes conjugated with fluorescence molecule for the X- and Y-sperm (Flaherty and Matthews, 1996). The application of double FISH using X- and Y-chromosome-specific probes has allowed a more accurate assessment than single label FISH (Han *et al.,*1993) The main advantage of FISH compared to flow cytometry reanalysis and single cell PCR evaluation is that it is highly qualitative and quantitative (Parrilla *et al.,*2003).

Flow-cytometric Sorting of Semen

The autosomes carried by X or Y spermatozoa have identical DNA content and the difference in DNA mass of the X and Y chromosomes has formed the basis upon which flow cytometric analysis of DNA in X and Y spermatozoa has been carried out. One of the first reports describing the analysis of sperm DNA using flow cytometry was made by Gledhill (Gledhill *et al.,* 1976). That report was followed by the ability to distinguish mouse X and Y spermatozoa using flow cytometry (Pinkel *et al.,* 1982).

Flow cytometers are the advanced cell sorters that use LASER to excite fluorescent dye that binds to the DNA in spermatozoa. The DNA percent and DNA specific dye are the major principle for sperm sexing through flow cytometry. In this method of sorting, the spermatozoa are treated with dye (e.g. Hoechst 33342), which is permeable to live and intact sperm membranes and binds to the DNA. Stained spermatozoa are transported to a point where they are exposed individually to a UV laser beam (wavelength of 351 – 364 nm) and the bright blue fluorescence emitted is detected and analyzed. Due to more DNA content in X

chromosome bearing spermatozoa, it takes more stain than Y sperm. On the basis of this fluorescence, spermatozoa are classified as X or Y chromosome bearing and sorted. Another dye, commonly called "red quencher food colouring dye", selectively penetrates into the damaged, dead and non – intact sperm membranes giving a red colour. Identification of live & dead sperm should be done before sorting process. Based on the excitation, spermatozoa are separated into discrete populations.

In domestic animals the differences in DNA content between X and Y bearing spermatozoa ranges from 3 – 4.5% (Johnson *et al.,* 1987; Johnson, 2000). Success rate in this method has been reported to be 85 – 95% (Pinker *et al.,* 1982; Johnson, 2000).

Limitations of flow cytometry

Since the DNA stain Hoechst 33342 can be mutagenic and may have cytotoxic effects, particularly at high dosage (Wiczoreck, 1984). Attention has been drawn to possible harmful effects of the dye on fertility, embryonic development and the normality of offspring.

There is little direct effect of the stain on the capacity to fertilize bovine eggs (Cran *et al.,* 1994). Unfortunately, this technique requires appropriate skills and expertise as well as it is not easily accessible, sex-sorted sperm using flow cytometric technique still has difficulties in terms of sperm damage, high economic cost, complexity of operation, and lower pregnancy rates than the traditional semen (Seidel, 2003; Boro *et al.,* 2016).

Fertility rate with sexed semen was also found lower than conventional semen (Norman Karakaya *et al.,* 2014). Sexed sperms of bull had 75-80% of the fertility of conventional non-sexed, frozen-thawed semen (Schenk *et al.,* 2009). Insemination of lactating dairy cows with low doses of sexed semen has not produced acceptable pregnancy rates (Seidel, 2003)

Effect of sexing process on sperm biochemical features, structure and function

Assessment of plasma membrane proteins

Intense manipulation of sperm during the sexing procedure may induce damage in plasma membrane and premature capacitation (Carvalho, 2013).

According to McNutt and Johnson (1996), the sexing procedure can remove cleavage or change the glycosylation of membrane proteins.

Because the membrane proteins have an important function, especially for events related to in vivo fertilization, it is possible that the lower fertility rate found when sexed sperm are used in vivo has correlation with modification on membrane protein profile of those sperm.

Assessment of motility

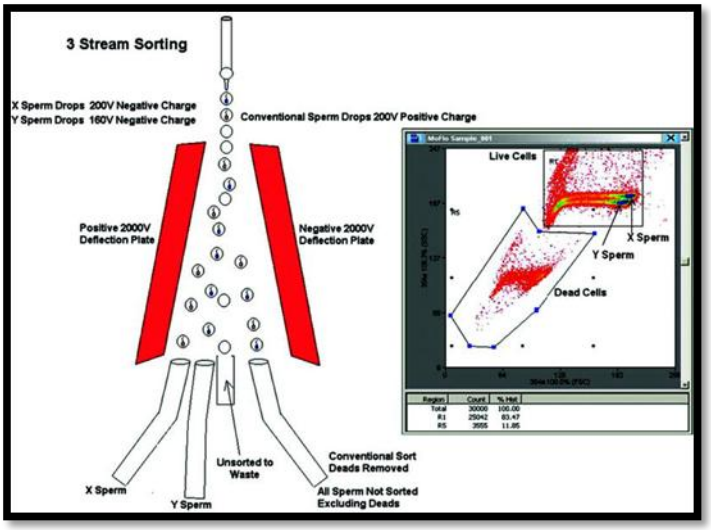
Among the characteristics affected by the sexing process, decreased motility has been reported by several authors (Carvalho *et al.,* 2010).

This change in the sexed sperm motility could have been caused by exposure to Hoechst 33342 stain, the laser light, or exposure in the droplets to electric charges (Watkins *et al.,* 1996).

According to Smith (1993), the effect of exposure to dye and then, the laser may reduce mitochondrial activity, causing a decrease in the production of ATP.

Differences in DNA content between X and Y spermatozoa in different species

Species	% of more DNA of x sperm than y sperm	references
Cattle	3.8	(Garner, 2006). (Johnson and Welch, 1999). (Johnson, 2000).
Buffalo	3.6	(Johnson, 2000). (Lu *et al.*, 2006).
Sheep	4.2	(Johnson, 2000).
Goat	4.4	(Parilla *et al.*, 2004).
Horse	3.7	(Johnson, 2000).
Swine	3.6	(Johnson, 2000).
Human	2.8	(Johnson, 2000).
Rabbit	3.0	(Johnson, 2000).
Camel	3.3	(Johnson, 2000).
Bison	3.6	(Johnson, 2000).
Yak	3.6	(Johnson, 2000).



Percentage of transferable embryos as affected by sorting and sperm dosage

	% Transferable embryos		Semen dosage (million)		
Experiment	**Sexed**	**Conventional**	**Sexed**	**Conventional**	**Heifers or cows**
Hayakawa *et al.*, 2009	53.4	68.1	5.0	5.0	Heifers
Peippo *et al.*, 2009 (Expt. 1)	70.3	75.0	6.0 to 8.0	30 to 45	Heifers
Peippo *et al.*, 2009 (Expt. 2)*	53.9	65.5	6.0 to 8.0	30 to 45	Heifers
Larson *et al.*, 2010*	39.5	60.5	8.4	80	Cows

*Effect of semen type on % transferable embryos (P <0.05)

Pregnancy rates after transfer are similar among embryos produced with sexed or unsorted semen (Schenk *et al.*, 2006; Hayakawa *et al.*, 2009).

Table.1 Dimensions and profiles of sperm heads and flow cytometric sorting indices for some domestic mammals and man[a]

Dimension	Bull	Boar	Ram	Rabbit	Cat	Dog	Horse	Man
Length (mm)	9.1	9.0	8.1	7.7	7.7	7.0	6.5	4.6
Head sagittal section Width (mm)	4.7	5.0	4.0	4.5	3.2	3.5	3.4	3.2
Head profile Area (mm^2)	34.5	37.5	26.6	28.0	19.0	20.9	15.2	10.8
X–Y difference (%)	3.8	3.6	4.2	3.0	4.2	3.9	3.9	2.8
Sorting index[b]	131	115	112	84	80	82	59	31

[a]Compiled (Mann, 1964; Mann and Lutwak-Mann, 1981; Johnson, 2000; Welch and Johnson, 1999; Garner, 2001; Garner and Seidel, 2003; Seidel and Garner, 2003).

Assessment of DNA, plasma membrane and acrosome integrity

Dye used for sorting procedures, sorting speed, pressure, laser light, electrical charging and deviation and changes in the medium collectively leads to defects in spermatozoa. Sexing procedure increases the percentage of sperm with plasma membrane damaged (Spinaci *et al.,* 2013). Besides plasma membrane, the acrosome can also be affected by the sexing process, which can substantially impair the ability of sperm cells to fertilize the oocyte since an acrosome intact is necessary to bind to the zona pellucida and fertilize the oocyte.

Dye Defects

Addition of DNA specific dye (Hoechst 33342) causes the chromatin decondensation (Johnson and Seidel, 1999). Among farm animals, boar has the stable chromatin compared to other farm animals (Bathgate, 2008). However, dye – mediated disturbances of heat shock proteins HSP70 and capacitation like changes in the sperm membranes has also been reported in boar sperms (Spinaci *et al.,* 2006).

Sorting Pressure and Speed

High sorting pressure of 40 – 60 psi and high speed (55 – 60 mph) makes the sperm more vulnerable for the damage of DNA during sorting (Suh *et al.,* 2005).

UV rays

Adverse effects of UV rays on DNA integrity is well known phenomenon. Laser power of 200 MW or higher had detrimental effect on the fertilizing ability of the rabbit sperm due to destruction of chromatin

integrity than sperm exposed to 125 MW (Silva and Gadella, 2006).

Electrical Charging and Electrical Deviation

Due to electrical charging and electrical deviation, the sperm membranes of mid piece and tail undergoe depolarization. Further, relative oxygen species produced by the electrical forces, reduced mitochondrial activity of sperm (Rath and Johnson, 2008). Stressors due to sorting process may damage the DNA to some extent which may potentially compete with spermatozoa having normal DNA and reduce embryonic viability (Tesarik *et al.,* 2004).

Changes in Medium

Changes in pH and osmolarity during sorting process decrease the sperm fertilizing ability (Harrison and Gadella, 2005). Although the sperm is normally exposed to different pH milieu in female reproductive tract to achieve fertilization, changes of pH during sorting process affects the fertilizing ability of sorted sperm.

Any alteration in the sperm physiology like modification of membrane stability, sperm motility or acrosome homeostasis, has a direct impact on the fertilizing capacity of sperm while the altered DNA quality affects the embryo quality leads to syngamy after fertilization of gametes. Removal of seminal plasma, sorting pressure, speed, electrical deviation, laser radiation all leads to membrane alteration (depolarization) and pre – capacitation like changes in the sorted sperm (Schenk *et al.,* 2009). Further, alteration of membrane proteinase due to sorting and freezing were reported by (de Graaf *et al.,* 2008). Overall, it is accepted that the sorting procedures reduce the life span of spermatozoa. Shorter life span cause reduced motility and thus reduced fertility of sex sorted spermatozoa (Peippo *et al.,* 2009).

Measures to Reduce the Defects in Spermatozoa during Sorting

Lowering the sorting pressure from standard pressure of 50 psi to 40 psi is useful to improve sorted spermatozoa quality without significant decrease in sorting efficiency in bull and stallion (Suh *et al.,* 2005).

UV laser with argon or solid state laser has been shown to reduce the defects associated with sperm membranes and DNA (Rath and Johnson, 2008).

Addition of seminal plasma (10% v/v) into the staining medium has been shown to improve the viability, motility and reduce capacitation like changes in boar and ram spermatozoa (de Graaf *et al.,* 2008) as it act as inhibitor of capacitation and maintains pH as alkalinity of spermatozoa in female reproductive tract.

Addition of bovine sheath fluid (197mM tris, 55.4mM citric acid, 47.5mM fructose) in the extender and addition of protamine before sorting process has been found to improve the sperm viability, motility and maintains the fertility of sperms (Gosalvez *et al.,* 2011).

Gradient centrifugation prior to sperm sexing also improves the resolution and sorting rates.

Sexing sperm in tropical livestock production

Currently, sexed semen has been carried out in temperate zones. Developing such a scheme for subtropical and tropical environments is a challenging task

constrained by small flock size, communally shared grazing, uncontrolled mating, and the absence of pedigree and performance recording. The technique sexing sperm can be used to increase reproductive rates in animals and subsequently increase rates of genetic gain through possible higher selection, intensity, and accuracy of selection. Indigenous cattle breeds, possessing high gene frequencies for adaptation, play a particular important role in livestock production systems in the tropics. The relatively low cost of skilled labor in tropical and subtropical countries enables consideration of artificial insemination of sexed semen for cattle and also identification and culling of males producing few sperm or sperm of inferior quality (Sørensen *et al.,* 2011).

Limitations of sperm sexing:

When identical doses of sexed and unsexed sperm were compared to normal dose controls, pregnancy rates of the low dose controls were close to the normal dose controls, indicating that the damage due to sexing sperm was also expressed as lower pregnancy rates (Seidel and Schenk, 2000).

Most equine sperm needs to be separated from its seminal plasma prior to processing for sperm sexing. This adds additional stress to the sample even prior to the sexing procedure.

Slow process. i.e. less number of spermatozoa sorted per hour due to sexing of one sperm at a time rather than multiple sperm and thus less number of sperm are being identified for its sex or only less number of straws are being produced (7 – 10 dose/hour) (Seidel, 2007).

Half of sperm sample are unsexable and go as waste (only 30% of sperm are sexable in which only 15% responsible for female offspring), leading to increased cost of sexed semen compared to unsexed semen.

Efficiency of sexing of sperm is best with fresh sperm, so sorters should be located near the bull's stations.

The equipment is extremely expensive and specially trained technicians are needed to assure sorting accuracy.

Require skilled manpower for operation and supervision of machine.

Management strategies when using sexed semen

If AI pregnancy rates with conventional semen are consistently 60% or better, then only sexed semen in herd should be considered.

It should be used only in healthy cycling females in good body condition. AI companies suggest using sexed semen only in heifers; however, research indicates that cycling mature beef cows are also good candidates.

Only the animals observed in heat, should be inseminated.

Only experienced and proven AI technicians should be allowed to inseminate cows or heifers.

Extreme care should be taken, during semen thawing and handling.

Numerous methods have been reported to separate X- and Y-chromosome bearing sperm. Improvements in semen technology have now contributed to a marked lift in fertility with sex sorted frozen semen. However, the common underlying problem

from these methods has been the lack of reproducibility. There is still a need in the animal breeding industry to develop a technique for sperm sexing that provides sufficient spermatozoa for AI doses, does not compromise sperm fertility, and is widely applicable to a range of species.

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