ISSN: 2456-2912
VET 2021; 6(1): 04-10

www.veterinarypaper.com
Received: 22-10-2020
Accepted: 05-12-2020

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A mini review on semen sexing in bovine

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DOI: https://doi.org/10.22271/veterinary.2021.v6.i1a.315

Abstract
Sexing of mammalian sperm is a long-standing goal in agricultural industries including cattle breeding. Being able to pre-select the sex of offspring at the time of conception, ranks among the most sought-after reproductive biotechnologies of all time. Sexed semen is commercially offered by most global A.I. companies yielding with 90% accuracy. Fertility of sexed sperm is typically lower compared with conventional sperm. Sexed semen is recommended for breeding only virgin heifers followed by 1st or 2nd lactational cow. The success of sexed semen industry depends upon the sorting speed, accuracy and the fertility of sorted spermatozoa. The use of sexed semen is expected to affect the structure of the dairy industry by creating a greater supply of replacement heifer if the reduction in conception rate can be minimized. Today, Flow cytometry is the only successful technique for the sorting of sperm.

Keywords: Semen sexing, flo cytometry, bovine, H-Y antigen, conception rate

Introduction

The aim of sexed semen is to supply a calf of a specific sex. In the livestock industry, predetermination of sex of animals has been a main goal of producers for generations, because of its financial advantage. Females are essential for dairy products and production of calves, while males are usually required for beef or meat production, because of the better feed conversion efficiency and lean-to-fat ratio. In addition, a male of high genetic merit is still required as sires in AI programs and by sexed semen fastens the genetic progress and allows the farm manger to increase selectively the amount of heifers or steers supported the necessity of the farm. It also reduces calving difficulty in first calvers (Seidel, 2007) [34] and reduces the replacement cost besides maintaining the biosecurity in the farm. There is an urgent need to expand the genetic merit of our cattle and buffaloes with the decreasing land productivity, crop yield and other feed resources; and also, due to a steady increase in the demand and consumption of high quality dairy products.

Importance of semen sexing

The most important application of sexing is to minimize sex-linked genetic disease in the population. The sex pre-selection to increase the chance of having a female child gives the reasonable assurance that they can avoid expression of the disease in their offspring. Some of the sex linked diseases are Haemophilia, Baldness and Eye colour in Drosophila flies.

Fertilization of ovum with Y sperm produces male off-spring and X sperm produces female off-spring. So if we could able to separate X and Y sperm then it would be possible to produce the calf of the desired sex. In progeny testing program sires are selected based on the performance of the female progenies. So if ovum could be fertilized with X sperm then we can eliminate the chances of production of male progenies. If only a few individuals of an endangered species are available, chance of production of a few males instead of a few females often would greatly diminish chances of survival of the species. Sex preselection for particular matings also becomes crucial in developing breeding systems that minimize inbreeding with few breeding animals. A commercial dairy farmer mates a heifer or cow for two reasons. The commercial beef cattle farmer produces calves for eventual sale as slaughter animals and for female herd replacements. Depending upon the role that is envisioned for a calf, males and females are of quite different value; so sexed semen potentially is a powerful technology to affect biological and economic efficiency (Hohenboken, 1999) [14].

Seedstock breeders produce breeding animals, semen and embryos for sale. The primary role of their animals is to produce profitable and efficient offspring. To be competitive in achieving this goal, they must maximize the rate of genetic improvement in their herds, control inbreeding accumulation, and accomplish these conflicting goals as economically as possible. They must seek the optimum balance between male and female selection intensities, selection accuracies and generation intervals (Hohenboken, 1999) [14]. Only live, membrane intact sperms sorted: With flow-cytometry sorting of spermatozoa, only live membrane spermatozoa takes the stain and remaining dead spermatozoa will not take colour of stain. So dead spermatozoa are discarded or eliminated at the time of sperm sorting.

The matured oocytes Sex-sorted bovine sperm were used for *in vitro* fertilization (IVF) to generate embryos from *in vitro*. Sex-sorted sperm is used in embryo transfer technology which will be useful to produce desired sex from genetically superior animals (Cran *et al*., 1993) [30].

Methods or approaches of sperm sexing

More sophisticated methods have been offered over the last few decades. Apart from recommendations concerning sexual behavior, most methods have relied on *in vitro* sperm-separation techniques. Successful separation depends on the existence of fundamental differences between sperm cells containing either X- or Y-chromosomes, such as Variations in relative survival rates in a different pH medium (Shettles, 1960) [33], Contrasts in sperm-surface charge that allow separation of spermatozoa according to their differences in electrophoretic motility (Engelmann *et al*, 1988) [4], Difference in weight, density, size and sperm swimming characteristics, Difference in the DNA content – Flow cytometric sorting of the semen.

Sexing on the basis of the difference in mass and density is Percoll Density Gradient Method. In a preliminary experiment, it was found that human X-bearing sperm tend to sediment faster than Y-bearing sperm in a density gradient centrifugation using Percoll (Kneko *et al*., 1984) [20]. Percoll is silica coated material with polyvinylpyrilidone used for density gradient. By more careful examination of the conditions for sedimentation of the two classes of sperm in a discontinuous density gradient with 12 steps of Percoll, selective isolation of human X-bearing sperm (purity of up to 94%) as the sediment has been achieved with the best recovery rate of almost 30% of the total sperm applied. The recovered X-bearing sperm showed active forward movement and were free of other contaminating cells found in the semen. Using the X-bearing sperm fraction for artificial insemination, at least six girls were born. The ratio of Y-bearing sperm in the upper layers of Percoll, however, did not increase remarkably under the same conditions. In spite of such a difference in sedimentation velocity between the two classes of sperm, their apparent densities showed little difference when examined by equilibrium sedimentation in a continuous density gradient of Percoll. This means some other factor (s) than apparent density affect the sedimentation velocity of X- and Y-bearing sperm. Density Gradient Centrifugation Density Gradient Centrifugation is a method of separation of X and Y chromosomes bearing sperm. In this procedure, sperm is centrifuged through increasingly dense layers of a solution, on the theory that heavier X-sperm will sediment to the bottom, and lighter Y-sperm will migrate to top. A density medium, a liquid solution available in various thicknesses, is layered in a conical centrifuge tube, with the densest layer at the bottom and the least dense layer at the top. In the standard procedure of preparing sperm for assisted reproduction, 2 layers are used. For gender selection, 3 to 12 layers are used. Diluted semen is layered on top, and centrifuged for 30 minutes. To conceive a woman, only rock bottom layer, or "pellet", is retained, during which the heavier X-sperm have sedimented. To conceive a boy, only the highest layer of the liquid "supernatant" is retained, containing the lighter Y-sperm. The remainder is washed and centrifuged again to remove the density medium.

Centrifugal countercurrent distribution is methods of separation of human X- and Y- chromosomes sperm on their centrifugal density. It's specially designed machine on the basis of the invention by Akerlund (1984) [1]. The apparatus contains 60 chambers arranged in a circle, which allows transfer of the upper (mobile) phases relative to the lower (stationary) phases. In this system, CCD is performed during centrifugation by keeping the denser (bottom) phases in the outer half while the lighter (upper) phases are in the inner half of each chamber. Because no elution or pumping of any phase takes place, the overall process consists of a circular multistep transfer of 60 uppers- over 60 bottom-batch phases. Each transfer in this centrifugal-enhanced CCD includes shaking the phases at unit gravity to thoroughly mix them and then separating them by centrifugation. (Ollero, *et al*., 2000). Seminal plasma was removed from the semen samples by a sucrose washing method. An aliquot of semen was diluted with 1.5 sucrose buffers (222 mm sucrose, 10 mm NaCl, 10 mm glucose, 20 mm HEPES, and 2.5 mm KOH). One milliliter of diluted sample was layered over 7.5 ml of the same medium and centrifuged for 5 minutes at 200 g and for 10 minutes at 1000 g; the supernatant layer was removed to leave a 1-ml volume in which the loose pellet of spermatozoa was suspended. Sperm samples were loaded in chamber 0 to 59 transfers were carried out. Shaking and centrifugation time was 60 seconds and all operations were performed at 20 °C. After the run, the solutions were transformed into a one-phase system by the addition of 1 volume of a dilution buffer (polymer-free medium). The fractions were then collected and the cells were counted under a light microscope. Partition results are expressed as the number of cells recovered in each fraction. Total viable cells in each fraction were assessed by the following: % viability recovered cells in each fraction/100. As a consequence of the separation procedure, sperm cell populations with a marked affinity for the lower dextran-rich phase (mainly due to a low hydrophobicity) partition in the left part of the profile. Sperm cells that partition almost equally in both phases distribute in the central sector. Finally, a sperm population with a high affinity for the upper PEG-rich phase (mainly due to a high hydrophobicity) and partition in the right sector of the profile was separated (Harrison *et al*., 1982) [13].

Sexing on the basis of difference in motility is Albumin Gradient Method, Successful separation of X- and Y-chromosome- human spermatozoa using an albumin gradient was first reported by Ericsson *et al*. (1973) [6]. The method is based on size that Y-chromosome spermatozoa are smaller in size and exhibit a greater downward swimming velocity than X-chromosome-bearing spermatozoa within vertical columns of high density human serum albumin (Ericsson *et al*., 1973) [6]. A fraction enriched with Y-chromosome bearing spermatozoa can be obtained by obtaining the first 22% of spermatozoa to swim to the bottom of the gradient, and discarding the remainder. The latest version of this technique improved the separation accuracy. (Ericsson and Ericsson, 1999) [5]. This technique has never been shown to sex spermatozoa accurately from mammals aside from humans (Beal *et al*., 1984; White *et al*., 1984) [2, 42].

Sephadex column separation: Sephadex is the methods of the separation of human X- and Y- chromosomes sperm. Sephadex column separation By means of the Sephadex gel filtration method we were ready to separate a fraction rich in X human spermatozoa, in contrast with the tactic of Ericsson *et al*. (1973) [6], which allows to obtain a fraction rich in Y sperm. The method, which is simple and not time-consuming, may allow control of sex in cases of expressing sex desire in off-spring. The separation of X- and Y sperm in human spermatozoa by means of Sephadex gel filtration was performed with Locke's solution at room temperature. Smears of semen and smears of the filtrate, and washed spermatozoa were stained with fluorochrome Quinacrine, in which fraction rich of X-bearing spermatozoa was obtained. The average number of F-bodies detected in semen before gel filtration was 38.8%, whereas the average number of F-bodies in filtrate samples was 4.7%, and 64.7% in washed spermatozoa. The concentration of spermatozoa in the collected fraction was about 25% of the sample concentrations prior to gel filtration. It might be due to that, immobile sperm cells are retained by the lack of an active passage, while Y-bearing spermatozoa are most likely attached to the gel particles by adsorption (Steeno *et al*., 1975) [35]. Sephadex column albumin gradient separation: Sephadex column albumin gradient is a method of separation of human X- and Y-bearing sperm. In which the semen was passed through the columns of Sephadex G50 in order to increase the proportion of X spermatozoa. The X spermatozoa increased from a mean of 60% to 74%. The degree of separation in individual samples showed considerable variation in this method, but in some samples only a 6% change in the proportion of X and Y spermatozoa could be obtained, whereas in others the increase was 37%. The proportion of motile spermatozoa showed a marked increase with the Sephadex method (Quinlivan, *et al*, 1982) [27].

Sexing on the basis of differences in surface charge is free flow Electrophoresis Method: Electrophoretic separation of X- and Y-bearing sperm has been tried by many investigators, on the basis of the difference in electric charge on their surface. Sperm moved towards the anode at pH 7.4 and were separated into two main peaks, the faster peak containing only X sperm and the slower peak consisting mainly (83-89%) of Y sperm. In other words, X sperm have a higher net negative charge on the cell surface than do Y-bearing sperm. Contamination of immature sperm and/or agglutinated sperm appeared to reduce the yield of Y sperm in the slower peak. The results were reproducible provided that normal semen samples were properly processed. Sperm motility was reduced after free-flow electrophoresis. Improvement of composition of buffers and other media would result in separation of the two classes of sperm without loss of motility. In fact, it was confirmed that bull sperm were fertile after the electrophoresis. The negative charge of the cell surface is mainly due to projection into the surrounding medium of glycoproteins with neuraminic acid and sulfate group. When human sperm were treated with neuraminidase prior to free-flow electrophoresis, the electrophoretic mobilities of the two classes of sperm were much reduced, and the two separated peaks finally joined into a single peak in which the yield of Y-bearing sperm was 43-46%. The observed difference in net negative charge appeared to be due at least in part to a larger content of glycoproteins possessing neuraminic acid on the surface of X-bearing sperm. Although X-bearing sperm have a larger surface area than Y-bearing sperm, it is possible that the surface difference between the two classes of sperm reflects the haploid gene expression. There was no remarkable difference in morphology of the separated human X- and Y-chromosome bearing sperm. Examination of separated X- and Y-sperm fractions by means other than quinacrine mustard staining is now in progress. Countercurrent galvanic separation: Convection Countercurrent galvanization is a method and apparatus for controlling the sex of mammalian offspring through separation of X-chromosome and Y-chromosome bearing sperm. The separation is accomplished by producing a thermal convection counter stream within a sedimentation column containing a universal medium with sperm suspended therein, and subsequently allowing the two sperm populations to sediment into different fractions according to different densities. A method of separating sperm cells of differing densities and electrical potentials from semen comprising to the mixing fresh semen with a liquid suspending medium and separating the mixture by thermal convection counter streaming sedimentation according to different sperm densities into three fractions, a first fraction containing predominately X-sperm of a normal genotype, a second fraction containing predominately Y-sperm of a normal genotype, and a third fraction containing both X and Y-sperm carrying defective genes; and subjecting a desired fraction of said medium to convection galvanization thus further separating and concentrating all X-sperm of a normal genotype and all Y-sperm of normal genotype (Gledhil, 1988) [7].

Sexing on the basis of immunological difference in sex chromosomes

Separation on the Basis of Presence of H-Y Antigen: Expose live, native spermatozoa to an excess concentration of an antibody that binds substantially exclusively to male determining spermatozoa. Specifically, freshly ejaculated spermatozoa are diluted. The diluent used varies depending on the species of the spermatozoa being separated. Specific examples of diluents include sodium citrate solution for bovine spermatozoa, creme gel buffer for equine spermatozoa and test buffer for human spermatozoa. The diluent used, however, should be free of protein as any protein in the diluent tends to coat the spermatozoa and block the H-Y antigen binding sites on the surface of the male determining spermatozoa. Thus, by substantially eliminating this blocking protein provides conditions for optimal cross reactivity between the antibody and antigen for the best possible immuno separation results. An excess concentration of the male specific antibody is contained in or added to the diluent and spermatozoa. The male specific antibody is a monoclonal anti-H-Y antibody (immunoglobulin G) prepared by outgrowth of primary hybridomas and recovered from culture medium by stepwise precipitation and dialysis. So only male determining spermatozoa express the H-Y antigen on their surface. The female determining spermatozoa do not express the H-Y antigen, believed associated with the Y or male determining chromosome. Since the monoclonal antibody is absolutely specific for the H-Y antigen, it binds only to the male determining spermatozoa and not any of the female determining spermatozoa. Following a short incubation period for immunoreaction of 45 minutes, the spermatozoa/monoclonal H-Y antibody dilution is centrifuged and collect the exposed spermatozoa from the diluent. The exposed spermatozoa are then washed using fresh, pure protein free diluent in order to remove unbound monoclonal antibody. After washing, the exposed spermatozoa are suspended in an equal volume of protein free diluent (as used during the exposing step) substantially without the presence of unbound H-Y-antibody. Immediately thereafter, antibody/Immuno absorbent conjugates are added to the dilution so as to form a conjugate/spermatozoa suspension preparation. The antibody is coupled to the Immuno absorbent material or agarose beads binds immunoglobulin G and, therefore, binds the monoclonal anti-H-Y antibody already bound to the male determining spermatozoa. Again, just as with the first antibody the female determining spermatozoa do not immunoreaction with the immunoglobulin G antibody bound to the beads. In order for the second, immunoglobulin G antibody, to properly react with the monoclonal H-Y antibody, the immunoglobulin G antibody must be species specific for the monoclonal antibody. Thus, if spermatozoa are exposed to monoclonal H-Y antibody produced by mice lymphocyte cells as described above, the second antibody bound to the conjugate must be anti-mouse immunoglobulin G. The anti-immunoglobulin G/agarose bead conjugate is added to the diluted spermatozoa at an approximate concentration of 0.4 milliliters of conjugate beads per milliliter of diluted spermatozoa. The conjugate/spermatozoa preparation is incubated at approximately 37 ˚C and agitated to prevent the beads from settling and thereby maintain the beads and spermatozoa in physical contact for complete immunoreaction. During this time, the anti-immunoglobulin G antibody of the conjugate binds the monoclonal H-Y antibody bound to the male determining spermatozoa. Thus, the antibody/bead conjugate agglutinates the monoclonal antibody/male determining spermatozoa complexes. Following immunoreaction and agglutination, the female determining spermatozoa are recovered. This step may be completed by draining the conjugate/spermatozoa preparation solution from the antibody/agarose bead conjugates now bound to the male determining spermatozoa. Since the female determining spermatozoa do not bind to the beads, the female determining spermatozoa are contained in this drained solution. The solution is then gently centrifuged to collect the female determining spermatozoa in a pellet at the bottom of the centrifuge tube without significantly adversely affecting spermatozoa viability. The pelleted female determining spermatozoa are then resuspended in a diluent and added into the filtering column. Viable spermatozoa pass through or are eluted from the column at a substantially faster rate than dead or weak spermatozoa and, therefore, excellent filtering results are obtainable. The spermatozoa eluted from the column may be collected in a protein containing medium for maintaining the spermatozoa for freezing and/or subsequent use in artificial insemination. An example of such a protein containing medium is the protein free diluent used above during the exposing and suspending steps mixed with egg yolk, 20% by volume. The male determining spermatozoa are also recovered from the agarose beads. The methods include competitive inhibition using a solution of male specific antibody, enzymatic digestion of the beads, and alteration of the pH or salt concentration of the diluent. Following recovery, the male determining spermatozoa is filtered through a sephadex column in the same manner as the female determining spermatozoa so as to increase the efficacy of the male determining spermatozoa fraction or sample. Sex Specific Proteins (SSPs): A viable immunological sperm sexing procedure which is non-invasive can be evolve using a more efficient method to isolate sex-specific proteins (SSPs) (Blecher *et al*., 1999) [3]. A large proportion of genes on the mammalian X chromosome are highly evolutionarily conserved; an X-linked genes in any one species predicts the existence of a homologue in all others. This proposition is known as Ohno‟s law (Ohno and Watchtel, 1979) [26]. The hypothesis is that 1) the sex chromosomes encode or control the expression of X- and Y- sex chromosome-specific proteins (X- or Y- SCSPs) that are expressed on the surface of somatic and sperm cells, and 2) as an extrapolation of Ohno‟s law, that these proteins are more highly conserved between species than non-specific molecules. As a consequence of the latter, when a same-sex recipient is immunized with tissue from a special species, the recipient’s system would mainly perceive SSPs as “self” and would not raise antibodies to these molecules. The letter would reflect species differences in the non SSPs; these antibodies could be used to immune precipitate non-SSPs and leave SSPs in a partially purified form. Based on this hypothesis the non-SSPs were removed immunologically before the attempted isolation of SSPs because they are likely to be more highly conserved than non-SSPs. Antibodies to SSPs were grown and used to identify SSPs by affinity chromatography (Blecher *et al*., 1999) [3]. In these methods Inject sperm and Freund”s incomplete adjuvant s/c in female and male rabbits respectively and semen of anti “X” or anti “Y” antisera and Incubated for 60 min at 38.5̊ C. Advantages of these methods are free swimming semen separated, only takes 20-30 minute and *in vitro* fertilization – 92% male embryos can produce (Moore and Thatcher, 1988). DNA Based Methods for Sperm Sexing Sorting based on volumetric differences: Spermatozoa containing an X chromosome are theoretically bigger than those containing a Y chromosome. Van Munster *et al*., (1999) [38] recently 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- chromosome carrying bovine spermatozoa. A method based on this principle has been evolved for sorting live spermatozoa by using interference microscopy optics with a flow cytometer (Van Munster, 2002) [37].

Flow-cytometric sorting of semen: It was first reported by Johnson, (1989) [16] in rabbits. Flow cytometry for selection of gender is based on variability in chromatin staining by the DNA-binding fluorescent dye Hoechst 33342, detection of fluorescence from individual cells, and sorting of individual cells based on fluorescence. Selected spermatozoa are then used for IUI, IVF or ICSI. However, purities of X- and Y-bearing sperm of 70-90%, as judged by PCR and FISH methods have been reported (Johnson, 1995, Vidal *et al*., 1998) [17, 39]. The difference of their DNA-content can be used by flow-cytometry to produce populations of either sex at high accuracy. As the method is predicated on single cell identification, the output of cells is restricted, although major improvements were made by high-speed flow-cytometry and improved cell orientation ahead of the UV-Laser.
Flow cytometer Principles and cell sorter use for semen sexing: The spermatozoon passes two fluorescence detectors at 90°angles to each other, each detector measures the intensity of fluorescence resulting from the excitation of the DNA-bound dye molecules by light, which is generated by a laser. The wavelengths of light used depend on the source of light. The strength of the fluorescence signals clearly depends

on the number of fluorescing molecules bound to DNA. This is the basis for sexing spermatozoa. In addition, the signal depends on a number of other parameters, including the laser intensity (Guthrie *et al*., 2002) [8], whether and how the laser is pulsed, optical properties of the entire system, the sensitivity of detectors and electronic noise. The main obstacle to accurate quantification of spermatozoa DNA with this approach is the geometry of the sperm head, which is paddle-shaped in most species of interest. The intensity of fluorescence is lowest if the flat face of the paddle is oriented toward a detector, and highest when the edge is so oriented. The most accurate discrimination of X and Y chromosome spermatozoa are results when its orientation is flat. The second detector at 90° to the laser is used to diagnose orientation (Senger, 2003) [32]. As the fluorescence signal is highest for spermatozoa oriented with their paddle edge toward this 90° detector, only the population of spermatozoa that emit peak fluorescence to the 90° detector are considered oriented appropriately for sexing by the contemporaneous signal to the 0°detector. A considerable effort, therefore, has been made to increase this percentage by modifying the cylindrical geometry of the fluid stream (Johnson and Welch, 1999; Rens *et al*., 2001) [18, 29]. DNA-binding dyes: Hoechst 33342 (H33342) may be a live cell stain that permeates the cell wall and binds selectively to A - T base pairs along the minor groove of dsDNA. H33342 usually is happy with the 351 or 364 nm lines of an argon-ion laser Most DNA stains intercalate between the base pairs of the DNA, thereby presumably increasing their mutagenicity. H33342 is carefully used as it might be toxic to workers at high doses. Dead or moribund cells in the population of spermatozoa stained with H33342 can be identified by adding propidium iodide (Johnson *et al*., 1994). More recently, this classical dead-cell stain has been replaced with red food dye (FD&C40) to avoid potential mutagenic effects of propidium iodide (Johnson and Welch, 2001; Schenk *et al*., 1999) [29, 30]. Other food dyes also are effective. Two basic sperm populations: Living (Stained with Hoechst 33342) Dead (Stained with Red food Dye)

These methods are independent of the type of cell sorter, sample preparation begins with the staining of spermatozoa using Hoechst 33342 stain at 34-38 °C for 60-90 minutes. Labelled spermatozoa are introduced into the flow cytometer across the sample line under high pressure. Two streams of the sheath fluid with pressure just above the sample pressure surround the core stream in the injection tube. Oriented spermatozoa exit through the vibrating nozzle tip, which produces small droplets, theoretically containing one labelled spermatozoa only. Immediately after their exit, the laser light excites the fluorescent dye and the emitted light is collected through detectors in position 0° and 90°. The electric signals of the photocells are processed in a computer. Cells failing to fulfill preset criteria are excluded from further analysis. The proper positioned cells are selected as X- or Y-chromosome bearing spermatozoa, according to the amount of emitted light from the flat sperm surface (0° detector). Based on this signal, the computer sends the signal to the wire loop to electrically charge the droplets accordingly. Charged droplets pass an electro-static field and are separately collected into tubes pre-filled with a collection extender. Sorted spermatozoa are then washed from the sheath fluid by centrifugation and the remaining sperm pellet is extended in a suitable medium. Composition of media differ in among species and also according to the following preservation process (Hollinshead *et al*., 2004; Lindsey *et al*., 2002; Rath *et al*., 2003; Seidel *et al*., 1996) [31, 15, 23, 28, 30]. Flow-cytometry based sperm sorting is the only available method that has been shown to produce high purity of sex chromosome selection. Several major improvements have been made in the past years, especially by invention of high speed cell sorting and improved orientation of cells in front of the Laser. Nevertheless, the limitations of the technology due to the principle of single sperm cell analysis have to be recognized and even with further technical improvements it is unlikely that the output will reach the amount of a normal insemination dosage, especially in the porcine. In combination with other biotechnologies, bovine sexed spermatozoa are already commercially available. In other species intensive research is required to provide sufficient sexed spermatozoa.

Limitations of Flow-Cytometry: It require specialized, expensive equipments and highly skilled operators, Risk of cyto-toxic mutagenic effect, Reduction in fertility, Reduction in rate of blastocyst formation (Merton *et al.*, 1997) [25], Cost of semen dose is higher, Present day methods do not result in 100% accuracy (Johnson, 1991) [19].

Evaluation of Sperm Separation Efficacy

Quinacrine Staining: Y-bearing spermatozoa exhibit a fluorescent spot or Y body. X-bearing spermatozoa remain unstained. However, quinacrine produces false positive (two Y bodies) and false negative results. This technique can produce misleading and inaccurate results with human spermatozoa. (Van kooij and Vanoost, 1992) [36].

Sort reanalysis: Sort reanalysis for DNA has an advantage over FISH and PCR since both techniques may take 3 to 4 h, whereas reanalysis in the flow cytometer requires less than 40 minutes. For reanalysis in the sorter 100,000 spermatozoa are taken preferable from the originally sorted material or can be taken from an extra sort for reanalysis. Cells are sonicated to remove tails and Hoechst 33342 is added at a 10^{th} of the original concentration in order to maintain staining uniformity. Spermatozoa are then analyzed, but not sorted at very low speed in order to maximize orientation. With the proper set up of the sorter and uniformly stained sperm population, more than 90% of the sperm sorted will be the desired sex. (Johnson and Welch, 1999) [18]

Polymerase Chain Reaction (PCR): It is based on using primers from putative testis - determining *SRY* on Y-chromosome to discriminate male DNA. It controls primers from sperm receptor gene on chromosome 7. It has been used to determine the ratio of X- to Y-bearing spermatozoa in the semen sample. It is very specific and highly sensitive. Its application to population of cells is of limited use in the evaluation of sex selection procedures (Lobel *et al*., 1993, Welch *et al*., 1993) [24].

Fluorescence In-situ Hybridization (FISH): FISH is a technique in which chromosome-specific DNA probes are hybridized to chromosomes in cells and are then visualized using reagents which produce a colored, fluorescent or radioactive signal. Fluorescence *in situ* hybridization (FISH) allows the detection of specific nucleic acid sequences on morphologically preserved spermatozoa, is an ideal method for quantitatively and qualitatively assessing the purity of sorted sperm samples (Piumi *et al.* 2001, Rens *et al.* 2001) [29]. The most important reagents on a FISH procedure are the fluorescent DNA probes. After or during DNA amplification steps by PCR, specific DNA must be labelled by incorporating fluorescent nucleotides. It is important to use a labelling method that allows the production of probes with good specificity that is easy to visualize producing optimal hybridization results. The type of probe and labelling method should be chosen according to the sensitivity requirements of the situation. FISH can be used on cells at either interphase or metaphase, so it is well suited to the detection of chromosomes in interphase sperm nuclei. Early studies used auto radiographic detection systems (Joseph *et al.*, 1984), but more recently, fluorescence in-situ hybridization (FISH) has become the method of choice for studying chromosomes in spermatozoa (Kawarasaki *et al*., 1998) [22].

FISH using a single probe to either the X or Y chromosome has been used to confirm that the ratio of X- to Y-bearing spermatozoa in human semen is 1:1 (Guttenbach and Schmid, 1990; Wyrobek *et al.*, 1990; Han *et al.*, 1992) [9, 43, 11]. Single label FISH has also been used to evaluate human sperm fractions recovered from albumin gradients and sephadex columns (Vidal *et al.*, 1993) [40]. However, when only a single probe is used, only some of the spermatozoa exhibit a hybridization signal and an assumption has to be made that the unlabelled spermatozoa carry the other sex chromosome. This is an unacceptable assumption because spermatozoa can remain unlabelled for several reasons: (i) they carry the other chromosome; (ii) they are aneuploid and do not carry either sex chromosome; or (iii) hybridization failed to occur. Furthermore, the overall hybridization efficiency cannot be determined using single label FISH, so the proportion of spermatozoa that were unlabelled due to hybridization failure cannot be determined.

To obtain unequivocal identification of X- and Y-bearing sperm, it is necessary to use a double label FISH procedure in which probes to both chromosomes are simultaneously hybridized to sperm nuclei (Han *et al.*, 1993) [10]. This enables accurate and unequivocal identification of the gender of each spermatozoon. Ideally, a third autosomal probe should be included to identify spermatozoa which are nullisomic for the sex chromosomes.

Advantages of FISH: Chromosome specific DNA probes are used, Individual spermatozoa are analyzed rather than population spermatozoa and large number of spermatozoa can be scored quickly and accurately.

Advantage of Semen Sexing over Embryo Sexing: ETT in remote areas - difficult as compared to AI, Less technical expertise needed in AI, Wastage of unwanted embryos and Time required for production of embryo animal has to cycle, the rest to be given.

Limitations of semen sexing

Low sorting rate: Full day's sort of intact spermatozoa will only yield 20 X 10^6 spermatozoa. It would take 1-2 hour to sort the sperm in a typical artificial insemination dose.

Expense: Flow cytometer is very expensive to purchase and maintain. Sex specific sperm will cost more and may be packaged differently for AI than conventional semen.

Required skill: as with many new technologies, effective commercial use of sex specific sperm will require greater management and labour skills, including well trained inseminator and properly design livestock handling facility (Kansara *et al*, 2009) [21]. Mutagenic effects of DNA binding dyes such as Hoechst 33342 bind loosely to the DNA in the genome but May still cause chromosomal aberrations. Fluorochrome dyes are a potential cause of low embryo viability and hence low pregnancy rates.

Low sperm viability/ fertility: To facilitate access of DNA stain to the genome, the integrity of the cell membrane has to be compromised either by digestion with papain or by light sonication, sonication increases cell survival rate but at the expense of sperm motility as sonication causes loss sperm flagellum, necessitating sperm microinjection. Fertilization and embryo development rates in IVF programs with sex semen are lower than the unsexed semen.

Low Conception: low conception rate achieved with sex semen in high producing lactating cow could be unsatisfactorily. Sexing semen having low fertility (70-80%) than the normal doses of unsexed semen. (Kansara *et al*, 2009) [21]. Sexing semen comes with two handicaps: limited number and stresses due to the number of steps and manipulation, they undergo during the sexing process.

It can conclude that sexed semen is commercially offered by most global A.I. companies yielding with 90% accuracy. Fertility of sexed sperm is usually lower compared with conventional sperm. Sexed semen is recommended for breeding only virgin heifers followed by 1st or 2nd lactational cow. The success of sexed semen industry depends upon the sorting speed, accuracy and the fertility of sorted spermatozoa. The use of sexed semen is expected to affect the structure of the dairy industry by creating a greater supply of replacement heifer if the reduction in conception rate can be minimized. Today, Flow cytometry is the only successful technique for the sorting of sperm.

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