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Review Article

SEXED SEMEN TECHNOLOGY IN CATTLE

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Gender sorting is one of the biggest outcome of research in livestock sector during last two decades of twentieth century. Sexed bovine semen came to the stage of broad commercial application within past few years. Use of sexed semen in dairy sector not only increase the number of superior heifers but also increase the genetic progress in a herd. It also helps in producing good male germplasm from elite bulls for future breeding. Several attempts have been made in different part of world and various methods are developed based on density gradient centrifugation or swim-up, sex specific antibodies, free flow electrophoresis and flow cytometry that efficiently separate bovine semen into fractions containing higher concentrations of X or Y chromosome bearing sperm. Among many proposed methods of sex sorting, flow cytometry is the only method proven to be commercially viable with around 90% accuracy to produce calves of desirable sex. However, during sorting of sperm by flow cytometry, sperm passes through nozzle with pressure, dyeing of DNA, passing through ultraviolet laser beam, electrostatic separation and centrifugation all leads to alteration of membrane and other changes like pre-capacitation in the sorted sperm leading to decrease in fertility. Despite these limitations, production of sexed semen usually followed by cryopreservation is being used commercially for cattle production. Advancement of instrument and enhancement of viable sperm count during sorting and also purity is still important area of investigation. With additional research, use of sexed semen with dairy heifers and cows could become widespread. Hence, this paper is reviewing the sexed semen methodology and applications in dairy cattle.

Key words: Dairy, Flow cytometry, Sexed semen, Spermatozoa

The livestock owner desired to search a suitable method to predetermine the sex of offspring for their herds for thousands of years. This goes back to the early Greeks when Democritus, 470-402 BC, believed that the male is produced due to right testis, whereas females by the left one. During first half of the 20th century, with the advancement in the biological sciences especially genetics, resulted in numerous discoveries including identification of the sex chromosomes. The history of development of commercial sexed semen was summarized by Garner and Seidel

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(2008). Briefly, the basic technology was developed in the early 1980s at the United States Department of Energy's Lawrence-Livermore Laboratory in California using procedures that required de-membraning sperm, resulting in non-viable sperm (Garner *et al*., 1983). With the advancement these methods were significantly improved to keep sperm live and fertile after sorting. This research was led by Dr Lawrence Johnson at the United States Department of Agriculture in Beltsville, Maryland (Johnson *et al*., 1989). Procedures were further improved by efforts at Colorado State University and made practical for use in artificial insemination.

Sexing of semen is separation of X and Y chromosome bearing sperm for selecting what type of sperm cell is to fertilize the egg cell. Several conventional techniques of sperm sorting have been routinely used including the method of density gradient centrifugation or swim-up, identification of H - Y Antigen and free flow electrophoresis. Flow cytometry is newly applied method which expands the possibilities of commercial sperm sorting. Sexed semen is semen in which the fractions of X chromosome bearing (female) and Y chromosome bearing (male) sperm have been separated from the natural mix through sorting and selection. Sorting is based on DNA content of sperm which is utilized in flow cytometrical cell sorting technique (Weigel, 2004 and Seidel Jr, 2007). The differences in DNA content in domestic animals between X and Y chromosome bearing spermatozoa ranges from 3% - 4.5% (Johnson, 2000). In cattle, both male and female chromosome bearing sperm can be electrically charged differently with the help of DNA-binding fluorescent dye. This allows for their separation by a fluorescence-activated cell sorter (Seidel Jr, 2007). The method is fairly accurate with about 90% of the sperm containing the desired sex (Garner and Seidel, 2003 and DeJarnette *et al*., 2008).

The sperm concentration in sexed semen straw is approximately 2 million which is lower than ordinary non-sexed semen straw which contains approximately 20 million (Sharpe and Evans, 2009). As a result of both sperm damage during the sorting process and lower sperm numbers included in each straw, use of sexed semen generally results in poorer conception rates compared with conventional semen (DeJarnette *et al*., 2009; Norman *et al*., 2010 and Borchersen and Peacock, 2009). Also, flow cytometry is relatively slow process. It takes 1-2 hours to sort the number of sperm in a typical artificial insemination dose (Garner and Seidel, 2003 and DeJarnette *et al*., 2008). Sexed semen is now widely available in dairy industries around the world, and is nearing commercial application in several other species (Seidel Jr, 2012). Despite reliably producing 80-90% gender bias (Cerchiaro *et al*., 2007; Borchersen and Peacock, 2009 and DeJarnette *et al*., 2009), the fertility of the sexed semen product is compromised (Seidel Jr, 2012).

The price of dairy heifers is usually high and also depends on supply and demand. Using this method, more dairy heifer calves to be born than are needed to replace culled cows from economically priced, genetically competitive sexed semen. The supply of dairy replacement heifers will increase with the use of sexed semen to meet this strong demand. The price of the cow is directly associated with the price of heifer. The average value of a cow in the herd would decrease with decrease in heifer prices. Moreover the availability of sexed semen will enable farmers to keep fewer heifers and cows which are needed to produce the next generation of replacement heifer calves. In general, the average level of inbreeding for the major dairy breeds continues to increase which can be controlled with the use of sexed semen. Thus, the use of sexed semen will lead to sustainable production, greatly enhancing the ability to make progress in cross breeding, marker assisted selection and genomic selection (Moore and Thatcher, 2006). Keeping all facts into consideration, the objectives of this paper is to review the sexed semen methodology and applications in dairy cattle.

Methods of sperm sexing

There are 60 chromosomes in each somatic cell of domestic cattle. The gametes contain half that number of total chromosome. Half of the gametes have haploid sperm that produce females carry 29 autosomes plus the X-chromosome. Similarly rest half haploid Y-chromosome bearing sperm have the same 29 autosomes plus the male determining Y-chromosome. It was observed that sperms bearing X-chromosomes contain approximately 4.2% more DNA than sperm containing Y-chromosomes in domestic cattle (Moruzzi, 1979). Besides difference in sex chromosome in gametes, many other differences were discovered during last four decades of 20th century. Size of spermatozoa with X chromosome is larger than the spermatozoa contains Y chromosome (Cui and Matthews, 1993 and Cui, 1997). Comparatively higher motility reported in spermatozoa containing Y chromosome than those bearing X chromosome (Shettles, 1960). There are difference in surface charges in spermatozoa as X chromosome bearing spermatozoa has a negative charge while Y chromosome bearing spermatozoa has a positive charge (Kiddy and Hafs, 1971). Difference in cell surface antigens among X and Y bearing chromosomes was also reported by Hoppe and Koo (1984). On the basis of these potential differences, various methods were proposed for sorting of spermatozoa.

Gradient swim down procedure of sorting of spermatozoa: The basic principle of sorting through this method is due to difference in ability of X and Y bearing spermatozoa to swim down in a gradient solution. Y chromosome bearing spermatozoa have high motility and

exhibiting greater downward swimming velocity due to its small size than X chromosome bearing spermatozoa. Hence the isolation of fraction of semen from specific part of albumin gradient shows higher proportion of X or Y spermatozoa at different gradients but the success rate is reported to be around 75% (Beernink *et al*., 1993).

Sperm sorting by swim-up procedure: Due to small size, Y chromosome bearing spermatozoa swim faster than X chromosome bearing spermatozoa and this difference was utilized by many researchers for sperm sorting (Van Munster *et al*., 1999 and Ollero *et al*., 2000). The success rate was recorded up to 81% by this method (Check *et al*., 1989). Sperm sorting by identification of H-Y antigen: Using specific antibody against surface protein of Y chromosome bearing spermatozoa (against H-Y antigen) is also an option for sperm sorting through affinity chromatography or magnetic bead. This sperm sorting is applied at large scale with efficacy reported to be more than 90% by many scientists (Hoppe and Koo, 1984; Hendriksen *et al*., 1996; Hendriksen, 1999 and Blecher *et al*., 1999).

Sperm sorting by free flow electrophoresis : Difference in surface charges in spermatozoa (as X chromosome bearing spermatozoa has a negative charge while Y chromosome bearing spermatozoa has a positive charge) is utilized for sperm sorting by electric field separation (Mohri *et al*., 1986 and Kaneko *et al*., 1984).

Sperm sorting by percoll density gradient method : Sedimentation density of X chromosome bearing spermatozoa is higher and settles in the bottom of column while the Y chromosome bearing spermatozoa remain in high proportion at the top of column during sperm sorting. The success rate is reported to be from 86% to 94% (Lizuka *et al*., 1987; Van Kooij and Van Oost, 1992).

Sperm sorting by flow cytometry : Flow cytometry is a method used to sort sperm and adaptations of this technique opens new opportunities in sperm sorting. Flow cytometry-based sperm sorting uses fluorescent dyes that stain DNA (Caroppo, 2013 and Ribeiro *et al*., 2013). This technique is able to determine the sex of future progeny by measuring DNA content of individual sperm cells as if they contain the larger X chromosome (giving rise to a female offspring) or smaller Y chromosome (leading to male progeny). It then allows separation of X and Y sperm (Johnson *et al*., 1989). The so-called Beltsfield sperm sexing technology was developed by USDA in conjunction with Lawrence Livermore National Laboratories, relying on the DNA difference between the X and Y chromosomes (Garner and Seidel, 2008).

In this method, first, sperm are stained with a non-toxic, DNA-binding dye (Hoechst 33342) and pumped in a stream in front of UV laser beam having wavelength of 351 - 364 nm and the bright blue fluorescence

emitted is detected and analyzed (Johnson and Welch, 1999). This stream is broken by crystal vibrator into individual droplets for facilitating analysis of individual spermatozoa. Bright fluorescence emitted by illuminated spermatozoa which is measured rapidly by a photo-multiplier tube as the sperm flow past in single file (Garner and Seidel, 2008). To ensure adequate illumination, the sperm stream is oriented at the appropriate angle for accurate measurement of a 4% difference in fluorescence (Sharpe and Evans, 2009). The relative fluorescence of X and Y chromosome bearing sperm population is analyzed by high speed computer, which are then sorted by DNA content by introducing opposite charges on droplets containing X chromosome bearing sperm than Y chromosome bearing sperm (Seidel Jr, 2007). These droplets falls on previously charged deflector plates thus separated into two streams and then collected separately. The separation of streams of X and Y chromosome bearing droplets are done by using electrostatic deflection and collected separately for further processing (Seidel Jr and Garner, 2002). A third stream of uncharged droplets passes through as waste and is discarded (Seidel Jr, 2007). Many preliminary results have since been confirmed by a number of larger studies that have demonstrated CR with frozen-thawed sexed semen (2×10^6 sperm/ straw) that were ~70% to 80% of the conception rate achieved with frozen-thawed conventional semen (15 to 20×10^6 sperm/ straw) in both virgin heifers and lactating cows (DeJarnette *et al*., 2010; Norman *et al*., 2010; DeJarnette *et al*., 2011 and Healy *et al*., 2013). This method of sex specific spermatozoa sorting is the most popular and consistently proven to be effective among various methods.

Challenges and limitations of sorted sperm by flow cytometry

The major limitation of flow cytometry is the slow speed of the process relative to the number of viable sperm required for artificial insemination in cattle. As individual sperm is passing through a nozzle which is analyzed by the detector, there are physical limits in how many sperm can be evaluated accurately (Garner *et al*., 2012). Currently the speed of passage is around 80Km/hr during which about 30000 sperm can be evaluated per second under ideal conditions. Thus, it would take 1-2 hr to sort the number of sperm in a typical artificial insemination dose (Seidel Jr, 2014). Furthermore, the process damages sperm, although to a lesser degree than current procedures for cryopreservation. A fringe benefit of sexing sperm is that dead and dying sperm, those with compromised cell membranes, are discarded, but sorter capacity is spent in evaluating these sperm.

Other challenge is the loss of high proportion of sperm cells. Dead sperm cannot be oriented for sorting, or cannot be accurately identified as bearing an X or Y chromosome and pass through without being sorted and these two factors may lead to loss of more than 75% loss (Seidel Jr

and Garner, 2002). Rest among 25% of sorted sperm, only half is the desired gender, consequently, only 10%-15% of the original sperm population entering the flow cytometer are recovered as marketable sexed semen (Seidel Jr and Garner, 2002). Due to very low availability of spermatozoa after sorting, centrifugation is done to concentrate the spermatozoa in small volume which cause further damage of already stressed sperm and further reduce the fertilizing ability (Maxwell *et al*., 1999) and shortens the life of spermatozoa (Sá Filho *et al*., 2010). Various steps during and after cryopreservation of sorted semen like dilution, cooling, freezing and thawing further damage the plasma membrane (Underwood *et al*., 2010).

The ordinary non-sexed semen straw contains approximately 20 million sperms but sperm concentration in sexed semen straw is approximately 2 million only (Sharpe and Evans, 2009). Because of lower doses of sperm per straw, it has a negative effect on conception. The use of sexed semen is not recommended in herds with poor reproductive performance and inherent infertility. It is also not recommended for cows as conception rates can be reduced by up to 20% with sexed semen (Hohenboken, 1999). Sexed semen is recommended for breeding only virgin heifers that have been well managed, are healthy with good body condition and are reproductively fit with clear heat signs. It is not recommended for lactating cows due to lower concentration of sperm in the straw. Various studies shows conception rate of sex semen in heifers are higher than lactating cows. Seidel Jr (2003) reported 70%-80% conception rate in heifers and 50%-60% conception rate in lactating cows while De Vries *et al*. (2008) reported 45% conception rate in heifers and 28% in lactating cows. Due to the loss of sperm sexed semen is more expensive than conventional semen (Fetrow *et al*., 2007).

Sexed semen is not economic in all production systems particularly tropics where cow is reared under nutritional challenge throughout year. There are a number of potential applications and benefits of using sexed semen in seasonal, pasture based production systems. There are, however, two primary negative factors that must be accounted for when considering the use of sexed semen. These are the price premium of sexed semen compared with conventional and the reduction in fertility performance of sexed semen compared with conventional. A number of authors have examined the economic effects of sexed semen use in a variety of scenarios, both in year-round (Seidel Jr, 2003; Olynk and Wolf, 2007 and McCullock *et al*., 2013) and seasonal production systems (McMillan and Newman, 2011 and Hutchinson *et al*., 2013a and 2013b). McCullock *et al*. (2013) described the economic advantage of using sexed semen as a function of interactions among three spheres of influence: the market environment, management practices and technological influence.

Also, lack of availability of sexed semen for all potential mating sires of interest, both progeny proven and young sires. Generally, the high demand for shorted semen from the most elite progeny proven sires is expensive due to the significant reduction in doses produced per ejaculate for sexed semen. Other important limitation with flow cytometry is high cost and maintenance of its patented technology. Also it requires skilled manpower for operation and supervision of machine.

Advantage of using sexed semen in dairy cattle

The principal benefit of sexed semen is to produce a calf of a specific sex. Normally, averaged over thousands of animals, 49% of calves born will be heifers, and a few of these will be sterile freemartins (Seidel Jr, 1999). Supply of replacement heifers in dairy farms is a major issue in commercial ventures. Among successful pregnancies resulting in number of calves born, approximately 90% heifer calf can be produced by using sexed semen. This may be utilized to expand herd size and production. With these increased availability of replacement heifers which thereby reduces dairy heifer purchase and sale prices. As milk production is a sex limited trait and males are not usually maintained by farmers for breeding purpose use of sexed semen will increase with in-herd growth and production. Alternatively these heifers may be sold as calves, which would increase incomes compared with the sale of lower value dairy bull calves. This also enables rapid herd expansion without the risk of introducing diseases that occur with purchased animals (Seidel Jr, 1999).

By using sexed semen, selection intensity can be increased by choosing genetically superior dams of replacements which accelerate the rate of genetic gain in dairy herds (Weigel, 2004 and Khalajzadeh *et al*., 2012). It is possible to reduce the incidence of difficulty in first calvers (heifer calves are lighter than male calves) and additional replacement heifers for herd expansion may offer benefits in terms of improved biosecurity by increasing herd size while maintaining a closed herd (Weigel, 2004).

Conclusion and recommendations

One of the biggest developments since the advent of artificial insemination over five decades ago is the commercialization of sexed semen. There are equal numbers of X or Y chromosomes bearing sperm in semen, resulting in female or male offspring, respectively. Separation of bovine X or Y chromosome bearing sperm by flow cytometry to get calves of owner's choice has improved considerably and is over 90% accurate with sexed semen. This unique technology has been widely and rapidly implemented by dairy producers to increase the proportion of heifers born and to capitalize on associated benefits. The main application is for dairy heifers to have heifer calves, either for herd expansion or for sale as replacements, often for eventual

export. The use of sexed semen has impact on increasing the genetic merit of a dairy herd and its level of productivity is critical for the dairy industry. However, as with any evolving technology, there are pitfalls and prior awareness of the advantages as well as the disadvantages of using sexed semen. Hence it is recommended before adopting this new technology- "Sexed semen is primarily recommended for breeding only virgin heifers that have been well managed and are reproductively fit with good body condition as conception rates in cows can be reduced due low sperm concentration in straw".

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