Sperm sexing of dairy cattle: economics, animal welfare and technological challenges

Jagdish Rai*

Institute of Forensic Science and Criminology, Panjab University, Chandigarh 160 014, India

There is a growing need for sexed semen of cattle in India. Farmers prefer female calves of cows or buffalos than male calves as they can produce milk in the future which is economically more beneficial. Very few bulls are required for breeding in herds and they are no more required in agricultural fields due to mechanization of cultivation and transportation. Therefore these extra bulls/oxen are often sold at low prices to the meat industry. This is an animal welfare concern and can also cause communal tensions due to differences in belief systems of religious sects. At present there is no indigenous technology for producing sexed semen although artificial insemination with non-sexed semen is a well-established method of breeding among cattle farmers. The imported sexed semen is not easily available and also not affordable for most Indian farmers. There is a need for the development of cost effective indigenous sperm sexing techniques as an alternative to fluorescence-activated cell sorting. Recent developments in microfluidics and BEMS (bio electromechanical systems) may hold the key to the development of a portable device for semen sexing with minimal tampering of the sperm structure.

Keywords: Animal welfare, chromosomes, sexed semen of cattle.

Need for sexed semen of cattle in India

BOVINE semen has two types of sperms, X and Y. The fertilization of bovine egg by X sperms results in the birth of female calves whereas Y sperms result in male calves. Fertilization is a matter of chance and therefore there are 50% chances for the birth of female calves and 50% chances for male calves. Getting a cub of the desired gender can be economically valuable. Due to mechanization of agriculture and transport, oxen or bulls are no longer required and only female calves are retained by farmers for milk production. Beef is generally produced from male calves of buffalos or cows. Farmers sell them at low prices or sometimes leave them orphan. This creates an animal welfare issue and moral compunction among animal lovers. Many religious sects also prohibit slaughter of bulls which has resulted in communal tensions. Improvement in semen sexing technology can bring down the price and increase success rate of fertilization. This will not only economically benefit cattle farmers but also avoid risk to communal harmony. At times, male calves are also desired to rapidly implement a genetic improvement program using genetically superior bulls.

According to the 19th livestock census 2012, there are 199 million breedable cattle in India[1]. Compared to the 2007 census, the milch animals (cow and buffalo) have increased from 111 million to 118 million in 2012, an increase of 6.75%. In 2012, there were 92 million female buffalos and only 16 million male buffalos, indicating preference for female buffalos in livestock[2]. Artificial insemination (AI) is technique in which preserved semen is introduced artificially into the reproductive tract of the female for conception. The semen is collected from males, processed in the laboratory and preserved in liquid nitrogen for later use in AI based on convenience of time and place. In India, the first AI was done in Mysore in 1939, using preserved semen of Holstein Friesian bulls for fertilization of Halliker cows. In 1950s, the Government of India introduced key village schemes involving AI for improvement of animals. AI can be utilized for improving breeds faster in a large population of cattle because semen can be transported more easily than animals. Using AI, one ejaculate of a good breed male can be utilized for conception of many females. This technique has also decreased the risk of transfer of venereal diseases. AI is now a popular, simple and inexpensive technique used quite often for cattle breeding in India. At present, in India, there are more than 50 semen stations, producing approximately 70 million doses which are enough for breeding around 25% of breedable cattle. However, currently there is no agency in India, producing sexed semen on a large scale, although many states import sexed semen. Sexed semen from Indian breeds of cattle is more suitable as the cattle are more adapted to the Indian climate. However, presently, sexed semen is mainly imported from the USA and Canada which is not from indigenous breeds. Development of an indigenous technology will also make sexed semen more affordable.

In 2009, PaschimBanga Go-Sampad Bikash Sanstha (PBGSBS), a Government of West Bengal, India organisation, initiated sorting of semen using flow cytometer with a production capacity of 40–50 sexed semen straws

*e-mail: jagdishrai@pu.ac.in

per day. Using this sexed semen, the conception rates observed were 20.7% in cows and 35.3% in heifers. The National Dairy Research Institute (NDRI), Karnal has also been funded with Rs 55 crore budget for semen sexing of cattle with an aim to multiply indigenous and crossbred cows in the country by providing sexed semen to farmers[3]. The animal husbandry department plans to breed sixty lakh cows for improving the breed of cattles every year, through the sexed semen program. The department of animal husbandry plans to establish 10 facilities for sexed semen production, including one at its premier institute CFSPTI (Central Frozen Semen Production and Training Institute). Indigenous breed will be used as a semen source and each year these facilities will produce 2 million doses of sexed semen. At present 200–300 crore rupees are required for establishing a semen sexing facility and it takes around 5 years to start production. A sexed semen facility can cost about Rs 200–300 crore over a five-year period. For 2016–17, an amount of 500 crore rupees were allocated for promoting semen sexing technology[4]. India is already importing sexed semen[5]. ABS India Inc alone imports 1 million straws of sexed semen every year[6]. The Punjab Progressive Dairy Farmers Association is the largest consumer in India with a purchase of around 20,000 doses. The cost to the farmer is around Rs 1500 per straw of sexed semen[7], while a straw of semen without sexing costs only Rs 50. The commercially most successful technology of semen sexing is patented by a US based company, Sexing Technologies (ST Genetics, Navasota, TX). This technology is based on unequal DNA amounts in the X-chromosome bearing sperm versus Y-chromosome bearing sperm. The fluorescence intensity of DNA binding dye (Hoechst 33342) is higher in X sperm than Y sperm which can be shorted using a flow cytometer. The claimed pregnancy rate of Sexing Technologies' SexedULTRA semen straw is 52% whereas conventional semen has 60% pregnancy rate. SexedULTRA semen has 90% X chromosome bearing sperms whereas conventional semen straw has 50% X-chromosome bearing sperms[8]. SexedULTRA semen shorting method involves orienting nozzle technique which is monopolized through exclusive patenting, causing increase in the price of the product. Orienting nozzle (HiSON) flow cytometer can separate the X-chromosome bearing frozen sperms at a speed of 11 million/h (ref. 9). A single dose contains 4 million sperms; therefore this machine can produce around 66 doses in 24 hours run time, which is very costly considering the 4–5 crore rupees cost of the machine itself. Development of alternative techniques not involving flow cytometry could bring the cost down.

Though most farmers are unaware of sexed semen, the supply often falls short. A survey in 2012, involving 871 farmers in Ahmed Nagar and Pune districts of Maharashtra showed that 66% of farmers will use sexed semen if it is available for Rs 500 per insemination. India has a ready market for sexed semen and has the potential to expand if the cost is reduced and sperm viability is enhanced resulting in more successful fertilization[10].

As per 2011 data by Jim McGrann, the value of a female calf at weaning stage is US$ 950 whereas the value of a male calf at weaning stage is US$ 750. Given the 50–50% chance of male and female calf birth using conventional semen, the average value of a calf at weaning stage is US$ 825. However, sexed semen will increase the chances of a female calf up to 87% and therefore the average value of a weaning calf will be US$ 918. This increase in value from US$ 825 to US$ 918 is more than enough to cover the extra cost of sexed semen that is US$ 15 (ref. 11). However, this difference in value will be more in India because the demand for beef in India is less and beef has to be exported. At present there is zero value for a bull calf and these calves are abandoned after they are no longer required for milking the cow. Rather, these stray bulls are a liability to farmers as they graze on crops[12]. However, demand for milk in India is more and very soon India may need to import milk. India was the largest beef exporter in 2016, with 20% in the world beef industry, as per the United States Department of Agriculture (USDA) review. Beef in India comes primarily from water buffalo meat[13]. This data excludes illegally smuggled of cattles across India–Bangladesh, which is estimated to be around 15 lakh cows every year[14]. This trend of preference of female cubs may go further because of increasing popularity of vegetarianism and higher carbon footprint of meat consumption which causes more global warming than vegetarian diet[15].

Techniques for semen sexing

The karyotype of buffalo has 25 pairs of chromosomes including the sex chromosome pair. The difference in size of the X and Y-chromosomes is the basis for most sperm sexing techniques. In buffalo, the size of the X-chromosome is approximately four-times larger than the Y-chromosome, resulting in total DNA content of the X sperm being more than that of the Y sperm. In *Bos indicus*, the average X-Y sperm DNA content difference is 3.73%. Similarly, Nili–Ravi and Murrah buffalo's X and Y sperms differ by 3.55% and 3.59% in terms of total DNA content in sperms[16]. This size difference in X and Y chromosomes can also result in difference in sperm shape and density. The size difference can be utilized to separate the two types of sperms. The sperms can be rendered immotile by cooling to 1°C. On the egg yolk medium, the X sperms sediment faster than Y sperms under the influence of gravity due to difference in density[17].

Antigen H-Y is a male specific protein on the cell surface, coded by Y chromosome. This protein has a role in gender determination during embryo development. However, there are also claims that X and Y sperms can be

separated using this antigen for immunolabelling[18]. It is claimed that monoclonal antibodies against antigen H-Y, binds preferentially to Y-chromosome bearing sperm which can then be separated using FACS. There have been various studies to find the difference between X and Y sperms in terms of gene expression during spermatogenesis, which can be used for an immunological method of separation of X and Y spermatozoa. But most of these gene products are shared between X and Y spermatids through inter-cellular bridges built during spermatogenesis. The X and Y sperms separated by FACS technique show no significant difference in terms of proteins on the surface, including antigen H-Y[19]. There have been several studies to determine X- and Y-sperm specific biomolecules on the surface of sperms. However low enrichment, low viability and high cost have been prohibitive in commercialization of immunological sperm sorting methods[20]. There have also been not so successful attempts to separate X and Y sperms through free flow electrophoresis, assuming that there is a difference in surface charge of X and Y sperms due to a difference in fatty acid composition of the membrane[21]. Rather, such differences between X and Y sperms are not well established.

Percoll density gradient centrifugation was also tried, but the success in enriching X chromosome bearing sperm was insignificant[22]. Swim up method of semen sexing is based on the assumption that the Y sperm is relatively smaller and therefore swims faster. However, subsequent studies have found it to be false[23]. Other methods utilizing DNA staining dyes are prone to mutagenicity and reduce the viability of sperms[24]. There have also been attempts to use thin layer counter current distribution (TLCCD) chromatography to sort sex sperms[25]. Even transgenics has been used to produce specific X or Y sperms[26]. In this method, the promoter of testis specific promoter gene, protamine 1 on Y chromosome is used to express an anti sense mRNA which is toxic to gamete. This anti sense mRNA can stop the expression of genes such as fertilin B, sperm adhesion molecule (spam-1), glyceraldehyde phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase which are crucial for a functional sperm. Therefore, sperms of undesirable types can be selectively made non-functional.

The separation efficiency of various techniques is checked by PCR, using Y-chromosome specific DNA sequence. The separated sperm solutions can be tested for their percentage of Y sperms using real time PCR. Y-chromosome-linked SRY gene specific primers and X-chromosome-linked PLP gene specific primers were designed to amplify markers for X and Y sperm content estimation in sexed semen[27]. Evolutionarily conserved sequences on Y chromosome have been utilized for designing Y specific primers R-IV and U-IV. These primers have been successfully used to detect the Y chromosome even in embryonic tissue of Zebu and Taurine[28]. A highly repetitive sequence S4, localized on the Y chromosome can be amplified using PCR primer set which gives a 178 bp male-specific product and a 145 bp product which appear both in X and Y sperms[29].

Challenges and upcoming developments in sperm sexing

The differences between X and Y sperms in terms of weight, size or density are very minute. Therefore a high precision technique is required to separate them on the basis of physical characteristics. Since several years, undoubtedly, various methods have been used to enrich the semen with X sperms, but all these methods are expensive and inefficient. Moreover, the X sperm enrichment procedure also makes it less viable for fertilization. The famous Ericsson's albumin layer based separation method for humans has been found ineffective[30]. The present technique of X sorting from bull semen involves the staining of DNA by Hoechst dye and further separation using FACS[31]. Hoechst 33342 is a DNA specific, non-intercalating dye. Therefore, it does not induce DNA damage and binds only in the minor groove of the A-T regions of the DNA[32]. The exposure of sperms to laser light and exposure of droplets to electric charge in FACS, reduces the motility of sperms as well as damages the acrosome and membrane[33]. Exposure to the dye in combination with the laser may reduce mitochondrial activity in bovine sperms. This results in reduced motility of sperms, because mitochondria produce ATP which is an energy source for sperm motility[34]. The shorted sperm in sheath fluid is then concentrated by centrifugation. Centrifugation also damages spermatozoa through lipid peroxidation. Due to high dilution in sheath fluid, the natural antioxidants present in seminal fluid are lost. The storage of shorted sperms in liquid nitrogen further increases peroxidation of membrane lipids. Various steps in FACS lead to reactive oxygen species (ROS) production causing damage to the membrane and mitochondria which can be reduced by a supplementing medium with antioxidants like sodium pyruvate and catalase[35]. Centrifugation and microfluidics may hold the key to the development of better techniques of sperm sorting with minimal tampering of cell structure. The miniature separation column has been used for continuous cell separation through density gradient centrifugation[36], which may be adapted for sperm sorting based on the density difference between the X and Y sperms. The seminal fluid itself can be a better medium for centrifugation, and optimization of centrifugation speeds, time and volumes can result in a more viable sexed semen. Microfluidic channels can be used as cell sorters and separation of X and Y sperms on the basis of the negligible difference they have is a fitting challenge for this emerging high precision technology[37]. Microfluidics can be explored for separation of sperms on the basis of their charge, density or shape. The

UV-absorbance spectroscopy can be coupled with microfluidic channels to sort the X sperms from Y sperms[38]. The Y sperm has less DNA content than the X sperm. This difference can be picked up by UV absorbance of DNA at 260 nm. The sperms move in a spiral path in a stagnant fluid. However in a flow stream, the angular velocity of the sperm is reduced and the path of the sperm movement becomes almost a straight line. This effect on angular velocity is more in the case of X sperm, probably due to more weight. Therefore, in a flow stream the X sperms moves comparatively straight whereas Y sperms move in a spiral path. This difference can be utilized for shorting X and Y sperms through microfluidics. The gradient of velocity across the axis of flow has been used successfully to short the X and Y sperms[39]. The difference in swimming behaviour of sperms is expected to be more pronounced in case of cattle sperms.

Advanced microfluidics may utilize this behaviour for X and Y sperm sorting in a setup similar to the one used for separating motile and nonmotile sperms (Figure 1)[35,41]. North Cyprus IVF Center has developed a microfluidic device to separate live human sperms from dead ones. Microfluidic channel may be designed to separate sperms on the basis that X sperms move in straight line in a flow whereas Y sperms move in an angular path. Microfluidic channels can also be coupled to various types of spectrophotometer[42], which can be used to detect Y sperms on the basis of DNA content, and then use high power laser for inactivation of individual Y sperms, leaving only X sperms alive. The dead sperms can be removed using a micro scale sperm sorter developed by Chung *et al.*[43] which is a point of care device driven by passive reservoir pumps. Size selective separation techniques used for nanoparticles in liquids may also be useful for picking up minute size differences between X and Y sperms[44]. Flow field-flow fractionation (FlFFF) has been used to separate carbon ink particles based on their size difference. Such high precision and delicate techniques also hold promise for the challenging task of semen sexing[45]. Nano size particles in natural samples have also been separated using symmetric as well as asymmetric FlFFF systems[46]). These techniques have a size resolution of ±10 nm and are very gentle, and therefore adapted for semen sexing. Microfluidic systems are emerging as a cost effective and portable alternative to FACS for cell sorting. Microfluidic devices have the potential to develop into on-site devices which will also avoid freeze-thaw of semen and preserve the viability of sperms.

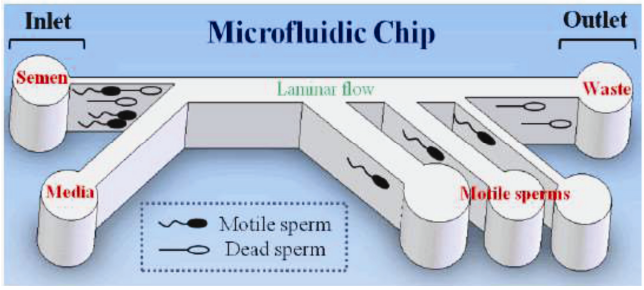

Figure 1. Multiple channel microfluidic chip. The dead sperms move straight with fluid whereas live sperms enter side channels due to their random motility. (Creative Commons: ref. 40).

Conclusion

Sexed semen for X chromosome bearing sperm of cattle can be beneficial for cattle farmers in India as female calves have high value due to milk production. However, male calves are loosing relevance as draught animals due to mechanization of agriculture. There have been many attempts to separate the X and Y sperms on the basis of surface protein marker, density, motility, etc. These techniques have been patented but are not effective because X and Y sperms do not differ significantly in these aspects. The presently used technique FACS is based on difference in DNA amount in X and Y sperms due to difference in the sizes of X and Y chromosomes. This patented technology uses a DNA binding dye which can cause DNA damage, and the instrument is also very expensive. Microfluidic devices are also being developed as cell shorter, which can result in cost effective, non invasive point of care devices for semen sexing. An indigenous technique for semen sexing can go a long way in decreasing cost, preserving genetic diversity of cattle and animal welfare.

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