Indian Journal of Animal Sciences **92** (7): 797–805, July 2022/Review Article
https://doi.org/10.56093/ijans.v92i7.105407

Use of sexed semen for genetic improvement of indigenous dairy cattle and buffaloes productivity

VINOD KUMAR YATA[1], SUMIT KUMAR SINGH[1], SUDARSHAN KUMAR[1], TUSHAR KUMAR MOHANTY[2] and ASHOK KUMAR MOHANTY[1]✉

ICAR-National Dairy Research Institute, Karnal, Haryana 132 001 India

Received: 30 September 2020; Accepted: 13 May 2022

ABSTRACT

The productivity of dairy animals in India needs to be improved to feed the growing population in the decades ahead. Sperm sexing offers a promising breeding strategy to meet the increased demand for food production and nutritional protection. Application of sexed sperm in the breeding of indigenous cattle and buffalo would help to protect endangered species, and it also helps to improve the productivity of indigenous milch animals which can survive long starvation and are well adopted to Indian climatic conditions along with disease resistance. The productivity of crossbreed milch animals is also required to meet the increasing food demand associated with population growth. This review discusses the sustainability of Indian dairy animals and provides sperm sexing strategies to boost dairy animal production in India.

Keywords: Animal breeding, Buffalo, Cattle, Dairy animals , Spermatozoa, Sperm sexing

Indian population crossed over 130 million in 2019 and is rising day by day (DESA 2019). To meet the food demand associated with this population growth, sperm or embryo sexing would entail the production of pre-sexed livestock, which offer a promising breeding strategy to meet the increased demand for food production and nutritional protection (Bhalakiya *et al.* 2018). So far, the application of sexed bovine sperm using artificial insemination (AI) is successful in altering the sex ratio and rapidly increasing dairy herds carrying animals of high genetic value. Therefore, the practical application of sexed sperm in breeding indigenous cattle and buffalo would be of great importance in both biological and economic terms. In addition to the long-term advantage, by generating optimum proportions of males and females in their animal production systems, farmers can directly profit from the use of sexed sperm. Using sexed spermatozoa increases the rate of genetic development, particularly in combination with sire genomic selection and easier culling decisions. This method can also enhance herd management by avoiding male calves and reducing the occurrence of dystocia. But the superior male can be produced by the superior dam's sex-sorted spermatozoa, which will be an excellent boost for the semen station that requires to increase the productivity of frozen semen in the country. Over the last few years, sexed semen has been processed and sold at a very high price in foreign countries. Countries such as the United States, New Zealand, Denmark and Australia have been using commercial sexed semen for several years. Off late, sexed semen straws containing only female sperms are available in India in recent years, to leverage its benefits; the department of animal husbandry has also capped this technology, the Government of India, which makes sexed semen straws available for insemination purposes at the field level (Khaja 2017). Farmers in some Indian states may make use of this technique at affordable range and subsidized rates. Sexed semen for X chromosome bearing cattle sperm may be beneficial to cattle farmers in India, as the high value of female calves is for milk production. Another benefit of using sexed semen is generating superior genetically suitable females for a substitution that will significantly affect a dairy enterprise's profitability. Nevertheless, due to agricultural mechanization, male calves are losing importance as draught animals.

Present address: [1]Animal Biotechnology Centre, National Dairy Research Institute, Karnal, Haryana. [2]Artificial Breeding Research Centre, National Dairy Research Institute, Karnal, Haryana. ✉Corresponding author email: ashokmohanty1@gmail.com

Sex-sorted semen allows farmers to skew the sex ratio of offspring of their animals substantially (Dasari 2013). Hence, herd reproductive management is no longer limited by balancing the need for a sufficient supply of replacement heifers against the need to achieve pregnancies to start new lactations (Holden and Butler 2018). A multi-dimensional approach is required to enhance the productivity of indigenous cattle to make them highly sustainable by enhancing their genetic capacity; selecting animals for overall adaptive value; reducing the number of non-described unproductive/low-production animals; improving infrastructure such as AI and veterinary services under field condition to lower down the calving interval; developing needs for farmers, especially small and marginal farmers, based and economically viable technologies; increasing farmers or rural women's knowledge of improved practices to enhance the production of milk and reproductive output by field/extension staff and; to educate and empower rural women (De *et al.* 2008). The sexed semen can also be used for the production of desired *in vitro* sex embryos at low doses. So it could also be an alternative to skewing the sex ratio of offspring.

Despite the advantages of sexed semen, growth of the sexed sperm market appears to have been slow. The problem with sperm sexing is the high cost of equipment and maintenance, lack of skilled human resources, about half of the sperm sample being unsexable, low sorting efficiency, low pregnancy rates, and slow pace of the process.

Indian dairy breeds

There are 145 million breedable cattle in India, and total milch animals (in milk and dry) in cows and buffalos rose from 118 million to 125 million in 2019 compared to the 2012 census, an increase of 6% (20th Livestock Census 2019a). In 2019, female cattle population increased by 18%, whereas male cattle decreased by 30.2%, suggesting a preference in livestock for female animals (DAHD 2018–19). The government's encouragement of artificial insemination in cattle encouraged this trend. In buffaloes also, the divergence between males and females is noticeable. Male buffalo population declined from 2012 to 2019 by 42.3%. India's size, complex geography and environment sustain a similar complex variety of cattle and water buffalo breeds that make up the world's largest dairy herd. While the average milk yield of India's dairy herd is low compared to more advanced dairy economies, many native breeds are capable of improved yields and also have beneficial characteristics in the form of disease resistance and the ability to successfully transform usable, relatively low-quality forages native to the area. Inventory data from the Indian Livestock Census divides the bovine herd into three categories: exotic/crossbred cattle, indigenous cattle and water buffalo. The river buffalo (*Bubalus bubalis*) common to India, is well adapted to heat and humidity and, unlike other ruminants, thrives on a diet of green and dry fodders. Most of the water buffalo in India is classified as non-descript or mixed, but there are several well-defined breeds, including, Bhadawari, which is also known as "Etawah,". Bhadawari is a dual purpose buffalo breed in central and northern India famous for their efficient ability to use low-quality raw food available in the region. While the average lactation yield is lower, the fat content of milk is as high as 14% (Ashish *et al.* 2017). The average milk yield of the breed is 1,294 kg per lactation with an average fat % of 7.88 kg (range from 540-1,400 kg per lactation and fat % of 6 to 12.8 kg per lactation). The other well-known water buffalo breed is Murrah and also known as "Delhi", "Kundi" and "Kali." The breeding area of "Murrah" covers the Hisar, Rohtak, Gurgaon and Jind districts of Haryana and Delhi. In addition, the breed is used for breeding non-descriptive buffaloes in many parts of India and has also played an important role in the livestock industry in many developing countries such as Bulgaria, the Philippines, Malaysia, Vietnam, Brazil and Sri Lanka (Kumar and Chitra 2019). The buffaloes are reared in a mixed form of housing with shelter during harsh weather conditions. Animals are often stall-fed with available seasonal green feed. Murrah buffalo is India's most efficient producers of milk and butterfat. Average lactation yield ranges between 1,500 and 2,500 kg per lactation and Butter is 7.83% fat weight. The Pandharpuri buffaloes, the native animal of Maharashtra are known to have been reared for more than 150 years in the breeding tract. The breed is famous for its better reproductive ability, producing a calf every 12-13 months. Under average management conditions and hot-dry climate, these buffaloes yield 6-7 litres of milk per day. Animals can let down milk multiple times (Yadav and Singh 2016). The farmer shall take the animals to the customer's door and the milk as needed. Then the animals are taken to the next customer and milked again. Buffaloes produce an average of 1,790 kg of milk per lactation, with 8% fat. Mehsana buffaloes are known to have developed through the crossing of Murrah and Surti buffalo breeds. The breed is named on the place of its birth, the Mehsana district of Gujarat and is also known as Mahesani or Mehsani. The breed has strong lactation endurance along with breeding regularity. The milk production of the breed ranges from 598 to 3,597 kg per lactation with 5.2 to 9.5% weight. The average milk yield is 1,988 kg, and the fat is 6.83%.

India also has several water buffalo breeds that combine the characteristics of high milk yield compared to most indigenous cattle (but not crossbred cattle), a high degree of climate and disease tolerance in many regions of India, and the ability to efficiently transform many of the available low-quality indigenous grasses and feeds. Buffalo milk is also commonly preferred by Indian consumers and processors due to its unique characteristics, including higher levels of skim milk solids (protein, lactose, and ash) than cattle or goat milk, and especially its high content of butterfat — typically 6-8% compared to 3-4% for cattle milk. The high content of buffalo milk solids increased its value while processing of finished goods. However, in addition to these attributes, water buffalo tends to have a higher age at first calving and longer dry periods and calving intervals than indigenous or crossbred cattle, all of which may result in lower overall productivity and higher costs; compared to dairy cattle (Presicce 2022). India has several indigenous cattle breeds, some of which are competitive milking breeds and others raised for draught (animal traction). Most indigenous herds are mixed breed animals with relatively low potential for milk yield (Patil *et al.* 1998). While the majority of the total bovine herd still comprises indigenous cattle, their share of the herd is decreasing, consistent with their relatively low milk yields and, with the advent of farm mechanization, the diminishing use of the draught animals (Joshi *et al.* 2005).

As in the case of buffalo, the majority of indigenous cattle are of mixed, non-described breeds, but the most productive dairy breeds include Sahiwal, which is considered to be one of India's best milk cattle breeds. The breed derives its name from the Sahiwal region of the Punjab district of Montgomery, Pakistan. The average lactation yield of Sahiwal cows is 2,325 kg and lactation yield ranges from 1,600 kg to 2,750 kg. However, the selected herds may have higher productivity. Milk yields of up to 6,000 L have been reported under coordinated farm conditions. Considering the merits of this breed, Sahiwal animals were imported by Australia and used for the production of a hybrid crossbreed called Australian Milking Zebu (AMZ) cattle.

Red Sindhi, also known as Malir, Red Karachi and Sindi is a well-known heat-tolerant milk cattle breed originating in the province of Sindh, Pakistan. The breed has a very high genetic potential for milk production and is equivalent to Sahiwal. In India, animals of this breed are not eligible in field conditions. The breed has been used for breeding production in many countries, including the USA, Australia, the Philippines, Brazil and Sri Lanka. The milk yield of cattle ranges from 1,100 kg to 2,600 kg per lactation, with an average yield of 1,840 kg per lactation. The fat content ranges from 4 to 5.2% in milk, with an average of 4.5%. Tharparkar is a dual-purpose and disease-resistant cattle breed also known as Black Sindhi, Grey Sindhi and Thari according to the place of its actual origin (Sind, Pakistan). The breed is medium-sized lightweight with a white and light grey coat. Animals can grow well on small bushy vegetation (Sewan grass) during drought and forage shortage and produce a fair amount of milk with an average yield of 1,749 kg/ per lactation. Healthy animals with good nutrition have provided even more than 3000 L/lactation in farm conditions. Males are also ideal for the function of draught. Due to improved heat tolerance and disease resistance, this breed was used for the production of the 'Karan Fries' breed at the National Dairy Research Institute (Landes *et al.* 2017). Gir is one of the best among indigenous cattle (also known as Bhodali, Desan, Gujarati, Kathiawari, Sorthi and Surati). This is a world-famous breed renowned for its resistance to stress conditions. Having faced scarcity for several years, it can produce more milk with less food and is immune to various tropical diseases. Due to unique qualities, the animals of this breed have been imported from countries such as Brazil, the USA, Venezuela and Mexico and are being bred successfully. The average milk production recorded is 2,110 L/lactation.

Crossbreeding of imported exotic breeds such as Holstein-Friesian, Jersey, and Brown Swiss with indigenous breeds started in the late 1960s. The crossbred herd rose to 51.36 million by 2019 (29%). Crossbreed milk production increased by 8.7%, and indigenous cattle increased by 5.7% compared to last year (Thiagarajan 2014). The milking herd includes dry and in-milk female water buffalo as well as indigenous cattle which have calved and are at least three years old, according to Indian data. The crossbred milking herd includes females who are at least two and a half years old and have calved. Generally, the crossbred cows have double or triple the yields of the native breeds, depending on the species crossed. Exotic heritage of about 50% is considered suitable for growth, and the best exotic breeds tend to be Holstein, Brown Swiss, Red Dane, and Jersey. The most rising crossbred combinations include Karan Swiss, which is a good milk-producing animal and especially suited for eastern, central, and the coastal regions of the country. The average age at first calving is 34 months, and 305-day lactation yield is about 3,400-3,600 kg. The other high milk-producing crossbreed is Karan Fries and especially suited for northern and northwestern regions of the country. The Karan Fries was developed by crossing Holstein Friesian with Tharparkar. The average age at first calving is 32-34 months, and 305-day lactation yield is about 4,000 kg. Frieswal cattle is one of the crossbred strains with 5/8 Holstein Friesian and 3/8 Sahiwal inheritance, established in collaboration with the Ministry of Defence by ICAR-Central Institute for Research on Cattle, Meerut. The project started in 1987 to establish a new crossbred national milch breed: Frieswal – (Holstein×Sahiwal), producing 4,000 kg of milk with 4% butterfat in a mature 300-day lactation. The dam's best lactation yield ranges from 4,000 to 6,000 kg, the highest value being 8,073 kg in lactation of 387 days. The growth trend in the milking herd conforms to the competitive qualities of the three animal groups. The crossbred or exotic herd consists of animals bred through state and central government initiatives initiated in the late 1960s and early 1970s by crossing indigenous breeds with a foreign exotic breed, mainly by artificial insemination. Though pure exotic breeds are not well adapted to the conditions of India's environment and diseases, crossbred cattle bring the higher milk yielding characteristics of exotic breeds with the indigenous breeds' environment tolerance and disease resistance traits (20th Livestock Census 2019b). The relatively fast growth in the crossbred herd is consistent with their relatively high milk yields, while crossbred animals are more involved in management than native breeds. Several studies have been conducted to evaluate crossbred results. Studies have shown that age in crossbreds at first calving (26-42 months) is much lower than that of some of the well-known native breeds (36-52 months). Average lactation yields ranged between 1,139 and 4,039 kg in crossbreds vs 1,438-2,326 kg in Indian cattle breeds which have strong milk production potentials such as Gir, Sahiwal, Rathi, Red Sindhi, Tharparkar, and Kankrej. Cows producing high-quality milk are registered under the Central Head Registration Scheme for breeds such as Gir (3,038-3,263 kg), Ongole (2,000-2,544 kg.) and Hariana (1,671-4,015 kg.). This data suggests that there is genetic potential for high milk production in indigenous breeds (Srivastava *et al.* 2019). The differences in milk production of crossbreds and indigenous milk breeds do not appear to be significant, provided that not much competition has resulted in improving indigenous breeds for milk yield. Crossbred efficiency also deteriorates from F1 to F2 and further generations. Even with relatively large population size, the indigenous cattle breeds are not only rapidly declining but are also subject to genetic erosion. This decrease in the population of indigenous cattle breeds and their neglect has been due to quickly shifting socio-economic rates of farmers, an ecological profile and an agricultural scenario (intensive and more mechanized agriculture replacing draft animal power), shrinking pasture areas, undue focus on crossbreeding with exotic cattle to increase milk production, and buffalo production (Ahlawat *et al.* 2019). Based on available data as well as the latest census, it can be concluded that the population of well-defined cattle breeds needs urgent action for the systematic conservation, genetic improvement and sustainable use of indigenous cattle.

Sperm sexing methods

Sexing semen is the method of splitting spermatozoa into two subpopulations of spermatozoa-bearing X-chromosome and Y-chromosome with higher than average concentrations (up to 90%) of either the 'X' or 'Y' sperm to produce offspring of the desired gender (Joshi and Singh 2005). Such alteration requires predetermining of offspring's sex before conception. It is a highly desirable technical resource for aided breeding programs primarily for the processing of milk and meat in females and males, respectively (Yadav *et al.* 2017). Both liquid and frozen sexed semen are used for superior germplasm and elite livestock development, enhancing the genetic advancement and protection of endangered species in wildlife using AI, embryo transfer technology and in vitro fertilization (Espinosa *et al.* 2012). Different methods used for sexing semen in various animals are given in Fig.1.

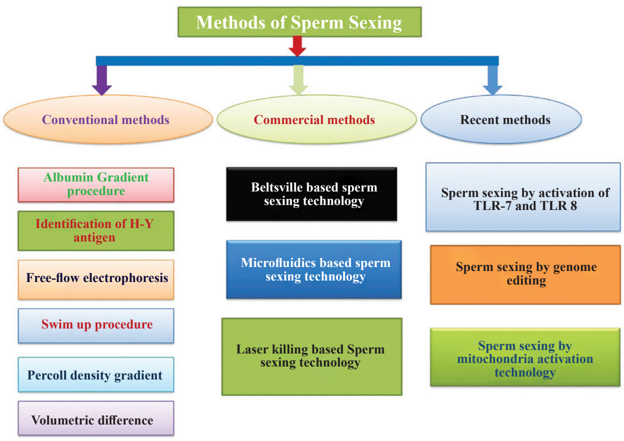

Fig.1. Methods for sperm sexing.

Conventional methods: Plenty of research has been devoted to the separation of X and Y sperm by assumptive differences. The physical characteristics of sperm are a possible means of distinguishing X from Y sperm. Albumin gradient (or) Gradient swim down Procedure is based on the differences between X and Y spermatozoa with swimming down ability in a gradient solution. because Y spermatozoa are smaller in size and have high motility, exhibiting a greater swimming speed downwards than the spermatozoa-bearing chromosome X. Therefore, all X / Y enriched fractions are assumed to be the fractions of semen derived from different albumin gradient parts. This method's success rate was reported to be around 75% (Sá Filho *et al.* 2010). Identification of surface proteins expressed in X or Y sperm chooses to use immunological methods to classify and distinguish X and Y bearing sperm. This sorting method can be used in large-scale sorting of sperm. Specific antibodies to H – Y antigen (expressed in Y-bearing spermatozoa) were used to sort sperm by affinity chromatography or magnetic bead with the efficacy of > 90% (Johnson 2000). Free-flow electrophoresis approach is based on electrical charges present on the surfaces of spermatozoa. X spermatozoa surface is negatively charged, whereas Y spermatozoa are positive (Johnson *et al.* 1989). Using the differences in surface charges, X and Y spermatozoa were separated based on the electric field of separation. Size mediated difference of Y spermatozoa was utilized by several researchers due to its smaller size, than X-bearing spermatozoa for sperm sorting by different methods. It was reported that the success rate in this method was 81%. Upreti *et al.* were unable to isolate bovine X and Y sperm after centrifugation when using a continuous Percoll gradient method (Upreti *et al.* 1999). Hamano and Uchiyama confirmed by chromosome analysis of sperm penetrated zona-free hamster oocytes that there is no difference in the X and the Y sperm ratio of the bull semen collected from bottom fraction separated by 8th fractions of Percoll density gradient centrifugation (Hamano 2007). Volumetrics difference approach uses sperm image analysis using interference microscopy to show a difference in sperm head volume based on the DNA content between sperm bearing X and Y chromosomes. A method based on this theory was developed using interference microscopy optics with a flow cytometer for sorting live spermatozoa. This method's success rate was reported to be < 80%. PCR uses the Y-chromosome-specific DNA sequence to test the separation efficiency of various techniques. The different sperm solutions can be tested using real-time PCR for their % of Y sperms. Y-chromosome-linked SRY gene-specific primers and X-chromosome-linked PLP gene-specific primers have been developed to amplify markers for estimating the X and Y sperm content in sexed semen (Parati *et al.* 2006). The differences in weight, size, or density between the X and Y sperms are very minute. Therefore, it requires a high precision technique to distinguish them based on physical characteristics.

As described above, there has been little reliable evidence to support the claims made for various physical, morphological, or non-DNA based separation procedures. The majority of attempts using these methods have failed. Although current sperm sex-sorting methods by flow-cytometry are well established and functional, the development of sex-specific probes would improve the efficiency of existing methods or facilitate the development of new sperm sorting strategies (Gledhill 1988). Previous efforts to resolve differences in X and Y-bearing sperm have ultimately failed to identify any sex-specific molecular marker or antigen in sperm.

Commercial technologies for semen sexing

Beltsville sperm sexing technology: Flow cytometers are the advanced cell sorters in which LASER is used to excite Fluorescent dye, which binds to spermatozoa DNA. The primary principle for sperm sexing through flow cytometry is DNA per cent and DNA specific dyes. In this sorting method, the spermatozoa are treated with dye (e.g. Hoechst 33342) that is permeable to living and intact sperm membranes and binds to the DNA. Stained spermatozoa are transported to a point where they are individually exposed to a UV laser beam (wavelength 351–364 nm) and the bright blue fluorescence emitted is detected and analyzed (Prakash *et al.* 2014) . It requires more stain in X than Y sperm, due to more DNA content in X chromosome bearing spermatozoa.

Based on this fluorescence, sperm are marked and categorized as X or Y chromosome bearing. The X-bearing sperm shines brighter than the Y-bearing sperm when exposed to light due to the difference in the amount of DNA (Rahman and Pang 2019). It allows the laser and detector of the cytometer to determine the sperm cell's gender, based on the amount of light it emits. On the droplet containing the single sperm cell, a positive or negative charge is then applied. One route is deflected positively charged drops, the other is deflected negatively charged drops, and uncharged droplets go straight through.

Sperm sorting through flow cytometer depends on the head of the sperm's orientation to the Laser beam. Because of its flat or oval-shaped head, which perfectly orients to the laser beam, bull spermatozoa is more efficiently sorted by flow cytometry among livestock species. The flow cytometer analyzes approximately 20,000 sperm per second with a sorting speed of up to 6,000 sperm per second, achieving purity at or above 90%. Under these conditions, in most farm animal species, approximately 15-20 million sorted X and Y spermatozoa could be produced per hour.

Microfluidics based sperm sexing: Engender Technologies, a joint venture between seed investment company Pacific Channel and the University of Auckland, has developed a new technique for the animal breeding sector to sort sperm by sex using microfluidic and photonic chips. The sperm sex sorting technology allows dairy farmers to select the sex of their calves and thus reduce waste and increase cost efficiencies in large animal reproduction. Engender takes semen samples and puts them in a microfluidic chip so that the all cells go straight, one after the other. They then use a laser to determine if the cells carry an X chromosome or a Y chromosome, whether the sperm will lead to a female or a male. They then use a second laser to nudge the cells within those channels so that they can sort them into different outflows. Engender is currently focussed on improving its microfluidic research chip into a commercial device suitable for mass production. In parallel, the company plans to develop and construct a prototype laboratory instrument that operates the microfluidic chip to perform IVF5 research and the first field AI research. Engender also seeks to monitor and refine technical innovations to ensure the product continues to out-perform the competition and intends to further develop the business by seeking further potential licensees. In November 2018, the global animal genetics company CRV bought Engender Technologies.

Laser killing based sperm sexing: LumiSort™ is the next generation semen sexing technology developed by Microbix for the livestock industry. Quality sexed semen is essential to herd management efficiency and genetic enhancement and will be a major contributor to productivity and global food security in the coming years. The technology will enable the widespread use of semen sexing by significantly improving the yield and quality of sexed semen, while greatly increasing sperm cell sorting speeds. The LumiSort™ instrument is a modern, flow cytometry-based technology (single-cell analysis and sorting). It uses a safe and widely accepted method to determine sperm cell sex by staining a semen sample's DNA with an innocuous dye, which binds to DNA and emits fluorescent light when a laser excites. Because female cells contain more DNA than male cells, the fluorescence emitted is slightly greater for female cells than male cells (only about 3.7%); this difference is used to determine the cell's sex. If the sex is determined, LumiSort uses laser killing to remove the unwanted sex cells, leaving the desired cells unchanged (Luscher 2013).

The LumiSort sperm sexing method combines a pioneering optical system for sperm cell sex detection with a fast and effective laser-based method of killing that eliminates sperm cells that are not of the desired sex. In this way, animal reproductive specialists can select sperm cells which will give rise to offspring of the desired sex, allowing reproduction with maximum efficiency for both herd replacement and genetic selection**.**

Recent advances in sperm sexing

Different techniques have indeed been used to supplement the semen with X sperms for many years, but all these techniques are costly and ineffective.

Sperm sexing by activation of TLR-7 and TLR 8: Previous studies suggested that sperm expresses specific toll-like receptors that can react to specific ligands. By regulating the expression of these genes, the X chromosome might have functional variations in sperm that are normally masked during fertilization. The overall gene expression is lower in sperm than in somatic cells, and the transcription is selectively activated in round spermatids. TLR7 is located at the tail, and TLR8 is situated in the midpiece of mice sperm. The ligand activation of TLR7/8 selectively inhibited the mobility of X chromosome-bearing sperm

(X-sperm) but not of Y-sperm without altering sperm viability or acrosome formation. The difference in sperm motility allowed the separation of Y-sperm from X-sperm. After *in vitro* fertilization using ligand-selected high-mobility sperm, 90% of the embryos were XY males. In the same way, 83% of the pups collected after embryo transfer were XY males. Conversely, the TLR7/8-activated, slow-moving sperm developed 81% female embryos and pups. The functional variations between Y-sperm and X-sperm motility were therefore established and attributed to different patterns of gene expression, specifically TLR7/8 on X-sperm. Based on this method, we can establish a new, quick and easy techniques for the determination of Y-sperm versus X-sperm that, without complex and complicated sorting systems, could be used to isolate Y-sperm or X-sperm in bovine selectively (Umehara *et al.* 2019).

Sperm sexing by genome editing: New technologies for genome editing, including finger nucleases of zinc (ZFN), transcript-activator like endonucleases (TALENs), and the CRISPR / Cas method have emerged with great potential for influencing genome-level sex determination (Kurtz and Petersen 2019). In the Y chromosome (SRY) sex-determination region is a significant genetic switch in the development of male genders. Knockout of the SRY gene in mice or rabbits has shown that the development of testis is suppressed in the fetal gonadal ridges leading to a phenotype of females. Such new products have great chances of pre-determining sex in bovines also. Further investigations are, however, needed to exploit their full potential for practical application.

Sperm sexing by mitochondrial activation technology: YKgen's technology claims a chemical approach to sperm mitochondria. YKgen's architecture is expected to achieve high sperm metabolic activation with increased motility and the predicted fertility thus, increased. The approach based on mitochondria will improve fertilization with higher purity, and higher cell counts to improve sperm sex selection. It is anticipated that the new technology would cost less capital than the current technology, which needs a dedicated laboratory plus capital equipment. In YKgen technology, this separation is possible by activating mitochondria with chemical material which specifically responds to gender. The technique is to assess the sex ratio of X or Y-bearing sperm by examining the difference in mitochondrial activity in sperm by Real-Time PCR. PCR results show that the ratio of X and Y for normal sperm is 50:50, but when analyzing reactant-treated sperm, the ratio of X and Y appears at 70:30.

Aptamer - A new hope for sperm sexing: Aptamers, a class of molecules used for molecular detection, have recently been shown to have out-performed clonal antibodies in target sensitivity and affinity and warrant an investigation into the development of sex-specific aptamer sperm probes. Aptamers are small, single-stranded DNA or RNA molecules that adopt stable, sequence-dependent secondary structures, which bind to different ligands. Sex-specific aptamer X- and Y-probes have been selected from a large randomized library to flow-sorted bull sperm using a modified systematic evolution of ligands by an exponential enrichment method (SELEX). The selected X- and Y-aptamer pools were monitored/assessed using flow-cytometry, fluorescent binding assays, and fluorescent microscopy over 20 SELEX cycles. The chosen X and Y-aptamer pools have substantially improved affinity to X- and Y-sorted sperm (relative to the naive aptamer library and the opposite aptamer pool selected). Based on these tests, aptamers can be easily chosen for sperm features, and similar methods can be useful for further work in the use of antibodies. Besides, X and Y-sperm can show a sex-unique marker that can be resolved using aptamer probes (Colley 2008). Research is underway to evaluate the aptamer binding target and to assess the feasibility of aptamers as sperm sexing probes.

Current status of sperm sexing in India and future prospective

Sexing Technology (ST) and ABS are the only two companies in India producing sexed semen. There are presently 51 semen stations in the country with an annual production potential of 81 million doses (Srinivastava *et al.* 2019). In contrast, current bovine semen demand is close to 119 million doses of 2019 million doses and is expected to grow to more than 150 million in the next five years. Farmers use the technology in pockets of Punjab, Haryana, Telangana and Andhra Pradesh, which mainly use imported semen of foreign species such as Holstein-Friesian (HF) and Jersey. Under the Rashtriya Gokul Mission, the Sex Sorted Semen project will undertake the development of indigenous breed semen straws such as Sahiwal, Gangatiri, Kankrej and Gir that are well suited to agro-climatic conditions and are immune to many tropical diseases (Rashtriya Gokul Mission 2019). This will also aid in the survival and spread of native breeds. There is currently no agency in India, however, which produces sexed semen on a large scale, though several states import sexed semen. Sexed semen from Indian cattle breeds is more suitable, as the cattle are more suited to the Indian environment. Nevertheless, sexed semen is primarily imported from the United States and Canada, which are not native breeds. An indigenous technology will also be built to make sexed semen more accessible. In 2009, Paschim Banga Go-Sampad Bikash Sanstha (PBGSBS), a West Bengal government organization based in India, initiated semen sorting using a flow cytometer with a production capacity of 40–50 sexed semen straws per day. Using this sexed semen, the rates of pregnancy observed for cows were 20.7% and for heifers, 35.3%. The National Dairy Research Institute (NDRI), Karnal, has also been working for cattle semen sexing to multiply the country's indigenous and crossbred cows by providing sexual semen to farmers (Kumar *et al.* 2016). The animal husbandry department plans to breed 60 lakh cows each year, through the sexed semen, to develop the cattle breed. The animal husbandry department plans to build 10 facilities for the production of sexed semen, including one at the CFSPTI (Central Frozen Semen Production and Training Institute) of its premier institute. Indigenous breeds would be used as a source of semen, delivering 2 million doses of sexed semen per year. Currently, ₹200–300 crore are required for the establishment of a sperm sexing facility, and starting production takes about five years. A total of ₹500 crore have been allocated to support semen sexing technology for 2016–17 (Rai 2018). India also imports sexed semen. ABS India Inc. alone imports one million sexed semen straws annually. The Punjab Progressive Dairy Farmers Association is India's largest customer with approximately 20,000 doses purchased. The farmer's cost is around Rs ₹1500 per sexed semen straw (Behera *et al.* 2018), while a semen straw without sexing costs just ₹20-50 from a certified semen station.

The most commercially active semen sexing technology is patented by Sexing Technologies, a US-based company (ST Genetics, Navasota, TX). This technique is focussed on unequal quantities of DNA in the sperm bearing X-chromosome over the sperm bearing Y-chromosome. In X sperm, the fluorescence strength of the DNA binding dye (Hoechst 33342) is higher than in Y sperm, which can be shortened with a flow cytometer. The reported pregnancy rate of Sexed ULTRA semen straw from Sexing Technologies is 52%, while conventional semen has a pregnancy rate of 60%. Sexed ULTRA semen has sperms bearing 90% X chromosome (Thomas *et al.* 2017), while traditional semen straw has sperms carrying 50% X chromosome. Sexed ULTRA semen shortening process involves orienting nozzle technique that is monopolized by proprietary patenting, causing the product's price to increase. Orienting nozzle (HiSON) flow cytometer will separate frozen sperms from the X-chromosome bearing at 11 million/h (Johnson and Welch 1990). A single dose contains 2-4 million sperms, and this machine can generate about 255-265 doses in 24 hours run time, which is very costly given the machine's cost of ₹4–5 crore. Developing alternative techniques that do not involve flow cytometry could reduce the cost. Even though most farmers don't know about sexed semen, the supply still falls short. A survey of 871 farmers of Ahmed Nagar and Pune in Maharashtra in 2012 found that 66% of farmers would use sexed semen if it is available for ₹500 per insemination. India has a ready market for sexed semen and has the potential to grow if the cost is reduced and the viability of sperm increases resulting in better fertilization (Jeyakumar *et al.* 2015). In today's dairy industry, farmers need to adopt strategies that will yield results and help them gain a competitive advantage in their business. The use of sexed semen is one such technique, which can help farmers grow their herds internally when appropriately used in a dairy. When small farms are converted into larger operations, there is a range of concerns that need to be resolved for a smooth transition. Using sexed semen technology, heifers will be born as many as 90-95% of the time, instead of 49% of the time while using non-sex semen. Modern techniques such as semen sexing are being used to control the sex ratio and to create large numbers of one-sex progenies. Female sex-sorted semen is made available to farmers in advanced dairy nations to generate more high genetic merit heifers and improve milk production and dairy farm profitability. The sex-assorted semen technology will be standardized in the near future for native breeds such as Sahiwal, Gir, Tharparkar, Red Sindhi, Hariana and Rathi .

One choice for the use of sexed sperm is embryo development *in vitro*. Approximately 10–15 oocytes (from ovaries originating from abattoirs) could be fertilized with 40,000–80,000 sexed buffalo sperm *in vitro*; the production rate of blastocysts (about 20%) was not significantly different from that achieved with unsexed sperm. In addition, the transfer of presumed X-embryos to a recipient resulted in the birth of female twins and proved conceptual. Nevertheless, abbatoir-derived ovaries are usually harvested from culled buffaloes, with low or unknown genetic merit. In comparison, ovum pick-up (OPU) technology may be used to recover large amounts of meiotically competent oocytes from antral follicles in live animals of proven genetic merit, thus significantly enhancing the value of *in vitro* embryo production programs. Adoption of sexed semen for AI in cattle was limited by cost, low levels of conception, and precision in sexing. Technology has improved by enhancing sorting levels, conception rates have risen over the past decade, sexing accuracy is close to 90%, and the premium for sexed semen is often less than $20 per dose (approximately double the semen costs). Consequently, this innovative and unique technology is rapidly used by the dairy industry. Nevertheless, several questions arise in which demand, production, and other circumstances result in a sexed semen advantage (SSA)—increased benefit relative to traditional semen use. Providers of sexing technology may alter their marketing activities with respect to consumer demand/ environment. Some studies have highlighted the economy of sexed semen due to the market price of milk or the cost of heifer/cows, or changes in the rate of sexing success. No one in our knowledge has discussed the consequences of using sexed semen over a wider range of input and output pricing, technology, and management options that can and do occur in the highly competitive and unpredictable dairy sector.

Conclusion

Available data indicates that the broad and uniquely developed dairy sector in India has sustained strong annual growth and appears to meet robust demand for milk and major dairy products at relatively stable real prices. Despite this growth, the dairy sector is characterized by low yields throughout the herd's crossbred, indigenous cattle and buffalo components, leaving substantial benefit opportunities through improved breeding, feeding, and management. Prospects for India's position in the world's dairy markets probably rely on its ability to boost its milking herd's low productivity, and on the growth and competitiveness of its emerging dairy product sectors. Continued expansion of India's relatively high-yielding indigenous dairy herd would be crucial to productivity gains, along with continued growth in buffalo milk production. The use of sexed semen is usually expected to have a beneficial impact on the farms' economies, retaining replacement heifers and the productivity of increasing dairy herds, resulting in increased milk yield. Sex pre-selection would improve genetic benefit, enhance herd biosecurity, and raise the incidence of dystocia and the number of extra bull calves, which is a significant welfare problem for the industry.

ACKNOWLEDGEMENTS

Indian Council of Agricultural Research supported the research through XII Plan Scheme Incentivizing Research on 'Semen Sexing in Cattle' (Project no: 1006660). The author would like to thank the Department of Biotechnology, Government of India for providing financial support from "DBT-RA Program in Biotechnology and Life Sciences".

REFERENCES

20th Livestock Census. 2019a. All India Report. Ministry of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India (www.dahd.nic.in).

20th Livestock Census. 2019b. Policy and report brief (www.vikaspedia.in).

Ahlawat, S P S and Singh P K. 2005. Conservation and improvement of Indian cattle breeds. *Proc. VIII National Conference on Animal Genetics and Breeding in India* (pp. 1-12).

Ashish Paliwal, Aslam Perwaiz and Saadia Taufiq. Report on Dairy Chain Opportunities India Pakistan and Bangladesh. Ministry of Foreign Affairs of Denmark.

Behera R, Singh L A and Mandal A. 2018. Sexed semen: A boon for Indian dairy farming. Research and Reviews. *Journal of Dairy Science and Technology* **6**(1): 10–6.

Bhalakiya N, Haque N, Patel D, Chaudhari A, Patel G, Madhavatar, M, Patel P, Hossain S and Kumar R. 2018. Sperm sexing and its application in livestock sector. *International Journal of Current Microbiology and Applied Science* **7**: 259–72.

Colley A J. 2008. Selection of sex-specific aptamer probes to sperm. Doctoral dissertation.

Dasari S. 2013. Breeding policy strategies for genetic improvement of cattle and buffaloes in India. *Veterinary World*, **6**(7).

De Vries A, Overton M, Fetrow J, Leslie K, Eicker S and Rogers G. 2008. Exploring the impact of sexed semen on the structure of the dairy industry. *Journal of Dairy Science* **91**(2): 847–56.

Department of Animal Husbandry, Dairying and Fisheries (DAHD). 2019. Basic Animal Husbandry Statistics.

DESA . 2019. World population prospects 2019. Online edition, Rev 1, United Nations, Department of Economic and Social Affairs. *Population Division*.

Espinosa-Cervantes R and Córdova-Izquierdo A. 2012. Sexing sperm of domestic animals. *Tropical Animal Health and Production* **45**(1): 1–8.

Gledhill B L. 1988. Selection and separation of X-and Y-chromosome-bearing mammalian sperm. *Gamete Research* **20**(3): 377–95.

Hamano K I. 2007. Sex preselection in bovine by separation of X-and Y-chromosome bearing spermatozoa. *Journal of Reproduction and Development* **53**(1): 27–38.

Holden S A and Butler S T. 2018. Applications and benefits of sexed semen in dairy and beef herds. *Animal* **12**(s1): s97–s103.

Jeyakumar S, Kataktalware M A and Ramesha K P. 2015, January. Invited lectures: Present status and future prospects of Sexed Semen in India. *National Seminar,* 24th January.

Johnson L A. 2000. Sexing mammalian sperm for production of offspring: the state-of-the-art. *Animal Reproduction Science* **60**: 93–107.

Johnson L A and Welch G R. 1999. Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. *Theriogenology* **52**(8): 1323–41.

Johnson L A, Flook J P and Hawk H W. 1989. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. *Biology of Reproduction* **41**(2): 199–203.

Joshi B K and Singh A V T A R. 2005, March. Indigenous cattle Milch breeds–their potential and improvement programs. *Proceedings of the VIII National Conference on Animal Genetics and Breeding in India* (pp. 21-28).

Joshi B K, Singh A and Mukherjee S. 2005. Genetic improvement of indigenous cattle for milk and draught: A review. *The Indian Journal of Animal Sciences* **75**(3).

Khaja Mohteshamuddin. 2017. Sexed semen technique: A revolution in Indian Dairy Industry. *Agrotechnology* (editorial) **6**(3).

Kumar A, Vineeth M R, Sinha R, Singh R K, Thakur A and Gupta S K. 2016. Current status, scope and constraints of sexed semen-An Indian perspective. *Agricultural Reviews* **37**(3): 240–44.

Kumar M, Dahiya S P, Ratwan P, Kumar S and Chitra A. 2019. Status, constraints and future prospects of Murrah buffaloes in India. *Indian Journal of Animal Sciences* **89**(12): 1291–1302.

Kurtz S and Petersen B. 2019. Pre-determination of sex in pigs by application of CRISPR/Cas system for genome editing. *Theriogenology* **137**: 67–74.

Landes M, Cessna J, Kuberka L and Jones K. 2017. *India's Dairy Sector: Structure, Performance, and Prospects*. United States Department of Agriculture.

Luscher M. 2013. *U.S. Patent No. 8,467,040*. Washington, DC: U.S. Patent and Trademark Office.

National Kamdhenu Breeding Centre. 2019. Ministry of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India (www.dahd.nic.in).

Parati K, Bongioni G, Aleandri R and Galli A. 2006. Sex ratio determination in bovine semen: A new approach by quantitative real time PCR. *Theriogenology* **66**(9): 2202–09.

Patil S S, Kale A M and Awati A A. 1998. Pandharpuri buffaloes for better persistency. *Buffalo Bulletin (Thailand)* **17**(3): 65–66.

Prakash M A, Arumugam K, Ayyasamy M, Joshi R K, Layek S S, Mohanty T and Divisha R R. 2014. Sexing of spermatozoa in farm animals: A mini review. *Advances in Animal and Veterinary Sciences* **2**(4): 226–32.

Presicce G A. 2022. *Biotechnological Applications in Buffalo Research,* pp. 319-332. Semen Sexing in the Buffalo. Springer, Singapore.

Rahman M S and Pang M G. 2019. New biological insights on X and Y chromosome-bearing spermatozoa. *Frontiers in Cell and Developmental Biology* **7**.

Rai J. 2018. Sperm sexing of dairy cattle: Economics, animal welfare and technological challenges. *Current Science* **114**(7): 1438.

Rashtriya Gokul Mission. 2019. Ministry of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India (www.dahd.nic.in).

Sá Filho M F, Ayres H, Ferreira R M, Nichi M, Fosado M, Campos Filho E P and Baruselli P S. 2010. Strategies to improve pregnancy per insemination using sex-sorted semen in dairy heifers detected in estrus. *Theriogenology* **74**(9): 1636–42.

Srivastava A K, Patel J B, Ankuya K J, Chauhan H D, Pawar M M and Gupta J P. 2019. Conservation of indigenous cattle breeds. *Journal of Animal Research* **9**(1): 1–12.

Thiagarajan R. 2014. *Textbook of Animal Breeding.* Satish Serial Publishing House, New Delhi.

Thomas J M, Locke J W C, Vishwanath R, Hall J B, Ellersieck M R, Smith M F and Patterson D J. 2017. Effective use of SexedULTRA™ sex-sorted semen for timed artificial insemination of beef heifers. *Theriogenology* **98**: 88–93.

Umehara T, Tsujita N and Shimada M. 2019. Activation of Toll-like receptor 7/8 encoded by the X chromosome alters sperm motility and provides a novel simple technology for sexing sperm. *PLoS Biology* **17**(8): e3000398.

Upreti G C, Hall E L, Koppens D, Oliver J E and Vishwanath R. 1999. Studies on the measurement of phospholipase A2 (PLA2) and PLA2 inhibitor activities in ram semen. *Animal Reproduction Science* **56**(2): 107–21.

Yadav A K and Singh J. 2016. Characteristics of registered indigenous horse and pony breeds of India: A review. *Journal of Progressive Agriculture* **7**(2): 161–64.

Yadav S K, Gangwar D K, Singh J, Tikadar C K, Khanna V V, Saini S, Dholpuria S, Palta P, Manik R S, Singh and Singla S K. 2017. An immunological approach of sperm sexing and different methods for identification of X-and Y-chromosome bearing sperm. *Veterinary World* **10**(5): 498.