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Recent advancement of frozen semen technology in India

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Abstract
India tops the livestock population in the world. Total livestock population in the country is 535.78 million showing an increase of 4.6% over Livestock Census-2012-the largest in the world. This is mainly because of recent advancements in frozen semen technology in India. With the advent of fluorescent microscopy, flow cytometry and advances in molecular biology, now it has become possible to evaluate the spermatozoa in terms of specific functions that are well related to fertility. However the emphasis on production of female calves is gaining popularity with a view to control the male calves' population. Fluorescence-activated cell sorting (FACS) and immunological method of semen sexing are the two commonly used sexed semen technologies. This article provides a concise review of progress in the advancements of frozen semen technology in India along with pioneer work carried by scientists in field of animal reproduction and breeding.

Keywords: India, sexed semen, technology, frozen semen

Introduction
With the inception of cryopreservation of semen, transmission of superior genetic material by artificial insemination has become rapid and wide spread crossing the geographical boundaries. Advances in frozen semen technology have improved post thaw semen quality which is reflected in improved fertility with frozen thawed semen over time. In spite of several developments in the semen freezing and thawing, approximately 50% of sperm are rendered immotile by cryopreservation and fertilizing capacity of spermatozoa is significantly leading to decreased conception rates with frozen semen compared to fresh semen. Therefore determining the quality of the frozen semen through certain *in vitro* test in relation to fertility is of prime importance in the dairy animal breeding industry.
With the advent of fluorescent microscopy, flow cytometry and advances in molecular biology, now it has become possible to evaluate the spermatozoa in terms of specific functions that are well related to fertility. The advances in terms of fluorescence microscopy, flow cytometry and molecular biology have provided more advanced and accurate tools to rapid detection of functional and structural assessment of spermatozoa. Development in flow cytometry has made the quality control measures more efficient and quick with accuracy.

Microscopy
- Fluorescent microscopy has been an essential tool in biology and reproductive sciences, because of wide array of fluorochromes. The use of fluorescence labeling enables identification of sub-microscopic cellular components. Fluorescent microscopy has been extensively used to analyze sperm viability, the sperm membrane, acrosome, and chromatin. In this microscopy method, cellular components of sperm function are stained with fluorescent probes to examine the DNA, membranes, or lectins. Sperm viability assay can be analyzed by fluorescence microscopy using LIVE/DEAD commercial kits, which are DNA-binding fluorescent stains (SYBR-14) and membrane-permeant stain (PI), respectively. Acrosome integrity can be analyzed using the sperm acrosome molecular marker Pisum sativum agglutinin linked to fluorescein isothiocyanate (FITC-PSA). Terminal transferase dUTP nick-end-labeling (TUNEL) can also be used to evaluate apoptosis by flow cytometry and fluorescence microscopy.
- Light Microscopy has been a commonly used tool to evaluate basic quality parameters of semen including sperm motility, morphology, membrane integrity, and concentration.
- Laser Confocal fluorescence microscopy is a technique that obtains three-dimensional

(3D) optical resolution with depth of focus and provides protein distributions in cellular compartments. The advantages of confocal microscopy are that it recognizes fluorescence in individual cells, provides multispectral flexibility, and avoids out-of-focus suppression.

- Electron Microscopy (EM) uses the electron as a tool to utilize a beam of accelerated electrons to develop a specimen image. This technique provides higher magnification and resolution than light microscopy. EM is performed in a vacuum and directly focuses an electron beam on the subject and images are magnified by the means of electromagnetic lenses. This microscopy technique has the advantage of using shorter wavelength of electrons at accelerating voltage. The two most common electron microscopes are Transmission electron (TEM) and scanning electron (SEM).
- Holographic microscopy and Raman spectroscopy have a holographic microscopy format where samples are visualized by laser light, and the obtained images are used to define the position, orientation, and the 3D structures of a microscopic sample. Raman microspectroscopy has the capability of evaluation of chemical changes and molecular features of human and bovine sperm cells. Additionally, it offers an analysis of live sperm with physiological status.

Computer-Assisted Sperm Analysis (CASA)

The CASA system, first established in 1980, has evolved into an accurate computer-based technique and software which provides quantitative measurements to assess the sperm motility and kinematics objectively and precisely. This technique uses the principle of capturing continuous images of motile sperm from a microscopic field and converts images into video images with different acquisition rates (frames s-1, Hz). Captured images are scanned to be visualized through dark field or negative-high-phase contrast in order to track motion of each individual sperm considering intensity of frame in pixels and the head.

Flow Cytometry

Flow cytometry (FC) is an outstanding system which has made it possible to analyze thousands of single cells in a short time. Flow cytometry permits analyses of large numbers of sperm cells as well as individual cells with physical characteristics of a single spermatozoon measured by a fluorescent compound. It is composed of fluidics, optics, and electronics systems, which uses the measurement of physical optics and chemical fluorescence characteristics of particles in a fluid when it is passes through a laser source (Ugur *et al*, 2019) [9]

Semen Sexing Technology

Fluorescence-activated cell sorting (FACS)

Over the past 40 years, considerable efforts have been made to develop a technology to separate X-and Y-bearing spermatozoa, the success is very limited particular in the cattle. The recent and most adopted technology of sperm sexing is based on exclusive DNA content of spermatozoa. X bearing spermatozoa carries almost 3.4% higher amount of DNA as compared to Y-bearing. So, X and Y-chromosome bearing florescent labelled sperm cells can be separated into fractions by fluorescence-activated cell sorting (FACS). However, this procedure is very slow, expensive and leads to sub-lethal damages to cells including sheep spermatozoa. This limits the standard insemination procedure of semen deposition in cervix or uterus of recipient female. It is believed that sex-sorted semen should deposit in the oviduct or deep within the uterus to obtain high success rates. In sheep or goat, transcervical deposition of semen is very difficult. In some special cases laparoscopically is required to deposit semen into the uterus. Thus, as compared to cattle's, the oviductal deposition of sex-sorted frozen semen in sheep is not practical in the farmyard.

Immunological method of semen sexing

The basis of the immunological method of semen sexing started when it was speculated that, if X-and Y-sperm differ in their genomic DNA content among different species then it must lead to the protein differences as well. Recently, Chen *et al*, 2015 reported that X-and Y-bearing bovine sperm differ in expression of at least 31 genes. Among these, in X-sperm 27 were up-regulated and 4 in Y-sperm. This leads to phenotypic variations in X-and Y-sperm proteins. If these proteins are expressed in sperm surface and if one can isolate/identify such a marker(s) then antibodies against that marker together with immunological methods (such as immunofluorescence labelling, immunoprecipitation and immunotoxicity approaches) can be employed to separate X-and Y-sperm. This can be much more adventurous than conventional FACS method of fast recovery of sex shorted sperms. However, the accuracy of detecting right sperm and possibly of its separation by using specific antibodies is linked to the accuracy of sperm surface protein marker identification and accessibility of antibodies to the selected protein targets (Gupta *et al*, 2018) [4].

What are the advantages of using sexed semen?

- Producing only female calves helps the farmers to save resources that would have been shared with unwanted males.
- Production of more female calves: increase supply of replacement heifers
- Opportunity to sell surplus heifers to other farmers/farms
- Speed up genetic improvement:
  - By increasing efficiency of progeny testing (PT) programme
  - By increasing efficiency of embryo transfer and IVF programme
- An economic way to increase herd strength with no risk of introducing diseases by purchasing heifers from outside (improves bio-security).
- As dead, dying or damaged sperm cells are removed during the sorting process, only viable sperm are available which helps the sexed semen to be successful even at a low concentration (than conventional semen).
- By producing more female calves using sexed semen, there will be less difficult births compared to male calves (dystocia). This is particularly useful for maiden heifers.

Gender selection using sexed semen from genetically elite bulls is imperative to meet the projected demand of 191.3 million tons of milk by 2020 in the country. The demand of sex semen in dairy cattle is also increasing in order to dispose the large number of unproductive males, to ensure required number of progenies per bull under progeny testing programme and to reduce the replacement cost on heifers. In India, this technique is gradually been adopted by many states like Punjab, Haryana, West Bengal, Kerala etc.

History of development of sexed semen technology

The first attempt to separate X and Y sperm by analytical flow cytometry was initiated in 1976 by Gledhill (1976).

Pinkel *et al*. (1982) [7] first time successfully separated mammalian sperm. But the methods were found to be destructive because the tails were removed by sonication leaving sperm biologically unusable. In 1980's a breakthrough in semen sexing technology was made by USDA researchers in the Lawerence Livermore Laboratory in California. This method works adequately since the X chromosome is larger than the Y and therefore takes up more of the DNA-specific stain.

Table 1: Potential differences between X and Y Spermatozoa (Source: Prasad *et al*., 2010)

Parameter	X spermatozoa	Y spermatozoa	Method
Size	Larger	Relatively smaller	Percoll method
Motility	Swim slower	Swim faster	Swim up
Surface charge	Migrate to cathode fast	Migrate to cathode slow	Free flow electrophoresis
Sperm surface	Absence of HY antigen	Presence of HY antigen	Immunological sexing
DNA	More DNA	Less DNA	Flow cytometry

The differences in DNA content between the X and Y-chromosome-bearing sperm in different species have been reviewed elsewhere (Garner and Seidel, 2003). In Bos indicus, the average X-Y sperm difference is 3.73%. Whereas, differences in DNA content for Murrah and Nili Ravi buffalo were 3.59% and 3.55% respectively (Lu *et al*, 2007) [6]. This means an optimum sorting accuracy and sorting rate would be more difficult for buffalo sperm compared to that of bovine sperm, whose difference in DNA content between X-and Y-sperm is slightly higher.

Status of semen sexing in India

In India, Paschim Banga Go-Sampad Bikash Sanstha (PBGSBS), a Government of West Bengal organisation, initiated sorting of semen using high speed semen sorter or flow cytometer (Influx, Becton Dickinson, Biosciences, San Jose, CA, USA) installed on 15th August, 2009 under RKVY with a total outlay of Rs. 2.90 crores, during 2007-08 and 2008-09 and completed in November, 2009 at Frozen Semen Bull Station, Haringhata. They reported first male calf named Shreyas, born on 1st Jan 2011 using sexed semen. Later female calves were also successfully born to sexed semen. They are currently in a position to produce 40-50 sexed semen straws per day. The conception rates observed were 20.7% in cows and 35.3% in heifers using sexed semen. The purity of X sorted semen was found to be higher compared to Y-sorted semen (Biswas *et al*., 2013). Kerala also reported birth of two sexed semen calves to Jersey crossbred heifers and Holstein Friesian crossbred cows respectively at Vakkavu in Nenmara, Palakkad under a pilot project jointly taken by Kerala Livestock Development Board (KLDB) and Department of Animal Husbandry. The sexed semen of HF and Jersey were imported by KLDB from Canada @Rs.1250 per dose. The Kerala Livestock Development Board has imported 650 doses of sexed semen doses. The ABS India is providing Holstein and Jersey sexed semen. Prime Bovine Genetics in collaboration with Sexing Technologies provides sexed semen of Holstein Friesian, Jersey, Brown Swiss and Gir crossbreds. But, the Planning commission, Govt. of India has recently assigned the responsibility for sorting of the sex of semen in cattle to NDRI, Karnal. Imported sexed semen is now available to farmers in some states with Punjab in forefront. The cost of one dose of sexed semen varies between Rs. 1000/-to 1500/-. In Punjab the farmers are being charged Rs 600/-per straw with the state subsidizing the remaining 50% of the total cost of Rs 1200 per straw. Moreover, Artificial Insemination co-operatives from USA are in collaboration with Progressive dairy farmers in Punjab for providing breeding solution that promises 90% female calves (Kumar *et al*, 2016) [5].

Semen stations in India

India is building its biggest frozen semen station, housing 300 bulls of indigenous breeds of buffalo and cattle, with construction costs estimated at Indian Rupees (INR) 640 million (US$9.7m) in Purnea, Bihar.

Under Rashtriya Gokul Mission, a unit under Department of Animal Husbandry, Dairying & Fisheries, Ministry of Agriculture, SSS production facilities are being developed at 10 A-grade (technically most advanced) bovine semen stations across the country. India has 56 semen stations. These stations collectively produce 70 million doses, which can cover 25 per cent of the breedable cattle (199 million, according to the 2012 livestock census) in India.

Of 56 semen stations in the country, 32 got Grade A and Frozen Semen Bank (FSB. Fifteen stations got Grade B and two were rated as non-graded by the Central Monitoring Unit (CMU). Two semen stations have not been evaluated.

The production of Frozen Semen Doses started in December, 2011.

Top ten semen stations are producing 50% of the total doses and top 25 semen stations about 85% of the total doses.

Table 2: A and B graded semen stations of the country and various breeds of Indigenous, exotic and crossbred cattle and Buffalo bulls maintained by them

SL. No.	Name of the Semen Station	State	Grade as per CMU evaluation (2012-13)	Indigenous	Exotic	Crossbred	Buffalo
1	Nandyal	Andhra Pradesh	A	Ongole		Jersey CB	Murrah
2	Banavasi	Andhra Pradesh	B	--	HF and Jersey	Jersey CB	Murrah
3	Vizag	Andhra Pradesh	A	Ongole	Jersey	Jersey CB	Murrah
4	Karimnagar	Telangana	A	--	HF	Jersey CB	Murrah
5	Anjora, Durg	Chhattisgarh	A	Gir, Tharparkar, Sahiwal, Red Sindhi and Ongole	Jersey	HF CB and Jersey CB	Murrah
6	Sabarmati Ashram Gaushala, Bidaj	Gujarat	A	Kankrej, Gir, Sahiwal, Red Sindhi, Khillar and Hariana	HF and Jersey	HF CB and Jersey CB	Murrah, Mehsana, Jaffarabadi, Banni and Pandharpuri
7	Mehsana, Jagudan	Gujarat	A	--	HF	HF CB	Mehsana
8	Banas, Dama	Gujarat	A	Kankrej	--	HF CB	Mehsana
9	Patan	Gujarat	A	Gir	HF	HF CB	Mehsana, Surti, Jaffarabadi and Banni
10	Amul (ARDA-Ode)	Gujarat	A	Gir	HF	HF CB	Murrah
1 1	Hissar	Haryana	A	Sahiwal	HF	HF CB	Murrah
12	Jagadhari	Haryana	A	Sahiwal	HF	HF CB	Murrah
13	Gurgaon	Haryana	A	--	--	HF CB	Murrah
14	Rohtak	Haryana	A	--	--	--	Murrah
15	Palampur	Himachal Pradesh	A	--	HF and Jersey	HF CB and Jersey CB	
16	Srinagar	Jammu & Kashmir	B	--	HF and Jersey	Jersey CB	--
17	Nandini	Karnataka	A	--	HF and Jersey	--	Murrah
18	CFSP&TI, Hessarghata	Karnataka	A	Tharparkar	HF and Jersey	--	Murrah
19	SSCC, Hessarghata	Karnataka	A	Hallikar and Amritmahal	--	--	--
20	SLBTC, Hessarghata	Karnataka	B	--	--	--	Murrah and Surti
21	CSCC, Dharwad	Karnataka	B	Khillar and Deoni	HF and Jersey	--	Murrah and Surti
22	Dhoni	Kerala	A	Kankrej	--	HF CB and Jersey CB	Murrah
23	Mattupatty	Kerala	A	--	HF and Jersey	HF CB and Jersey CB	--
24	Kulathupuzha	Kerala	A	Gir	--	HF CB and Jersey CB	--
25	Central Semen Station, Bhadbhada, Bhopal	Madhya Pradesh	B	Gir, Tharparkar, Sahiwal, Malvi and Nimari	HF and Jersey	HF CB and Jersey CB	Murrah, Jaffarabadi and Bhadawari
26	BAIF, Uruli Kanchan	Maharashtra	A	Tharparkar, Sahiwal, Khillar, Hallikar, Amritmahal, Red, Kandhari, Nimari and Krishna Valley	HF and Jersey	HF CB and Jersey CB	Murrah, Surti, Jaffarabadi, Bhadawari and Banni
27	Chitale	Maharashtra	B	--	HF and Jersey	HF CB	Murrah
28	Kirkee, Pune	Maharashtra	A	Khillar	HF and Jersey	HF CB	Murrah, Surti and Pandharpuri
29	Nagpur	Maharashtra	B	Sahiwal, Gaolao and Nagori	Jersey	HF CB and Jersey CB	Murrah
30	Cuttack	Odisha	A	Red Sindhi and Hariana	Jersey	Jersey CB	--

31	Bhattian	Punjab	B	Sahiwal	HF and Jersey	--	Murrah
32	Nabha	Punjab	A	Sahiwal	HF	HF CB	Murrah and Nili Ravi
33	Ropar	Punjab	A	Sahiwal	HF and Jersey	HF CB	--
34	Bassi	Rajasthan	B	Kankrej, Rathi, Gir, Tharparkar, Sahiwal, Nagori and Hariana	HF and Jersey	HF CB and Jersey CB	Murrah and Surti

Sl. No.	Name of the Semen Station	State	Grade as per CMU evaluation (2012-13)	Indigenous	Exotic	Crossbred	Buffalo
35	NJF, Ooty	Tamil Nadu	B	--	HF and Jersey	HF CB and Jersey CB	--
36	Eachankottai	Tamil Nadu	A	Umblachery	Jersey	Jersey CB	Murrah
37	FSPS, DLF, Ooty	Tamil Nadu	B	--	HF and Jersey	HF CB and Jersey CB	--
38	DLF, Hosur	Tamil Nadu	A	Red Sindhi and Kangayam	--	Jersey CB	--
39	ABC, Salon	Uttar Pradesh	A	Rathi, Tharparkar, Sahiwal, Red Sindhi and Hariana	HF and Jersey	HF CB and Jersey CB	Murrah and Bhadawari
40	FSBS, Babugarh	Uttar Pradesh	B	Hariana	Jersey	HF CB	Murrah
41	Rishikesh	Uttarakhand	B	Sahiwal and Red Sindhi	HF and Jersey	HF CB and Jersey CB	Murrah
42	Haringhata	West Bengal	A	Sahiwal and Gir	Jersey	HF CB and Jersey CB	Murrah
43	Salboni	West Bengal	A	Gir	Jersey	Jersey CB	Murrah

Sl. No.	Name of the Semen Station	State	Grade as per CMU evaluation (2012-13)	Indigenous	Exotic	Crossbred	Buffalo
44	Beldanga	West Bengal	B	Gir and Sahiwal	Jersey	Jersey CB	--

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