# Gearing up superior quality semen production: breeding soundness evaluation of bulls and beyond

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India is the foremost milk producing nation of the world, and homes the largest domesticated bovine population. But the average productivity of indigenous/non-descript animals is far below than that of Taurine cattle. Crossbreeding of low producing indigenous and nondescript cattle with high producing exotic breeds like Holstein Friesian and Jersey is followed as the breeding policy of the country for the last four decades, to meet the ever-increasing demand for milk and milk products. Artificial insemination (AI) with frozen semen from superior bulls is the major tool behind the success of crossbreeding, which is witnessed by the increase in crossbred cattle population from 8.8 million in 1982 to 39.73 million in 2012. Female cross bred cattle are growing at the rate of almost 10 per cent per annum; the rate of growth would have been much higher had the AI service in India been of better quality.

Presently, India has one of the largest breeding infrastructures in the world (48 frozen semen stations, 3297 bulls and 93,283 AI centres) with total production of about 67 million frozen semen straws and 54 million AI per year, covering 25 per cent of the breedable population[1].To achieve national target of 50 per cent AI coverage by 2021-22, high number of superior bulls are required and semen production must reach at least 140 million doses. The major limiting factor in producing required numbers of frozen semen straws is the availability of quality breeding bulls. Moreover, among those bulls selected after routine bull

and semen evaluation and regularly used for breeding, 20-25% differences in pregnancy rates were observed[2]. These low performing bulls can be identified at a very later stage only, upon large scale use of semen samples through AI and subsequent feedback from the field, hampering the genetic progress and economic loss. This clearly indicates that the present system of bull selection require a major reviewing and remodelling. In this paper, we attempted to review various traditional methods of bull evaluation and selection, recent advances in this area and our findings on testicular cytogram and proteomic biomarkers, for fair determination of latent fertility of bulls.

**Breeding Soundness Evaluation of Bulls**

The saying 'bull is more than half of the herd' itself signifies the value of selecting the best bull in a population. Bull fertility may be plainly defined as the ability to produce and ejaculate normal fertile sperm to impregnate the cow. But while going into deeper dimensions fertility reveals being itself associated with various components and stages, which require the male and female to be functionally capable of producing young ones for the next generation. Thus, predicting this particular ability of an individual animal unrolls to various physical, biological and molecular factors; and hence evaluation of parameters representing those related factors would be the most suitable approach for predicting the fertility. Even in case of a single animal, the need for continuous evaluation is warranted before breeding as the fertility of an individual animal can vary from year to year or with advancing age. Breeding soundness evaluation (BSE) is an amalgamation of various physical, biological and microscopic examinations to predict the fitness and fertility of bulls. Thus proper evaluation of breeding bulls can improve reproductive efficiency and overall profitability of dairy farm enterprises.

The practice of BSE started when the 'Society for Theriogenology' developed standardized procedures and published the manual for Breeding Soundness Examination of bulls in 1983. On the basis of numerical scoring system, bulls

were classified into satisfactory potential breeder, questionable potential breeder, and unsatisfactory potential breeder. Later on in 1992, the new guidelines for evaluating and classifying bulls for breeding soundness was formulated based on the latest scientific information. It was repeatedly observed that, whenever this procedure had been followed accurately, the results were consistent irrespective of breed, location and examiner. The major challenge faced in evaluating BSE for the young bulls is the greater variation in age of puberty and sexual maturity in various breeds. Some animals attaining puberty at 11-13 months whereas; some bulls will not attain puberty until 15-16 months. The young bulls found unsatisfactory at the time of evaluation may turn satisfactory breeder if re-tested after 3 months. This is because spermatogenesis process takes about 60 days and extra 15 days required to transport from reproductive tract and maturation. It should be noted that the BSE is a single day observation and it may change over a period of time due physical defects or injury to the bull.

**Components of Bull Breeding Soundness Evaluation**

**A. Detailed medical history of bull**

The bull should be evaluated for past medical history before beginning of the BSE. The basic information includes the age, breed, identification number, vaccination given and site of administration, fever, retained testicle, hernia and incidence of chronic debilitating diseases. All these factors significantly affect the sperm production ability and quality of semen of particular bull.

**B. Physical examination**

The physical examination includes general body confirmation, eye, teeth and legs. Any physical defect may lead to decreased fertility under natural mating conditions. The bull should be free of warts, ringworm and other epidermal ailments. Vision plays an important role in identifying potentially receptive females in natural service as well as during mounting on the dummy. The eye

should be clear and free from any discharges and injuries. The teeth should be examined for age as well as wearing and missing. It is also helpful in assuring proper chewing of the food to maintain the normal body condition for breeding bulls.

The structural and conformational soundness have an important role in breeding soundness, because any leg deformities lead to lameness. The bull must be able to physically support a large portion of his weight on rear legs to accomplish copulation or ejaculation in the artificial vagina (A.V). The bull should be free from any swollen stifle and knee joints, scars around the coronary band, longed hoof and foot rot or hoof cracks. Various conformational defects like cow hock, sickle hock, crooked legs and post-leggedness, or splayed toes can lead to stifling or injuries to the feet during the breeding season.

**C. Examination of reproductive system**

It includes the examination of –

i. External reproductive organs- Testis, penis, prepuce and scrotal circumference
ii. Internal accessary glands - Seminal vesicles, prostate and ampulla for detection of any abnormalities

***External reproductive examination***

The external reproductive organs are examined by visual observation as well as palpation by hand. The testes of bull should be well placed, symmetric and freely movable within the scrotum. Any asymmetry may be an indication of orchitis, testicular degeneration, hernia or hydrocele. The testes should also be examined for consistency and size. The consistency of testes should be like flexed bicep muscle. The size of the testis is directly correlated with sperm production ability and early puberty in female. The penis and prepuce should be thoroughly examined for prolapses, lacerations, adhesions of the prepuce,

abscesses, phimosis (unable to extend the penis from prepuce), paraphimosis (unable to retract the penis into the prepuce), hematomas along the shaft of the penis and persistent frenulum.

Testicular consistency is generally estimated using tonometer, which gives an idea of consistency of testicular parenchyma. There are studies that relate the tonometer reading with quality semen production ability. It has been reported that the tonometer provides a simple quantitative means of predicting semen quality and potential fertility in dairy bulls. However, there has been no consensus that it can be used as a tool to predict bull fertility. Further investigation of the usefulness of the tonometer in evaluating breeding potential in young bulls is to be carried out.

Testicular dimensions, especially the scrotal circumference (SC) have been extensively used in breeding soundness examinations to determine the semen production ability of bulls. The relationship of testicular biometry with male reproductive traits and semen quality parameters, such as the number of normal sperm, sperm concentration, sperm motility, and total daily sperm production are very well studied. Many reports have demonstrated that scrotal circumference of bull is positively related to conception and/or pregnancy rate[3-5]. Sperm production potential of bulls is directly related to the scrotal circumference (SC), testicular volume (TV), testicular weight (TW) and shape of the testis[6]. A larger than average SC measurement has typically been related to greater testicular mass and volume, and to higher sperm production[7,8], whereas a small SC often associated with small testis, has been related to infertility[9].

However, measuring the SC only would not give a clear picture about the fertility status of bulls[10], whereas TV and TW are highly correlated to sperm production[11]. Bulls with smaller than average SC and longer testis, may have a larger TV or TM than bulls with higher SC and shorter testis[6]. Hence

combination of SC with TL and TW would give fairly reliable picture of TV and TM. SC is highly heritable[12, 13] and provides an indirect measurement of testicular weight and size, which are highly correlated to the sperm output[14]. Our experiences with crossbred bulls at NDRI also proved that, even though SC had a positive relationship with initial semen quality, combination of SC, TL, TW, TV and TM may yield superior results for the selection of crossbred bulls[11]. The Society for Theriogenology has set up certain standards for the scrotal circumference of *Bos taurus* animals (Table 1), for selecting as breeding bulls[15]. But this has not been standardized so far with respect smaller breeds like zebu and crossbred bulls, making difficult to follow in our country. So it is of utmost priority to make a uniform standard for scrotal circumferences in different breeds considering the Indian conditions.

**Table 1.** Minimum scrotal circumference requirement for HF breeding bulls

| **Age (months)** | 15 | >15-18 | >18-21 | >21-24 | >24 |
|---|---|---|---|---|---|
| **SC (cm)** | 30 | 31 | 32 | 33 | 34 |

***Internal reproductive examination***

The internal accessary glands are examined per-rectum after proper removal of dung. The prostate gland is palpated like band across the urethra and it should be free from any abnormalities. The ampulla is present on the distal part of the ductus deference and chance of any disease incidence with this gland is not normal. Seminal vesicles are present in a pair and are palpated like a bunch of grapes on either side of pelvic urethra. Any asymmetry and pain associated during palpation may be associated with vesiculitis, and obviously it is the most common diseases in bulls. The inguinal ring should also be palpated and if it is found that there is a large opening or presence of a loop of intestine through this, it may lead to breeding related issues in the future.

**D. Semen collection and evaluation**

For collection of semen from bulls, various methods are available. Electro-ejaculation is used for young bull, whereas artificial vagina is utilized in trained bulls. The electro- ejaculator having the probe connected with electric supply provides rhythmic pulsation on accessory gland to ejaculate the semen. After ejaculation the semen is evaluated for individual motility and morphology. Routine or classical subjective semen analysis using simple microscopy is used as a surrogate measure for *in vitro* determination of the fertility potential of bulls used in artificial insemination[16]. This provides an outlook of the functional testicular mechanism; hence it is routinely used in semen labs to grade the quality of semen samples or fertility status of bulls. Various procedures are being used for semen evaluation like mass activity, volume, colour, sperm concentration, consistency, individual motility, sperm viability, sperm abnormalities and sperm membrane integrity tests. But there lacks a uniform protocol for standardizing the quality of semen samples produced from different laboratories all over the world, as in the case of WHO guidelines for human semen evaluation[17]. Besides, each technique is related to different physiological and biochemical aspects of sperm qualities[18] and the results are not consistent or correlating to each other[19-21]. Furthermore, bulls that passed the BSE and routine semen evaluation shows differences in non-return rate (NRR) by 20-25%[2, 22-23]. The advanced techniques used for semen evaluation like computer assisted semen analysis (CASA) and different sperm function tests are discussed in another paper by the corresponding author of this paper.

**Endocrine profile and fertility of bulls**

It is generally believed that the libido or sexual drive in male animals is associated with the testosterone level. Since the role of testosterone on the spermatogenic process is well established, it is expected that the concentrations of testosterone have positive correlation with semen quality. But conversely, many researchers have reported that the circulating blood testosterone level is not related to libido[24, 25] and semen quality[24, 26], whereas

some studies reported positive association of testosterone with semen quality[27, 28]. In a study recently conducted at NDRI, we observed that various seminal attributes did not have any significant correlation with peripheral blood testosterone level in crossbred bulls[29].

**Testicular cytology as a tool for fertility evaluation**

Production of fertile sperm is a consequence of normal mitosis and meiosis of germ cell and proper function of germ and Sertoli cells. Since the quality of spermatozoa reflects the health of seminiferous tubule, studying the components of seminiferous tubules, especially the germ cells and Sertoli cells would give an idea about the status of seminiferous tubule, and hence the fertility status of bulls. Testicular functions can be appraised by determining various cell indices of spermatogenic cells, Sertoli cells and Leydig cells. In humans, the testicular cytogram is being evaluated through many methods, like open method, split needle biopsy, needle punch biopsy and fine needle aspiration cytology (FNAC). Compared to other techniques, testicular FNAC is considered as simple, quick and less invasive method to evaluate spermatogenesis. Representative samples obtained using testicular FNAC is routinely used to study the details of testicular cytogram and to identify the fertility status of a male in humans. But in animals very limited reports are available about these techniques, especially the use of FNAC for studying testicular cytogram. Some workers have developed certain standard indices for assessing the spermatogenic function potential of men[30, 31], as mentioned below.

**Testicular cytology indices**

Spermatic index = Total no. of spermatozoa per 100 spermatogenic cells

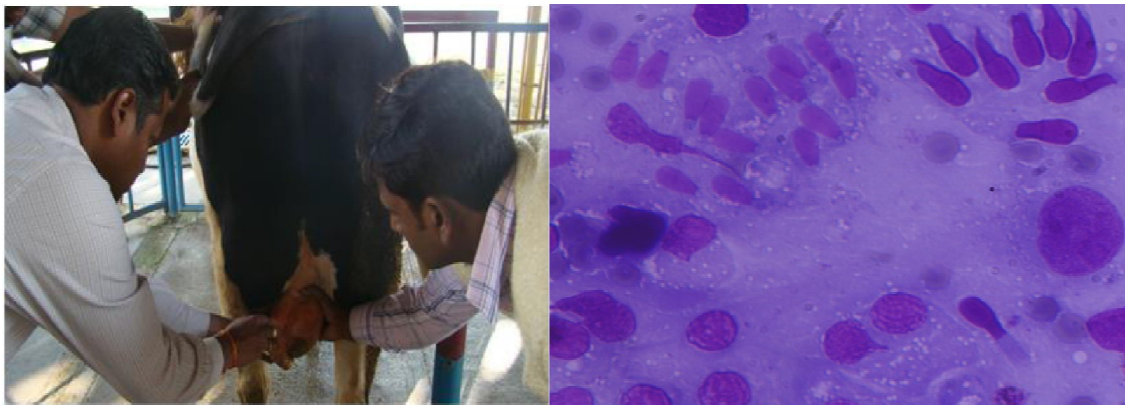
Sertoli cell index = Total no. of sertoli cell per 100 spermatogenic cells

Sperm- Sertoi cell ratio = Ratio of total no. of spermatozoa to sertoli cells

Spermatogram = Proportion of particular developmental forms of spermatogenic cells per 100 spermatogenic cells

Mitotic index = Number of spermatogenic cells undergoing division per 100 spermatogenic cells

Spermatogenic: Sertoli cell ratio = Ratio of total no. of spermatogenic cells to total no. of Sertoli cells



Percutaneous needle aspiration in bulls (Left) and cells at 100x magnification (Right)

These indices can be adopted in case of bulls also, after proper validation and standardization. Recently we standardized the technique for percutaneous needle aspiration biopsy (PNAB) in bulls and tried to find out the correlation of various cytological indices with fertility status of the bulls. We observed that in bulls with high fertility, the number of Sertoli cells, Sertoli cell index and the spermatogenic: Sertoli cell ratios were significantly higher than that of low fertile bulls[32]. Our preliminary experience supports the potential of testicular cytology and their indices in bull fertility prediction. However these results need validation in larger number of bulls and once proved this simple tool can be used in bull selection program.

**Effect of PNAB procedure on semen production and reproductive health of bulls**

We studied the SC and semen profile in bulls 4 weeks before to 4 weeks after testicular PNAB at weekly interval and observed that there was no significant difference observed in scrotal circumference and testicular length before and after the PNAB process. There was no significant changes observed in ejaculate volume (4.63±0.33 Vs 4.59±0.26), mass activity (2.32±0.33 Vs 2.44±0.30) and individual motility (53.15±4.29 Vs 58.07±4.42) in bulls during pre-PNAB and post-PNAB period. Similarly, the percentage of live spermatozoa, membrane intact spermatozoa and acrosome intact spermatozoa did not differ significantly between pre-PNAB and post-PNAB period. These results indicate that testicular percutaneous needle aspiration technique can be used as a routine diagnostic method to detect sub-fertility and infertility in crossbred bulls without affecting their reproductive health.

**Proteomic biomarkers in sperm and seminal plasma**

The advent of proteomic studies in systems biology has thrown light into many of the hitherto unanswered aspects of biological functions. Proteomics can be broadly defined as the systematic analysis and documentation of the proteins in biological samples. It provides fascinating insight in to the pathway of cellular function by systematic study of protein expressions. It can be seen as a mass screening approach to molecular biology, which aims to document the overall distribution of proteins in cells, identify and characterize individual proteins of interest, and ultimately to elucidate their relationships and functional roles. The proteomics approach has been gaining importance in andrological studies in humans and animals, for understanding the biological mechanisms of sperm function and its interaction with oocyte. Moreover, mature spermatozoa are transcriptionally inactive, so the only comprehensive method to understand the molecular functions in spermatozoa is via proteomics. Seminal plasma and sperm contains many proteins that are responsible for various functions such as motility, capacitation and acrosome

reaction, immune modulation in uterus, formation of tubal sperm reservoir, zona penetration, sperm-egg fusion and development of embryo.

Earlier, Killian *et al.*[22] identified four fertility associated proteins in the seminal plasma of Holstein bulls using 2D-PAGE technique. They reported that two proteins (26 kDa, pI 6.2; 55 kDa, pI 4.5) occurred with greater frequency and density in bulls of higher fertility and another two proteins (16 kDa, pI 4.1; 16 kDa, pI 6.7) were more prominent in bulls of lower fertility. They also developed a multi-linear regression equation to describe the relationship between the presence of the fertility associated proteins and bull fertility. Later these protein, that were over expressed in high fertile bulls were identified as osteopontin[33] (55 kDa) and Lipocaine type prostaglandin D synthase[34] (26 kDa). It was reported that bovine seminal plasma protein (BSP) 30 kDa[35, 36] and phospholipase A2[37] are more prevalent in high fertility bulls, whereas amounts of spermadhesin Z13 were inversely related to fertility[38]. Likewise, comparative proteomic analysis of spermatozoa proved that the expression levels of many proteins are related with the fertility status of animals[39-41].

Various techniques used in proteomics studies of animals are SDS-PAGE, Two dimensional electrophoresis (2D) and Difference Gel Electrophoresis (DIGE). For identification of differentially expressed proteins, Mass spectrometry methods like MALDI TOF and LC MS/MS are being used[42]. The results of the experiments are generally validated using techniques like western blotting[40]. The DIGE has an advantage over other electrophoretic techniques in terms of high sensitivity and linearity of the dyes utilized, and its significant reduction of inter-gel variability, which increases the possibility to unambiguously identify biological variability and reduces bias from experimental variation. Moreover, the use of a pooled internal standard, loaded together with the control and experimental samples, increases quantification accuracy and statistical confidence[43].

The fertility related proteomic profiling of bull spermatozoa started with the pioneer work of Peddinti *et al.*[39] in 2008. They employed differential detergent fractionation multidimensional protein identification technology (DDF-Mud PIT) and identified 125 putative biomarkers of fertility. They reported that the spermatozoa from high fertile bulls were having over expression of proteins involved in energy metabolism, cell communication, spermatogenesis, and cell motility. Later, D'Amours *et al.*[44] compared the proteomic profile of high fertile, medium fertile, and low fertile HF bulls using 2D-PAGE technique and validated the results using western blotting. Park *et al.* [40] also used the same technique for identification of fertility related proteins in bulls. Recently, Soggiu *et al.* [41] reported that some proteins are linked with sperm fertility, after analysing the sperm samples from Italian breed of bulls with varying fertility.

Recently, we (Theriogenology Lab, NDRI) along with technical collaboration from All India Institute of Medical Sciences (AIIMS), New Delhi made initiative to identify some putative biomarkers in the spermatozoa and seminal plasma of crossbred bulls having association with fertility, to formulate some measures to determine the latent fertility of bulls accurately. The study identified some potential molecules in sperm and seminal plasma of crossbred bulls, which may perform as a panel of as biomarkers of fertility[45]. Out of 18 differentially expressed proteins ($P<0.05$), 9 were over expressed in seminal plasma of high fertile bulls and 9 were over expressed in seminal plasma of low fertile bulls. The differential expressions ranged from 1.5 to 5.5 fold between the two groups, where Protection of telomeres-1 protein (POT1) was highly over expressed (2.9 fold) in high fertile group and Prostaglandin E2 receptor EP3 (PTGER3) was highly abundant (5.5 fold) in low fertile group. We also performed the proteomic analysis of spermatogenic and Sertoli cells from Taurine, Indicine and crossbred bulls and identified some potential molecules under/over expressed in the testicular cells of crossbred bulls[46].

**Epilogue:**

A major factor limiting the genetic progress in India is the limited availability of quality breeding bulls. Presently we do not have consistent and reliable markers for prediction of bull fertility. Growing an infertile bull up to the age of donating semen and then declaring the bull as infertile based on semen analysis or *in vivo* fertility trials costs huge to the farmer/semen stations. It is imperative that all the semen stations should induct advanced semen evaluation tools to grade their bulls. Testicular percutaneous needle aspiration cytology is a good tool for evaluation of the sperm production efficiency of bulls. With the advancements in "omics" era, now it is possible to identify proteomic differences in sperm and seminal plasma of bulls with varying levels of fertility. Thus, incorporating all these parameters, a uniform protocol should be formulated for the evaluation of breeding bulls, taking into consideration various breed wise differences and geographical peculiarities.

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