

# Relationship of conventional and fluorescent microscopic technique to assess *in vitro* semen quality status of Murrah buffalo males

Shivahre, P. R.<sup>1\*</sup>; Gupta, A. K.<sup>2</sup>; Panmei, A.<sup>1</sup>; Yadav, B. R.<sup>2</sup>; Bhakat, M.<sup>2</sup>; Mohanty, T. K.<sup>2</sup>; Kumaresan, A.<sup>2</sup>; Kumar, V.<sup>3</sup>; Dash, S. K.<sup>4</sup> and Singh, S.<sup>5</sup>

<sup>1</sup>Ph.D. Scholar in Animal Genetics and Breeding, Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal, 132001, India; <sup>2</sup>Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal, 132001, India; <sup>3</sup>Department of Animal Genetics and Breeding, Pt. Deen Dayal Upadhyaya Veterinary and Animal Sciences University (DUVASU), Mathura, Uttar Pradesh, India; <sup>4</sup>Department of Animal Genetics and Breeding, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India; <sup>5</sup>M.V.Sc. Scholar in Livestock Production and Management, Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal, 132001, India

\*Correspondence: P. R. Shivahre, Ph.D. Scholar in Animal Genetics and Breeding, Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal, 132001, India. E-mail: drpr06@gmail.com

(Received 25 May 2015; revised version 19 Sept 2015; accepted 5 Oct 2015)

## Summary

*In vitro* fertility assessment using fluorescent technique is a better predictor of fertility status of bulls as compared to traditional semen quality assessment techniques, therefore, the study was planned to assess *in vitro* fertility status of bulls based on conventional and fluorescent techniques. Seventy-three ejaculates were collected from 12 Murrah buffalo bulls maintained at Artificial Breeding Research Centre, NDRI, Karnal, India for the experiment and subjected to statistical analysis using SYSTAT. The mean values of ejaculate volume (ml), mass activity, individual motility (%), sperm concentration (millions/ml), live sperm (%), total abnormalities (%), HOST (%) and acrosomal integrity (%) were  $2.70 \pm 0.28$ ,  $2.8 \pm 0.14$ ,  $63.8 \pm 2.16$ ,  $1749.7 \pm 122.24$ ,  $77.3 \pm 2.48$ ,  $6.2 \pm 0.51$ ,  $75.1 \pm 1.81$  and  $84.5 \pm 2.26$ , respectively. The repeatability estimates were significant ( $P < 0.05$ ) for ejaculate volume ( $0.34 \pm 0.137$ ), acrosomal integrity ( $0.29 \pm 0.134$ ) and live percentage ( $0.28 \pm 0.133$ ), indicating sufficient bull to bull variation for the parameters. The mean values of seminal attributes of fluorescent based criteria of CMA3 (Chromomycin A3), SYBR-PI and FITC-PNA (fluorescent isothiocyanate-conjugated peanut agglutinin) were  $5.25 \pm 0.41$ ,  $67.91 \pm 1.24$  and  $82.00 \pm 1.25$  percent, respectively. Bulls were ranked on the basis of expected producing ability (EPA) for semen characteristics assessed by conventional and fluorescent criteria. Rank correlations were found to be significant for FITC with most of the parameters evaluated by conventional methods. In conclusion, among the conventional criteria, individual motility (%) revealed ranking of bulls almost similar to that of fluorescent criteria.

**Key words:** Conventional method, Fluorescent method, Murrah buffalo bull, Semen quality

## Introduction

Buffalo is the black gold of India and spread over all parts of the country with varying population density, but the majority (72%) of the milch breeds of buffaloes are found in Haryana, Punjab, Uttar Pradesh, Rajasthan, Gujarat and Maharashtra. The higher growth rate of buffalo in north and western states may be due to the increasing demand for buffalo milk, meat and superior buffalo germplasm. The strength of India lies on the supremacy in terms of the largest buffalo population (57% of the world), huge buffalo germplasm diversity and the world renowned Murrah breed of buffalo. Buffalo is a multipurpose animal and produces milk, meat and draught power, and buffalo can efficiently utilize the roughages and crop by-products into high quality milk and meat as well. Further, genetic improvement of buffaloes to achieve the desired level of production of artificial insemination (A.I.) has proven to be the most effective tool with better fertilization capacity or fertility of male animal, which can be evaluated based on the conception rates on large number

of AI, but the method is time consuming. Therefore, there is a need for alternative methods of assessing the functional capacity of spermatozoa *in vitro* to predict fertility. Amann (1989) have reported a relationship between different laboratory tests and fertility, but that is dependent on the use of accurate and specific laboratory tests with fertility data. Assessment of *in vivo* fertilizing capacity of semen becomes challenging as it is influenced not only by semen-related factors but also by female fertility and by many other factors that may or may not be determinable (Amann and Hammerstedt, 2002). Functional analysis of sperm organelles has gained importance in recent decades as conventional techniques are not able to estimate the fertility of a semen sample accurately and repeatedly (Correa *et al.*, 1997). Thus, the development of techniques that help to evaluate the functional status of sperm organelles (acrosome and mitochondria) or the integrity of cellular components (membranes and chromatin), allows an alternate approach to the problem (Graham, 2001). Therefore, it is important to conduct a comparative study to assess *in vitro* fertility status of bulls on the basis of

conventional semen quality criteria vs. recently developed fluorescent techniques of semen quality assessment.

## Materials and Methods

A total of 73 ejaculates of 12 Murrah buffalo (around 6 replicates of each bull) breeding bulls (3.0-5.0 years age group) maintained at Artificial Breeding Research Centre, NDRI, Karnal, were collected and semen quality was analyzed by conventional and fluorescent techniques. Loose housing system was followed, and bulls were kept in open paddocks with roof over mangers. This system provides adequate exercise to the animals, which are exposed to all types of climate. The average age and body weight of the bulls were 5 years and 643 kg, respectively. Semen was collected from the bull using sterile bovine artificial vagina (IVM model-005417; maintained between 42-43°C) over a male dummy in the morning, twice a week schedule.

### Conventional technique

Immediately after collection, semen was evaluated for colour, sperm concentration (by haemocytometer or Neubaur's chamber), volume, mass activity (Tomar, 1984), eosin-nigrosin staining (Barth *et al.*, 1989), HOST (Jeyendran *et al.*, 1984) and acrosome integrity (Watson, 1976).

### Fluorescent technique

Each semen sample from each bull has been analyzed with fluorescent techniques (CMA3 for chromatin integrity, SYBR-PI for plasma integrity and FITC-PNA for acrosome integrity). Chromatin damage of each sperm was quantified by fluorescent microscopy (olympus) after staining with CMA3 fluorescence stain as described by Bianchi *et al.* (1993). Chromatin damaged spermatozoa reacted positively to the above stain whereas normal spermatozoa did not show any response to the stain. About 400 spermatozoa were assessed for chromatin integrity. To assess sperm viability and membrane integrity, SYBR-14 in combination with PI (propidium iodide) was used as described by Januskauskas *et al.* (1999). In randomly selected fields at least 200 spermatozoa were analyzed twice under an epifluorescent microscope. Viable spermatozoa show fluorescent bright green colour of SYBR-14, whereas dead sperm nuclei stained red with PI. Acrosome integrity was assessed using FITC-PNA (fluorescent isothiocyanate-conjugated peanut agglutinin) by a method modified from that described by Roth *et al.* (1998). Acrosome non reacted spermatozoa react with FITC-PNA and glow bright green while acrosome reacted spermatozoa do not show fluorescence signal in acrosomal region.

### Statistical analysis

Data were analyzed using SYSTAT. The repeatability of various seminal attributes of Murrah bulls was estimated as intra class correlation from the

analysis of variance (Becker, 1986) using records of the same animal in successive collections. The standard error of repeatability was estimated by using formula given by Swiger *et al.* (1964). Expected producing ability (EPA) is a measure of future performance potential and was computed for breeding bulls for semen quality parameters using the formula given by Lush (1945). Differential number of records and repeatability of the traits are used for estimation of EPA, which is calculated based on the following formula:

$$EPA = \bar{H} + \left[ \frac{nr}{1 + (n-1)r} \right] (\bar{I} - \bar{H})$$

where,

$\bar{H}$ : Herd average for given trait (semen parameter)

$\bar{I}$ : Individual average for given trait (semen parameter)

n: No. of semen ejaculate

r: Repeatability of a given trait

The correlation between rankings of sires based on conventional and fluorescent based semen evaluation criteria were calculated by Spearman's rank correlation coefficient (Steel and Torrie, 1960).

$$r_s = 1 - \left[ \frac{6 \sum d_i^2}{n(n^2 - 1)} \right]$$

where,

$r_s$ : Spearman's rank correlation coefficient

$d_i$ : Difference between the ranking of a sire by two techniques used in semen evaluation

n: Number of sire

## Results

The results of the semen quality parameters of Murrah buffalo bulls by conventional and fluorescent technique are presented in Table 1. The mean values of ejaculate volume (ml), mass activity, sperm concentration (millions/ml), individual motility (%), live sperm (%), total abnormalities (%), HOST (%) and acrosomal integrity (%) were  $2.70 \pm 0.28$ ,  $2.8 \pm 0.14$ ,  $1749.7 \pm 122.24$ ,  $63.8 \pm 2.16$ ,  $77.3 \pm 2.48$ ,  $6.2 \pm 0.51$ ,  $75.1 \pm 1.81$  and  $84.5 \pm 2.26$ , respectively.

**Table 1:** Mean and repeatability of semen quality parameters of Murrah buffalo bulls (n=12) based on conventional technique

Parameters	Mean $\pm$ SE	Repeatability $\pm$ SE
Volume (ml)	2.70 $\pm$ 0.28	0.34 $\pm$ 0.137
Mass activity	2.8 $\pm$ 0.14	0.21 $\pm$ 0.125
Concentration (million/ml)	1749.7 $\pm$ 122.24	0.18 $\pm$ 0.120
Individual motility (%)	63.8 $\pm$ 2.16	0.26 $\pm$ 0.131
Live (%)	77.3 $\pm$ 2.48	0.28 $\pm$ 0.133
Abnormality (%)	6.2 $\pm$ 0.51	0.10 $\pm$ 0.103
HOST (%)	75.1 $\pm$ 1.87	0.16 $\pm$ 0.116
Acrosomal integrity (%)	84.5 $\pm$ 2.26	0.29 $\pm$ 0.134

The repeatability estimates of the ejaculate volume, mass activity, sperm concentration, individual motility, live sperm, total abnormalities, HOST and acrosomal

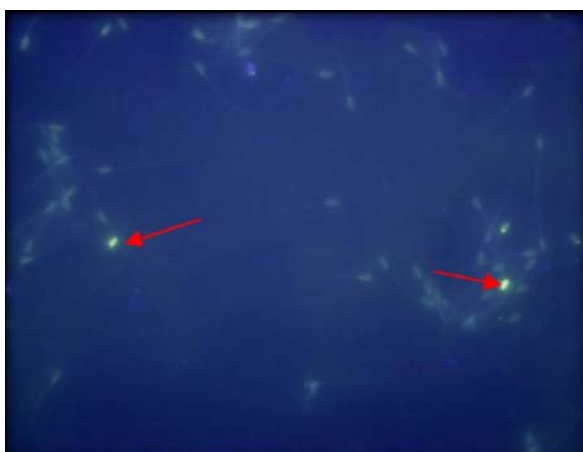
integrity were  $0.34 \pm 0.137$ ,  $0.21 \pm 0.125$ ,  $0.18 \pm 0.120$ ,  $0.26 \pm 0.131$ ,  $0.28 \pm 0.133$ ,  $0.10 \pm 0.103$ ,  $0.16 \pm 0.116$  and  $0.29 \pm 0.134$ , respectively. The repeatability estimates were significant ( $P < 0.05$ ) for ejaculate volume, acrosomal integrity and live percent, indicating bull to bull variation for the parameters.

The mean values of seminal attributes of fluorescent based criteria of CMA3 (Fig. 1), SYBR-PI (Fig. 2) and FITC-PNA (Figs. 3 and 4) were  $5.25 \pm 0.41$ ,  $67.91 \pm 1.24$  and  $82.00 \pm 1.25$  percent, respectively (Table 2).

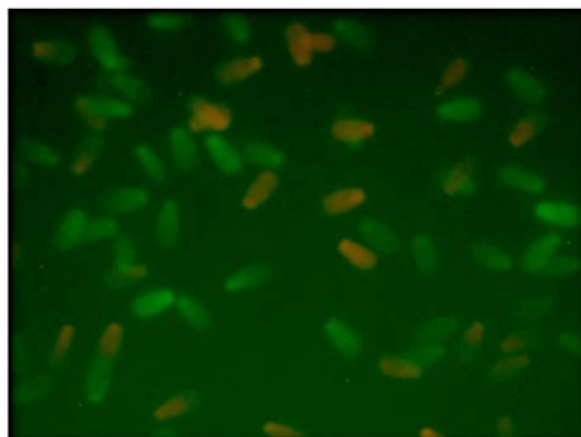
**Table 2:** Mean of semen quality parameters of Murrah buffalo bulls (n=12) based on fluorescent technique

Fluorescent criteria	CMA3 (%)	SYBR-PI (%)	FITC-PNA (%)
Mean $\pm$ SE	5.25 $\pm$ 0.41	67.91 $\pm$ 1.24	82.00 $\pm$ 1.25

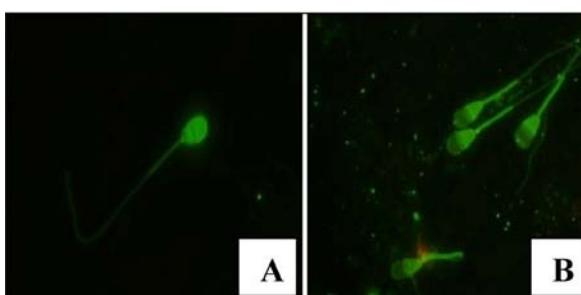
Bulls were ranked on the basis of EPA for semen quality parameters assessed by conventional and fluorescent criteria. EPA estimate of bulls are expressed as a deviation from herd mate. Rank of the bull represents the position of bull among them with respect to semen quality parameters. Rank correlations were found to be significant ( $P \leq 0.05$ ) for FITC with most parameters evaluated by conventional methods. This may be due to better accuracy of work to evaluate the semen quality parameters by both conventional and fluorescent techniques and all the tests are basically depicting qualitative picture of sperm or similar type of functional status. Overall, among the conventional criteria, individual motility (%) revealed ranking of bulls almost similar to that of fluorescent criteria (Table 3). Mass activity and individual motility are the basic criteria among the semen quality assessment to get an overall idea about the fertility. The basic semen evaluation



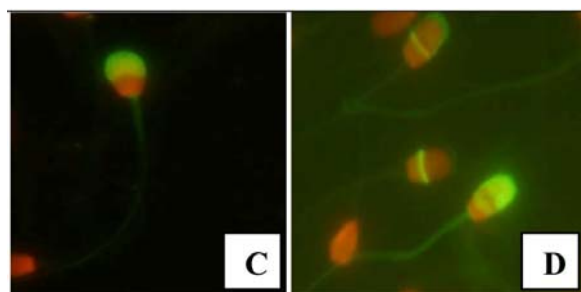
**Fig. 1:** CMA3 staining-bright spermatozoa (protamine deficient) shown with arrows



**Fig. 2:** SYBR (live-green) and PI (dead-red) spermatozoa



**Fig. 3:** FITC-PNA with PI staining. Live spermatozoa A) Non-reacted, and B) Reacted



**Fig. 4:** FITC-PNA with PI staining. Dead spermatozoa C) Non reacted, and D) Reacted

criteria of motility, which is followed in all the semen station throughout the world for cryopreservation is still important in the absence of facilities for fluorescent based semen quality assessment.

**Discussion**

Some studies have been conducted to identify fertility status of bulls by conventional and florescent based *in vitro* fertility tests in cattle bulls but in the case of buffalo

**Table 3:** Rank correlation based on ranks assigned to the bulls

Fluorescent criteria	Conventional criteria					
	MA (+)	IM (%)	Live (%)	Morph. abnormality (%)	HOST (%)	Acrosome integrity (NR) (%)
CMA3 (chromatin integrity)	0.999**	0.878**	0.556	0.339	0.692*	0.535
SYBR-PI (membrane integrity)	0.434	0.675*	0.381	0.318	0.909**	0.388
FITC (acrosome integrity)	0.640*	0.727*	0.846*	0.531	0.674*	0.888**

\* t ( $P < 0.05$ ), and \*\* t ( $P < 0.0$ )

bulls there are very few reports available therefore, in the present research, we focused on semen characteristics by conventional semen techniques and fluorescent techniques and their correlation. Similar estimates for volume were reported by Suryaprakasam and Rao (1993). However, comparatively higher values were reported by Jindal and Jain (1992), Tomar and Singh (1996) and Shukla and Mishra (2005) whereas lower values were observed by Bhosrekar (1980), Tuli (1984) and Kumar (2011). The result of mass activity was similar to those of Tuli (1984), and Tomar and Singh (1996). However, lower values were reported by Kumar (1993), Bhakat (2008) and higher values by Bhosrekar (1980) and Raizada *et al.* (1988). The findings of individual motility values of the present experiment were in consonance with these of Bhakat (2008). The present estimates were lower than the values reported by Bhosrekar *et al.* (1994), Shukla and Mishra (2005) and Kumar (2011). The present sperm concentration values were higher than those reported by Rattan (1990), Pratap (1999), Murugan and Raman (2003), Shukla and Mishra (2005), Bhakat (2008) and Kumar (2011). Higher sperm concentration may be due to all the samples being creamy and thick creamy. The values of non-eosinophilic spermatozoa were lower than those reported by Bhosrekar (1980), Bhalde *et al.* (1991) and Bhakat (2008) and higher values were found by Kumar (2011). Shukla *et al.* (2005) had reported higher values of morphological abnormality (12.57%). For HOST and acrosomal integrity, the estimates were comparatively higher than the previous finding of Bhakat (2008), and lesser than the values found by Aguiar *et al.* (1994). The slight consistency in the results may be due to variation in age, breed, geographical location, technique, instrument used, type of chemical used, sample size and seasons (Bhakat *et al.* 2009). Fluorescent based criteria of CMA3, SYBR-PI and FITC-PNA of semen represent reliable and objective methods of assessment of semen quality and provide additional information of acrosome intactness and sperm chromatin structure integrity, which cannot be assessed by routine sperm quality assessment. The fluorescent staining technique plays an important role in detecting sperm damage, which reflects the fertility, infertility and integrity of sperm (Farah *et al.*, 2013). Rajak (2012) found  $2.5 \pm 0.85\%$  CMA3 positive sperms in Karan Fries bulls, whereas Singh (2014) reported  $1.93 \pm 0.90\%$  CMA3 and  $67.08 \pm 0.97\%$  SYBR positive spermatozoa.

The results of repeatability estimates and correlation based on EPA and rank are not comparable with the findings of others as there is no published data available in literature.

From the results it can be concluded that although assessment of *in vitro* fertility of breeding bulls is more accurate on the basis of fluorescent based tests of chromatin integrity, membrane integrity and acrosome integrity, yet the results indicated that amongst conventional criteria, mass activity, individual motility (%) and HOST (%) can be fairly used in absence of fluorescent method because the conventional tests also resulted in ranking of bulls almost similar to that of

fluorescent criteria.

## Acknowledgements

The authors are thankful to the Head, DCB Division and Incharge, ABRC of NDRI, India for providing necessary facility. The authors are also thankful to the Director, NDRI, India for financial assistance provided during the research work.

## Conflict of interest

The authors declare that they have no competing interests.

## References

- Aguiar, PHP; Andrade, VJ; Abreu, JJ and Gomez, NBN (1994). Physical and morphological semen characteristics of buffaloes aged from four to eight years old. *Proceedings of the 4th World Buffalo Congress*. Sao Paulo, Brazil. 3: 486-488.
- Amann, RP (1989). Can the fertility potential of a seminal sample be predicted accurately? *J. Androl.*, 10: 89-98.
- Amann, RP and Hammerstedt, RH (2002). Detection of differences in fertility. *J. Androl.*, 23: 317-325.
- Barth, AD and Oko, R (1989). *Abnormal morphology of bovine spermatozoa*. Ames, IA, USA, Iowa State University Press. PP: 130-266.
- Becker, WA (1986). *Manual of procedures in quantitative genetics*. Pullman, USA, Publication of Washington State University.
- Bhakat, M (2008). Studies on low grade ejaculates for augmenting semen quality and preservility in dairy bulls. Ph.D. Thesis, NDRI Deemed University, Karnal, Haryana.
- Bhakat, M; Mohanty, TK; Gupta, AK and Raina, VS (2009). Effect of season and management on semen quality of breeding bulls- a review. *Agric. Rev.*, 30: 79-93.
- Bhalde, RM; Hukeni, VB and Deopaskas, VL (1991). Post thaw keeping quality of frozen bull semen maintain at room/chilled temperature. *Indian J. Anim. Reprod.*, 12: 87-90.
- Bhosrekar, M (1980). Studies on buffalo semen. *Indian Vet. J.*, 57: 806-810.
- Bhosrekar, MR; Mokashi, SP; Purohit, JR; Gokhale, SB and Mangurkar, BK (1994). Comparative study on conventional and control (programmable) freezer on the quality of buffalo semen. *Indian J. Anim. Sci.*, 64: 583-587.
- Bianchi, PG; Manicardi, GC; Bizzaro, D; Bianchi, U and Sakkas, D (1993). Effect of deoxyribonucleic acid protamination on fluorochrome staining and *in situ* nick translation of Maurine and human mature spermatozoa. *Biol. Reprod.*, 49: 1083-1088.
- Correa, JR; Pace, MM and Zavos, PM (1997). Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology*. 48: 721-731.
- Farah, OI; Cuiling, L; Jiaojiao, W and Huiping, Z (2013). Use of fluorescent dyes for readily recognizing sperm damage. *J. Reprod. Infertil.*, 14: 120-125.
- Graham, JK (2001). Assessment of sperm quality: a flow cytometric approach. *Anim. Reprod. Sci.*, 68: 239-247.
- Januskauskas, A; Gil, J; Soderquist, L; Haard, MGM;

- Haard, MC; Johannisson, A and Rodriguez-Martinez, H** (1999). Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bull semen used for artificial insemination in Sweden. *Theriogenology*, 52: 641-658.
- Jeyendran, RS; Van Der Ven, HH; Perez-Pelaez, M; Crabo, BG and Zanaveld, LJD** (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, 70: 219-228.
- Jindal, SK and Jain, G** (1992). Daily sperm output of Murrah buffalo bulls based on depletion trails. *Indian Vet. J.*, 69: 562-567.
- Kumar, A** (2011). Subfertility and its detection with fragile X-chromosome in cattle and buffalo bulls. M.V.Sc. Thesis in Animal Genetics and Breeding, National Dairy Research Institute, Karnal, Haryana.
- Lush, JL** (1945). *Animal breeding plans*. Iowa State College Press. Eleventh Printing, Ames, Iowa.
- Murugan, RT and Raman, KS** (2003). Influence of age and body weight on semen production traits in Murrah bulls. *Indian J. Anim. Sci.*, 3: 767-768.
- Pratab, N; Reddy, VN; Sharma, PA; Honnappa, TG; Devraj, M; Krishnaswamy, A and Arora, VK** (1999). Spermogram and biochemical studies in Murrah buffalo bulls. *Indian J. Anim. Reprod.*, 20: 156-158.
- Raizada, BC; Sattar, A and Pandey, MD** (1988). A comparative study of freezing buffalo semen in two dilutions. *Proceeding of 2nd World Buffalo Congress*. New Delhi. 3: 66-74.
- Rajak, S** (2012). Testicular fine needle aspiration (FNA) cytology to evaluate fertility in crossbred (KF) bulls. M.V.Sc. Thesis, NDRI Deemed University, Karnal, Haryana.
- Rattan, PJS** (1990). Physio-chemical constituents of buffalo bull semen. In: Acharya, RM; Lokeshwar, RR and Kumar, S (Eds.), *Recent Adv. Buffalo Res.*, 3: 26-30.
- Roth, TL; Weiss, RB; Buff, JL; Bush, LM; Wildt, DE and Bush, M** (1998). Heterologous *in vitro* fertilization and sperm capacitation in an endangered African antelope, the scimitar-horned oryx (*Oryx dammah*). *Biol. Reprod.*, 58: 475-482.
- Shukla, MS and Mishra, AK** (2005). Correlation between seminal characteristics in Murrah bulls. *Indian J. Anim. Sci.*, 75: 263-266.
- Singh, S** (2014). Selected management interventions and bio-stimulation to augment libido and sperm production in Sahiwal bulls. M.V.Sc. Thesis, NDRI Deemed University, Karnal, Haryana.
- Steel, RGD and Torrie, JH** (1960). *Principles and procedures of statistics*. New York, McGraw Hill Book Co., P: 481.
- Suryaprakasam, TB and Narsimha Rao, AV** (1993). Studied on breeding and disposal pattern of A. I. sire in Andhra Pradesh. *Indian Vet. J.*, 70: 1022-1024.
- Swiger, LA; Harvey, WR; Everson, DD and Gregory, KE** (1964). The variance of intraclass correlation involving groups with one observation. *Biometrics*. 20: 818-826.
- Tomar, NS** (1984). *Artificial insemination and reproduction of cattle and buffaloes*. 3rd Edn., UP, India, Saroj Prakashan Publishers Allahabad.
- Tomar, SS and Singh, SP** (1996). Studies on reaction time and score of the seminal attributes and their interrelationship in Murrah buffalo bulls. *Indian J. Anim. Res.*, 30: 49-54.
- Tuli, RK** (1984). Comparative study of various physical characteristics of buffalo, Red Dane and Holstein Friesian bull semen. *Livestock Advisor*. 9: 13-17.
- Watson, P** (1976). The protection of ram and bull spermatozoa by low density lipoprotein fraction of egg yolk during storage at 5°C and deep freezing. *J. Therm. Biol.*, 1: 137-141.