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

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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 ± 0.28, 2.8 ± 0.14, 63.8 ± 2.16, 1749.7 ± 122.24, 77.3 ± 2.48, 6.2 ± 0.51, 75.1 ± 1.81 and 84.5 ± 2.26, respectively. The repeatability estimates were significant ($P<0.05$) for ejaculate volume (0.34 ± 0.137), acrosomal integrity (0.29 ± 0.134) and live percentage (0.28 ± 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 isothiocynate-conjugated peanut agglutinin) were 5.25 ± 0.41, 67.91 ± 1.24 and 82.00 ± 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 Pl. 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:

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

where,
$\overline{H}$: 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).

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

where,
rs: Spearman's rank correlation coefficient
di: 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 ± 0.28, 2.8 ± 0.14, 1749.7 ± 122.24, 63.8 ± 2.16, 77.3 ± 2.48, 6.2 ± 0.51, 75.1 ± 1.81 and 84.5 ± 2.26, respectively.

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

Parameters	Mean ± SE	Repeatability ± SE
Volume (ml)	2.70±0.28	0.34±0.137
Mass activity	2.8±0.14	0.21±0.125
Concentration (million/ml)	1749.7±122.24	0.18±0.120
Individual motility (%)	63.8±2.16	0.26±0.131
Live (%)	77.3±2.48	0.28±0.133
Abnormality (%)	6.2±0.51	0.10±0.103
HOST (%)	75.1±1.87	0.16±0.116
Acrosomal integrity (%)	84.5±2.26	0.29±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 ± 0.137, 0.21 ± 0.125, 0.18 ± 0.120, 0.26 ± 0.131, 0.28 ± 0.133, 0.10 ± 0.103, 0.16 ± 0.116 and 0.29 ± 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 ± 0.41, 67.91 ± 1.24 and 82.00 ± 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 ± SE	5.25±0.41	67.91±1.24	82.00±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≤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 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.

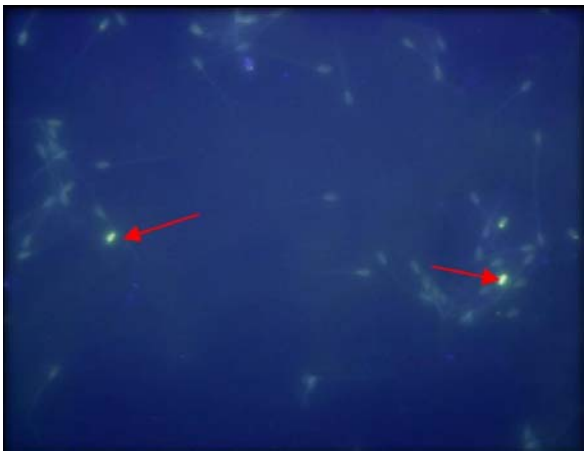

Fig. 1: CMA3 staining-bright spermatozoa (protamine deficient) shown with arrows

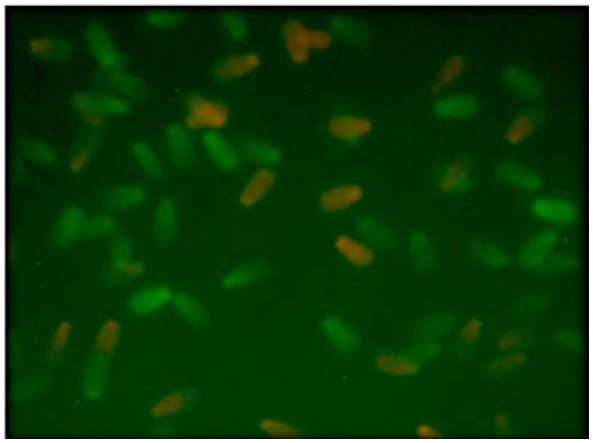

Fig. 2: SYBR (live-green) and PI (dead-red) spermatozoa

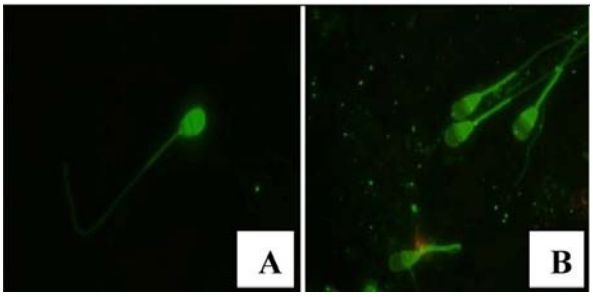

Fig. 3: FITC-PNA with PI staining. Live spermatozoa A) Non-reacted, and B) Reacted

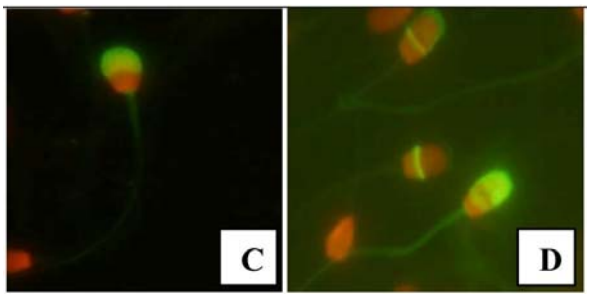

Fig. 4: FITC-PNA with PI staining. Dead spermatozoa C) Non reacted, and D) Reacted

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 florescent 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-eosiniphilic 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 ± 0.85% CMA3 positive sperms in Karan Fries bulls, whereas Singh (2014) reported 1.93 ± 0.90% CMA3 and 67.08 ± 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.

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Conflict of interest

The authors declare that they have no competing interests.

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