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Prediction of buffalo bull fertility on the basis of sperm motion traits, viability, membrane integrity, heat shock protein (HSP70) expression and fertility associated antigen (FAA)

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ABSTRACT

The present study was conducted on 20 buffalo bulls to predict the fertility on the basis of: (a) Computer assisted semen analysis (CASA) based sperm motion traits, (b) viability, (c) membrane integrity, (d) expression for HSP70, and (e) assessment of fertility associated antigen (FAA). Six frozen semen straws from each buffalo bulls were analyzed for sperm motion traits, viz. individual motility, progressive motility, average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head deviation (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and sperm size. Viability and hypoosmotic swelling tests (HOST) were also conducted. Heat shock protein 70 (HSP70) expression and quantification was conducted using a real time PCR. Fertility associated antigen (FAA) was assessed in fresh semen from buffalo bulls using test. Fertility trial was conducted on 250 normal cycling buffaloes following estrus synchronization with GnRH-PGF-GnRH protocol. It was concluded that buffalo bull fertility could be best predicted on the basis of sperm motion traits (VCL, VAP, VSL, ALH, LIN), motility, viability, HOST and HSP70 expression. However, FAA assessment in fresh semen did not indicate fertility.

Key words: Buffalo bulls, Fertility associated antigen, Fertility prediction, HSP70, Membrane integrity, Sperm motion traits, Viability

Sperm concentration, motility and morphology are the most common semen metrics used at breeding stations throughout the world. Yet these semen parameters have limited value for predicting a bull's fertility (Dogan *et al*. 2013). The advent of sophisticated instruments like computer assisted semen analysis (CASA) has increased the efficiency of sperm motion trait analysis (Sellem *et al*. 2015). However, reliability of CASA based sperm motion traits is still controversial (Arman *et al*. 2006). The live sperm count indicates membrane intactness and HOS reactive sperm detects capability of osmotic regulation across plasma membrane (Petrunkina *et al*. 2007). The HSP70 also helps in sperm oocyte interaction at the time of fertilization (Kamaruddin *et al*. 1996), sperm capacitation, zona adhesion and penetration (Asquith *et al*. 2005). However, there is little information on gene expression for HSP70 in relation to semen quality and fertility in buffalo bulls. Fertility associated antigen (FAA) positive bulls are 17–19% more fertile than their contemporary herd mates (Bellin *et al*. 1998). However, the accuracy of FAA based fertility prediction is still not validated (Park *et al*. 2012). Hence, the present work was planned to predict fertility of breeding buffalo bulls on the basis of sperm motion traits, viability, HOST, expression for HSP70 and FAA assessment.

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MATERIALS AND METHODS

Selection of buffalo bulls: Buffalo bulls (20) were randomly selected from a herd of 57 breeding buffalo bulls maintained at Government semen bank, Nabha, Punjab. Bulls were being maintained under loose housing system (covered area - 12 ft × 10 ft and uncovered area - 25 ft × 10 ft) and standard feeding schedule along with *ad lib* green fodder and standard managemental conditions. Fifty mini straws (0.25ml) from each buffalo bull frozen on the same date were collected and ear marked for study.

Evaluation of sperm motion traits: Sperm motion traits

(individual motility (%), progressive motility (%), VAP (µm/s), VSL (µm/s), VCL (µm/s), ALH (µm), BCF (Hz), STR (%), LIN (%), SIZE (µ), were analyzed through computer assisted semen analysis (CASA) at Division of Buffalo Physiology and Reproduction, Central Institute for Research on Buffalo, Hisar. Five straws from each buffalo bulls were thawed and 5µl semen was placed on the CASA slide (Leja4) and sperm motion traits were evaluated (Kathiravan *et al.* 2008).

Viability and hypoosmotic swelling test: Frozen thawed semen samples (5) were evaluated for viability through Eosin Nigrosin staining method and hypoosmotic swelling test (Kumar *et al*. 2004).

Gene expression for HSP70 in buffalo bull sperm

Washing of frozen thawed sperms with Percoll density gradient method: To eliminate the dead and abnormal sperm, frozen thawed buffalo bull sperms were washed with Percoll density gradient method (Parrish *et al.*1995). RNA was extracted from washed sperm samples by Trizol method (Pawar *et al*. 2014). The first strand cDNA was synthesized from total RNA using a kit as per the instruction manual.

Quantification of HSP70 expression by real time PCR: Total RNA extracted from sperms was reverse-transcribed and quantification of HSP70 was performed using real time PCR. Both the primers and probes were designed using the Primer Express software package. The primers and probes were as follows:

Forward primer	Reverse primer	Probe
HSP70 (Acc No U02892)		
ACGCGACGCCA AGCT	CCCCACCAGGAC CAGGT	AAGGCGCAGA TCCAC
18S (Endogenous control)		
GGTTGATCCTG CCAGTAGCATAT	TGAGCCATTC GCAGTTTCACT	CCGTGCGTAC TTAGACATG

The gene expression for the target gene (HSP70) was quantified by normalizing against the reference gene (18S; endogenous control). Briefly, following reaction mixture was prepared on ice by using Taqman Universal PCR Master mix: (a) 20× primer probe mix- 1µl; (b) 2× Taqman master mix-10 µl; (c) distilled water- 5 µl and (e) cDNA- 4 µl. The reaction mixture was dispensed in triplicate and put into the real time PCR instrument for further quantification using following reaction:

Stage 1	Initial denaturation	95°C	10 min
Stage 2	Denaturation	95°C	45 sec
Stage 3	Annealing	60°C	1 min

The above reaction was carried out for 40 cycles. Initially, even before carrying out the quantification experiment, for all genes tenfold serial dilutions were run in the study to estimate the efficiency of PCR of the primers, and the percentage efficiency ranged between 95 and 100%.

Assessment of fertility associated antigen (FAA): Fertility associated antigen (FAA) were detected in fresh semen from each buffalo bull by test. Freshly ejaculated semen (1 ml) was diluted with 1 ml of dilution buffer in a sterile dilution vial supplied with the kit, gently mixed and 4–5 drops were poured in the sample well of the Reprotest, kept on a level floor. The Reprotest was allowed to stand for 20–30 min at room temperature. A negative test for FAA was indicated by one line at 'C' position (Fig. 1) and positive test indicated by two lines at 'C' and 'T' positions (Fig. 2).

Fertility trial: Fertility trial was conducted on 250 buffaloes selected from 4 organized dairy farms in Punjab over the period of 3 years. The feeding and managemental conditions were similar in all the dairy farms. The buffaloes were examined for their body condition scores, physical problems like lameness, vaginal discharge and conditions of genitalia. Animals with physical or genital tract problems were excluded from this study. Selected buffaloes were treated with Inj. Ciprofloxacin (4mg/kg) b.i.d. for 3 days to rule out the possibility of genital tract infections. The estrus was synchronized using standard ovsynch protocol (GnRH-PGF-GnRH on day 0, 7 and 9th, respectively) followed by fixed time inseminations on 10th and 11th day, 14–16 h after the last GnRH injection. In this study, all the buffaloes were inseminated by only one person. Ten to twelve buffaloes were inseminated using frozen semen from each buffalo bull. The pregnancy diagnosis was done on day 60 post-insemination and confirmed after day 90 using ultrasonography.

Data analysis: The data in per cent values were subjected to arc sine transformation followed by analysis of Pearson correlation between dependent variable pregnancy and predictors like sperm motion traits, viability, HOST and heat shock protein expression. The backward linear regression model was applied between dependent variable pregnancy and predictors. Variables having no significant impact were excluded from the model. Further, zero order, partial and part correlations were compared. Since, there was significant covariance among independent variables, and relationship with dependent variable pregnancy was non linear, principal component analysis (PCA) with Oblimin with Kaiser Normalization was applied on correlation matrix to show the relationship of different

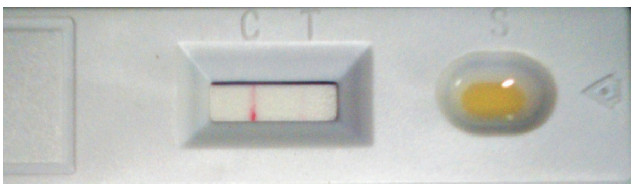

Fig. 1. Only one line at 'C' position-FAA negative.

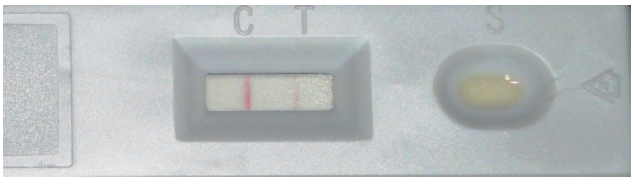

Fig. 2. Two lines at 'C' and 'T' positions-FAA positive.

components with pregnancy rate. The relationship of FAA data with pregnancy was analyzed using cross tabulation and Pearson Chi square test.

RESULTS AND DISCUSSION

The present work was carried out to predict the buffalo bull fertility on the basis of sperm motion traits, viability, membrane integrity indicated by HOST, HSP70 expression and FAA assessment. Pearson correlation coefficient between pregnancy rate and various laboratory tests are presented in Table 1. The pregnancy rate is significantly (P<0.05) correlated with motility (r=0.481), followed by sperm size (r=0.426) and straightness (r=-0.418). The prediction of pregnancy on the basis of sperm motion traits, viability, membrane integrity and HSP70 expression was analyzed through backward regression model. The backward regression analysis yielded two models and summary is presented in Table 2. All the predictor variables were entered in Model 1 and BCF was removed from Model 2 without any significant change in R^2 value indicating that BCF had not much impact in the model. The R^2 values of Models 1 and 2 were 0.914 and 0.890, respectively, which indicated that 91.4 and 89.0% of variance in pregnancy rates could be explained by various laboratory tests. The ANOVA of two regression models are presented in Table 3 (Model 1, P=0.031; Model 2, P=0.024). The regression coefficients are presented in Table 4. The unstandardized coefficients i.e. B weight for motility was 5.712, which indicated that after controlling other test parameters, a unit increase in motility would increase 5.712 units in pregnancy rate. Similarly, unstandardized coefficient B for other predictors had significant influence on pregnancy rate except LIN, Size and HSP70. The partial and part correlations between pregnancy and laboratory tests are presented in Table 4. The partial correlation between pregnancy and motility was 0.785, which indicated a correlation between 2 variables by removing the effects of other variables. The part variable between pregnancy and motility was 0.421 which indicated that 17.72% i.e. $(0.421)^2$ of variation in pregnancy was uniquely contributed by motility. To check the normal distribution of residuals that is the difference between observed and expected pregnancy, a normal P-P plot of regression standardized residual was plotted (Fig 3). Since, the residuals were not closely distributed along the diagonal, the data was considered non-normally distributed. Due to the large covariance between predictors, and non-normal

Table 1. Pearson correlation coefficients between various predictors and dependent variable pregnancy rate

Parameters	PREG	MOTILE	PROG	VAP	VSL	VCL	ALH	BCF	STR	LIN	SIZE	LIVE	HOS	HSP
PREG	1.000													
MOTILE	0.481*	1.000												
PROG	0.356	0.922*	1.000											
VAP	–0.116	0.070	0.244	1.000										
VSL	–0.204	–0.052	0.187	0.969*	1.000									
VCL	–0.112	0.039	0.158	0.971*	0.910*	1.000								
ALH	–0.102	0.092	0.125	0.866*	0.764*	0.950*	1.000							
BCF	0.095	–0.191	–0.011	–0.215	–0.021	–0.329	–0.509*	1.000						
STR	–0.418*	–0.488*	–0.226	–0.126	0.110	–0.245	–0.421*	0.721*	1.000					
LIN	–0.206	–0.283	–0.146	–0.496*	–0.308*	–0.649*	–0.793*	0.643*	0.812*	1.000				
SIZE	0.426*	0.397*	0.218	0.152	0.016	0.187	0.276	–0.287	–0.684*	–0.479*	1.000			
LIVE	0.050	0.360	0.187	0.124	0.022	0.154	0.211	–0.218	–0.348	–0.232	0.208	1.000		
HOS	–0.018	0.247	0.039	–0.328	–0.403	–0.306	–0.189	–0.332	–0.350	–0.077	0.176	0.052	1.000	
HSP	0.267	0.239	0.120	–0.299	–0.355	–0.274	–0.176	–0.258	–0.128	0.064	–0.197	0.191	0.298	1.000

*Values are significant (P<0.05).

Table 2. Regression model summary

Model	R	R square	Adjusted R square	Std. error of the estimate	Change statistics		
					R square change	F change	Sig. F change
1	0.956[a]	0.914	0.726	11.47270	0.914	4.879	0.031*
2	0.943[b]	0.890	0.701	11.99850	–0.024	1.656	0.246

*Values are significant (P<0.05). a. Predictors, (Constant), HSP, LIN, PROG, HOS, LIVE, SIZE, VSL, BCF, STR, ALH, MOTILE, VCL, VAP. b. Predictors, (Constant), HSP, LIN, PROG, HOS, LIVE, SIZE, VSL, STR, ALH, MOTILE, VCL, VAP (BCF removed from model). c. Dependent variable, Pregnancy.

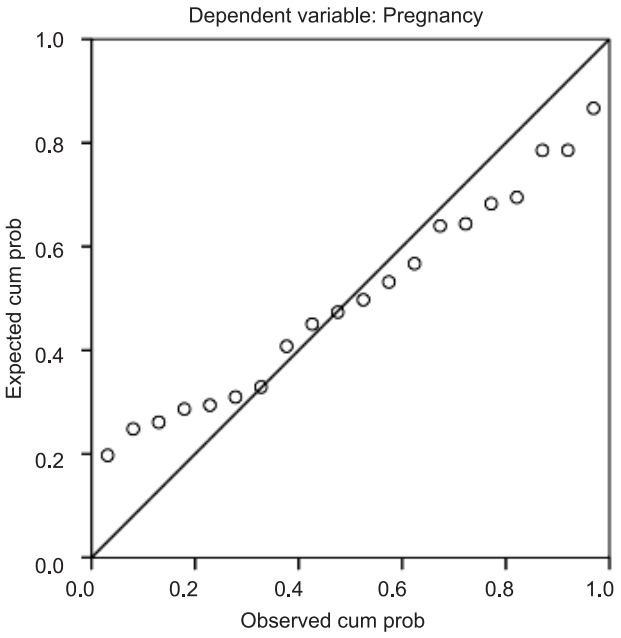

Fig. 3. Normal P-P plot of regression standardized residual.

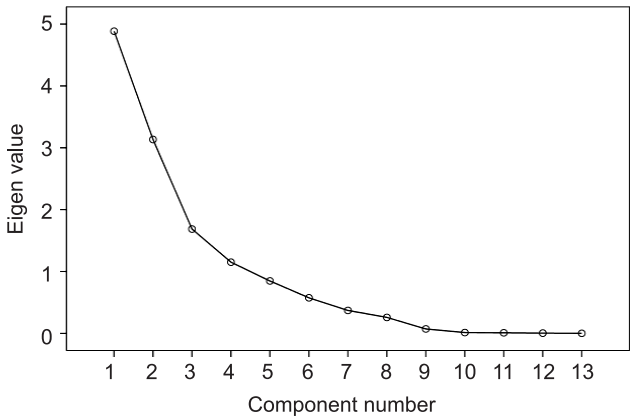

Fig. 4. Screen plot of principal component analysis.

distribution of residuals, data were analyzed and interpreted using principal component analysis.

Principal component analysis (PCA) results are shown in Table 5. The number of components extracted during PCA depends on the number of variables in the model. In our study, 13 semen evaluation parameters were included

Table 3. ANOVA in two regression models

Model	Sum of squares	Df	Mean square	F	Sig.
1. Regression Total	8347.726	13	642.133	4.879	0.031*
	9137.463	19			
2. Regression Residual Total	8129.715	12	677.476	4.706	0.024*
	1007.748	7	143.964		
	9137.463	19		4.706	0.024*

*Values are significant (P<0.05). 1. Predictors, (Constant), HSP, LIN, PROG, HOS, LIVE, SIZE, VSL, BCF, STR, ALH, MOTILE, VCL, VAP. 2. Predictors, (Constant), HSP, LIN, PROG, HOS, LIVE, SIZE, VSL, STR, ALH, MOTILE, VCL, VAP (BCF removed from model).

Table 4. Regression coefficients of pregnancy on laboratory tests in Model 2

Model 2	Unstandardized coefficients		Standardized coefficients	T	Sig.	Correlations		
	B	Std. error	Beta			Zero-order	Partial	Part
(Constant)	3334.912	1107.605		3.011	0.020			
MOTILE	5.712	1.701	4.006	3.357	0.012*	0.481	0.785	0.421
PROG	–6.566	2.434	–2.844	–2.698	0.031*	0.356	–0.714	–0.339
VAP	–39.812	14.146	–22.940	–2.814	0.026*	–0.116	–0.729	–0.353
VSL	33.685	10.925	16.820	3.083	0.018*	–0.204	0.759	0.387
VCL	9.046	3.560	10.878	2.541	0.039*	–0.112	0.693	0.319
ALH	–59.540	16.741	–3.080	–3.556	0.009*	–0.102	–0.802	–0.446
STR	–45.480	16.338	–6.247	–2.784	0.027*	–0.418	–0.725	–0.349
LIN	17.569	8.369	3.394	2.099	0.074	–0.206	0.622	0.264
SIZE	–53.580	27.998	–0.800	–1.914	0.097	0.426	–0.586	–0.240
LIVE	–1.477	0.472	–0.667	–3.125	0.017*	0.050	–0.763	–0.392
HOS	–1.409	0.364	–0.747	–3.873	0.006*	–0.018	–0.826	–0.486
HSP	28.337	12.528	0.378	2.262	0.058	0.267	0.650	0.284

*Values are significant (P<0.05). Dependent variable: Pregnancy.

and data were resolved into 13 components. The first component had highest eigen value (4.885) which accounted for the maximum variance, followed by 2nd component (3.134) and so on. This model explained 83.5% of variations in the principal components. The screen plot also indicated that first 4 components explained the maximum variations as indicated by cumulative variance (Fig. 4).

The structure matrix indicated the weight of different variables in the respective components (Table 6). In first component, the maximum impact (37.57%) was of sperm motion traits: VCL (r=0.979), VAP (r=0.974), VSL (0.952) and ALH (0.898) on pregnancy rate. Similarly, in second component, STR, BCF and LIN had 24.10% impact on pregnancy. However, motility and progressive motility had 12.9% contribution to the pregnancy as indicated by third component followed by minimum in fourth component.

Table 5. Principal component analysis between pregnancy and various laboratory tests

Component	Initial eigen values			Rotation sums of squared loadings [a]
	Total	% of variance	Cumulative %	Total
1	4.885	37.576	37.576	4.291
2	3.134	24.105	61.681	3.508
3	1.686	12.971	74.652	2.551
4	1.151	8.852	83.504	1.379
5	0.847	6.515	90.019	
6	0.574	4.419	94.438	
7	0.370	2.848	97.286	
8	0.258	1.983	99.268	
9	0.071	0.545	99.814	
10	0.012	0.094	99.908	
11	0.008	0.063	99.971	
12	0.004	0.028	99.999	
13	0.000	0.001	100.000	

Extraction method, Principal component analysis.

Table 6. Structure matrix of principal component analysis

	Components			
	1	2	3	4
VCL	0.979	-	-	-
VAP	0.974	-	-	-
VSL	0.952	-	-	-
ALH	0.898	–0.490		
STR	-	0.952	–0.417	-
BCF	-	0.831	-	–0.365
LIN	–0.525	0.816		
SIZE	-	–0.643	0.405	–0.501
HOS	–0.452	–0.464	-	-
MOTILE	-	–0.350	0.981	-
PROG	-	-	0.942	-
LIVE	-	–0.337	0.422	0.312
HSP	–0.317	-	-	0.863

Extraction method, Principal component analysis, Rotation method, Oblimin with Kaiser normalization.

The overall average individual motility (%), progressive motility (%), VAP (μm/s), VSL (μm/s), VCL (μm/s), ALH (μm), BCF (Hz), STR (%), LIN (%), size (μ), viability (%), membrane integrity (%) and HSP70 expression ($Log_{10}RQ$) were 45.20±3.43, 23.55±2.12, 94.48±2.82, 79.07±2.44, 160.06±5.89, 6.56±0.25, 35.06±0.41, 83.32±0.67, 52.68±0.94, 6.00±0.07, 67.31±2.21, 57.47±2.60 and 0.43±0.65, respectively. The overall average pregnancy rates (%) in buffaloes were 43.55±4.90. The pregnancy rates in estrus synchronized bovine females depend upon semen handling, number of sperm deposited, site of insemination, semen quality, fertilization status, embryo quality, bull effect and time of AI (Dalton *et al*. 2010). In our study, all the buffaloes were inseminated by the same person to minimize the variations due to the insemination technique, semen handling and site of semen deposition. The time of insemination after the last GnRH injection was also kept similar in all the buffaloes. Hence, the variations in the conception rates may be due to the semen quality or the bull effect in the present study. Sperm motility is related with its energy status (Quintero *et al*. 2004) and sperm with high progressive motility have more chances of fertilization (Muino *et al*. 2008). Post-thaw sperm motility is correlated (r = 0.81) with sperm penetration distance in cervical mucus (Anilkumar *et al*. 2001). In the present study, high impact of sperm motion traits in first and second component on pregnancy rate as indicated by 61.68% of cumulative variation. Mukherjee *et al*. (2006) also observed a difference in VAP, VSL, VCL, LIN, ALH in high and low fertility cow bulls. On the contrary, it was reported that sperm motility and the kinetic parameters cannot be a reliable fertility marker (Holt *et al*. 1997) as high VCL and ALH have tendency to follow a curved path and might not reach at the site of fertilization. Changes in sperm motion traits (VAP, VSL, VCL and ALH) are less sensitive indicators of frozen semen quality than the motility and progressive motilty (Arman *et al*. 2006). The decline in motility characteristics (VAP, VSL, VCL and ALH) is relatively much smaller than the fall in per cent individual and progressive motility of sperm during processing or storage (Arman *et al*. 2006). In our study, sperm size also influenced pregnancy rates in second component. Larger sperm head have higher progressive motility (Irvine *et al*. 1994) and higher fertility (Muino *et al*. 2008). Sperm viability and membrane integrity indicated by HOS was associated with fertility as in first and second component, respectively. Viability and HOST has been associated with semen quality (Zodinsanga *et al*. 2015). HSP70 expression was also associated with bull fertility as explained in 1st component. Expression for HSP70 is associated with sperm motility (Lewis *et al*. 1996), cryofreezing stress (Spinaci *et al*. 2005), intracellular protein homeostasis (Shi *et al*. 1998), protection from oxidative stress (Fukuda *et al*. 1996) capacitation (Asquith *et al*. 2005). Hence, our study

indicates that sperm motion traits (VCL, VAP, VSL, ALH, LIN), HOST, motility, viability and HSP70 are indicators of buffalo bull fertility.

The Reprotest has been devised as a chute side test for the identification of FAA in bull semen. In our study, FAA was investigated through Reprotests in the fresh semen of buffalo bulls. The cross tabulation and Chi square test indicated no significant difference (P=0.576) between the FAA negative (20%) and FAA positive (80%) buffalo bulls. In our study, FAA did not indicate fertility in buffalo bulls. Several authors reported similar incidence of FAA in cow bulls (Bellin *et al*. 1998, McCauley *et al*. 2004). Hence, it was concluded that buffalo bull fertility could be predicted on the basis of sperm motion traits (VCL, VAP, VSL, ALH, LIN), motility, viability, HOST and HSP70 expression. However, FAA assessment did not indicate fertility.

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