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Profiling of sperm gene transcripts in crossbred (*Bos taurus x Bos indicus*) bulls

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ABSTRACT

Crossbred cattle in some sectors of the world have a significant role in enhancing milk production thereby enhancing the per capita milk availability as a human food source. However, there are certain constraints associated with crossbred animals, such as disease susceptibility, increased reproductive problems, repeat breeding and poor seminal quality. The semen of crossbred bulls has a poor freezing capacity, increased cryo-damage, poor mass cell motility, greater percentages of dead/abnormal sperm and poor initial and post-freeze cell motility. The rejection rate of crossbred bulls for cryostorage of semen has been reported to be as great as 50% as a result of unacceptable semen quality. The identification of superior bulls using molecular technologies is needed which necessitates identification of the genes having a role in sperm function. The present study was, therefore, conducted to gain information on identification and expression of genes having a role in sperm motility in crossbred bulls. The gene transcripts in bulls with sperm of superior and inferior quality were profiled in Vrindavani crossbred cattle by microarray analyses and the results were verified by real time-quantitative PCR. Microarray analyses revealed 19,454 genes which were differentially expressed. At a two-fold cut off, 305 genes were differentially ($P < 0.01$) expressed with 160 genes upregulated and 145 genes down regulated. Some of the upregulated candidate genes were further validated by RT-qPCR. These genes had a four to 16 fold upregulation in sperm with inferior motility as compared to sperm of crossbred bulls with superior motility.

1. Introduction

Conception requires transport of sperm, initially in the male and subsequently female reproductive tract. To assess the fertilizing ability of males/bulls/sires, the semen ejaculate is evaluated for various physical variables such as volume, color, consistency, concentration, percent live sperm, percent sperm abnormalities, acrosomal integrity, mass sperm motility, initial progressive sperm motility and post thaw sperm motility, before initiation of a breeding program. Among the different seminal variables, sperm motility is foremost in determining a bulls' fertilizing capacity. However, mere visual evaluation of semen sam-

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ples may not be sufficient to assess the fertility potential of bulls. Numerous *in vitro* analyses methods have been proposed which determine the fertilizing capacity of ejaculates (Rodriguez-Martinez, 2006; Rodriguez-Martinez and Barth 2007). The sperm motility is controlled by many genes that have a role in flagellar structure, energy metabolism, mitochondrial functions and ion exchange channels. With the rapid progress made in genomics, the profiling of mRNA transcripts in sperm could be a rapid technology for the assessment of semen's fertilizing potential.

Crossbred (*Bos taurus* x *Bos indicus*) cattle, have a major role in the White Revolution in India that relates to increased milk production thereby enhancing the per capita milk availability. However, there are certain constraints associated with crossbred animals such as greater disease susceptibility, increased reproductive problems, repeat breeding and poor seminal quality (Venkatasubramanian et al., 2003; Dhanju et al., 2006; Martin et al., 2012). The semen of crossbred bulls has a compromised freezing capacity, increased cryoinjuries, poor mass sperm motility, a greater percentage of dead/abnormal sperm and less initial and post-freeze cell motility (Dhanju et al., 2006; Ghosh et al., 2007). The rejection rate of crossbred bulls for cryopreservation of semen has been reported to be as great as 50%, owing to their unacceptable semen quality (Mathew et al., 1982; Sahni and Mohan, 1988; Rao and Rao, 1991; Kumar, 2006; Ghosh et al., 2007). The crossbred cattle population in India is 39.732 million (Livestock Census, 2012) indicating more crossbred bulls need to be used that have superior quality semen to satisfy the need for increasing the crossbred cattle population. This identification of bulls that have greater semen cryopreservation capacity using molecular techniques is needed and identification of the genes that have a role in sperm function could help to address this need. The present study was, therefore, conducted to gain information on identification and expression of genes having a role in sperm motility of crossbred bulls.

2. Materials and methods

2.1. Animals

The present investigations were conducted on bulls (*Bos taurus* x *Bos indicus*) of composite breeding which were developed and given the name, Vrindavani, at the Indian Veterinary Research Institute, Izatnagar, India. The Vrindavani cattle are the crosses of Holstein Frisian, Brown Swiss, Jersey (exotic breed) with Hariana (indigenous breed). The exotic inheritance level of these crosses is between 50% and 62.5%. These bulls were maintained at the Germplasm Centre (GPC) of the institute.

2.2. Semen collection and evaluation

Semen was collected from the bulls during the early morning hours, using an artificial vagina maintained at 42–45 °C. Immediately after collection, each ejaculate was kept in a water bath (Memmert, Minitube International, Germany) and maintained at 37 °C and subjected to volume and mass sperm motility evaluation. For motility evaluations, 10× and 40× magnifications occurred using a phase contrast microscope fitted with a thermostatically controlled warm stage at 37 °C (Motic B1 Series, Motic Asia, Hongkong).

Mass sperm motility (MM) of the semen was recorded by placing a small drop of freshly collected neat semen on clean grease free, pre-warmed glass slide at 37 °C and the sample was examined without cover slip under low power magnification (10X). The mass motility of sperm was graded on a 0–5 scale based on the presence of waves and swirls in the sample (Salisbury et al., 1978). After diluting the fresh semen with Tris egg yolk glycerol extender at 37 °C, initial progressive motility of sperm was assessed subjectively (scale = 0% to 100%), by placing a drop of diluted semen on a glass slide covered with a cover slip and viewing fields under high magnification (40×). The motility was recorded as the percentage of progressively motile sperm and it was designated as initial progressive motility (IPM). Post thaw motility of sperm (PTM) of frozen semen was assessed after thawing the semen at 37 °C for 30 s and placing a drop of semen on a slide and covering it with a cover slip at 37 °C with high magnification (40X) and recording the motility as a percentage of progressively motile sperm. These seminal variables were estimated to identify normal (good) and motility impaired (poorly motile) sperm producing bulls. The 55% IPM and 35% PTM values were considered as the threshold/criterion for classifying the semen as freezable (good) or non-freezable (poor). The semen samples which had a value above the threshold were considered as good/freezable whereas below the threshold, were designated as poor/non-freezable semen. Based on semen variables, the two most desirable and two worst bulls were shortlisted for subsequent microarray analyses. Semen samples for RNA isolation from four shortlisted/selected bulls were collected in sterile and DEPC-treated eppendorf tubes and transported to laboratory at 4 °C in an icebox containing ice packs.

2.3. Sperm purification

To ensure RNA isolation exclusively from normal sperm, it was imperative to discard the somatic cells, immature/damaged sperm and other contaminants from the semen. To achieve this, the semen samples were purified with BoviPure™ (Nidacon International, Gothenburg, Sweden). Semen samples were layered gently on three volumes of pre-warmed (37 °C) Bovipure™ in a 15 mL centrifuge tubes. These were then centrifuged at 1500*g* for 30 min at room temperature (18–24 °C). The supernatant was discarded and the sperm pellet obtained at the bottom of the tube was topped up with 8 mL 1X PBS (Ambion, USA). Similar centrifugations were again conducted on the sperm pellet. Another wash with 1 mL 1x PBS was performed and the sperm pellet was suspended in 0.5 mL 1 X PBS and mixed with 0.5 mL RNA Later (Ambion, USA) and stored at −80 °C until the isolation of RNA.

2.4. Isolation of total RNA from sperm

Isolation of total RNA from bull sperm was conducted by using TRIzol Reagent (Invitrogen, USA). The purified sperm samples were washed with 1x PBS to enable removal of RNA later. The final suspension of sperm pellet in PBS was accomplished in such a way that each sample had a sperm concentration of 400×10^6. Equal volumes of the sperm suspension were used for RNA isolation from each bull. This suspension was then mixed with pre-heated TRIzol (60 °C) and passed through 24G needle using a 20 mL syringe for 25–30 times. This was followed by homogenizing the mixture using a tissue homogenizer (HG300, MRC Laboratory Equipments, Israel) for 3 min to ensure complete dissociation of the sperm membranes. The mixture was then incubated for 30 min at room temperature. The RNA isolation was performed as per Das et al. (2010) with slight modifications. The quality of RNA was analyzed by gel electrophoresis, NanoDrop spectrophotometer (NanoDrop1000, Thermo Scientific, USA) and Bioanalyzer (Experion, Bio-Rad, USA). The total RNA concentrations of the samples and ratios ($OD_{280/260}$ and $OD_{260/230}$) were also evaluated using a Nanodrop spectrophotometer. The samples classified as having good motility were considered as 'control' while the poor motility samples were considered as 'case' in a case-control study format.

2.5. cDNA synthesis and determination of genomic DNA contamination

The cDNA was synthesized by using the total RNA isolated from sperm. The RNA was transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, USA) as per the manufacturer's recommendation employing the M-MuLV Reverse Transcriptase and Random Hexamers. Contamination of the RNA samples by genomic DNA was assessed by PCR amplification of protamine 1 (*PRM1*: Accn No. NM_174156.2) gene using intron-spanning primers (forward: AGA TAC CGA TGC TGC CTC AC and reverse: GTG GCA TGT TCA AGA TGT GG) and a bovine genomic DNA was used as a positive control. The PCR was performed in a total volume of 25 μL with 1X Taq buffer, 200 μM of dNTP mix, 1.5 mM $MgCl_2$, 6 pico moles each of forward and reverse primers specific to PRM1, 1U of *Taq* DNA polymerase, 100 ng of cDNA and quantum sufficient nuclease free water. The PCR was conducted in a Thermocycler (iCycler, Bio-Rad, USA) and the thermal profile comprised of an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 58.8° C for 45 s and extension at 72 °C for 45 s, followed by one cycle of final extension at 72 °C for 10 min. This amplification produces an amplicon of 235 bp for cDNA and 315 bp for genomic DNA. The sperm cDNA samples that were free from genomic DNA contamination were stored at −20 °C until use. However, in case of genomic DNA contamination, DNase-I treatment (Ambion, USA) was performed as per the manufacturer's recommendations.

2.6. Linear amplification of RNA

The total RNA samples, if confirmed for the absence of genomic DNA contamination, were subjected to linear amplification of RNA by using Ramp Up Two Round Linear Amplification kit (Genisphere, Hatfield, PA, USA) following manufacturer's instructions. This was required to multifold enhance the quantity of RNA, so as to enable its use in microarray application.

2.7. Microarray experiment and analyses

Microarray analyses were performed by outsourcing (iLife Discoveries, Gurgaon, India). This was conducted on a bovine microarray with a 4×44 K slide format (Agilent Technologies, Santa Clara, CA, USA) and with a dye swap (cyanin 3 and cyanin 5). The total RNA obtained from two sperm sample of bulls classified as good (highest motility) was pooled to serve as controls. Similarly the semen sample from two bulls classified as poor (poorest motility) was pooled to serve as case, under the case-control study. There were 1500 ng of RNA taken as the starting material for the microarray experiment with three technical replicates. The differences in gene expression were analyzed with a *t*-test. The fold change ($P < 0.01$) in gene expression in case samples was evaluated and genes having the greatest fold changes were short listed and further validated by real-time PCR to further confirm the relative expression.

2.8. Real time gene expression analyses

Real time-quantitative PCR of selected genes was performed to validate the microarray results in another set of bulls comprising two bulls that had sperm that were classified with good motility from a freezing capacity perspective and two sperm samples that were classified as having a poor freezing capacity. With some sperm samples where the total RNA yield was small, the cDNA was prepared using a QuantiTect® whole transcriptome Kit (Qiagen, Germany). The abundance of transcripts of selected genes in sperm of bulls that were classified as having good (normal) and poor (impaired motility) sperm motility were investigated by Real Time- quantitative PCR (Stratagene MX 3005P qPCR Systems, Agilent Technologies, Santa Clara, CA, USA) in a case-control format using three technical replicates from each cDNA sample. The primers for each gene for RT-qPCR were designed using the Primer Express software. The primer matrix and primer efficiency were calculated to ascertain optimum titer of primers and template.

The mRNA abundance was expressed as relative amounts of transcript using the B_2M gene as an endogenous control. For determination of mRNA abundance, the PCR reaction was conducted in duplicates using 2 x concentrated SsoFast™ Eva Green® Supermix (Bio-Rad, USA), which is a ready-to-use reaction cocktail that contains Sso7d-fusion polymerase and all other necessary components except primers and template. As per the manufacturer's recommendation, 0.5 μL of cDNA template, 5 μL 2x Eva Green® Supermix, 0.25 μL each primers and 4 μL Nuclease Free Water were mixed in the final reaction vol-

Table 1
Least square means (±SE) of mass sperm motility (MM), initial progressive motility (IPM) and post thaw motility (PTM) in ejaculates of Vrindavani bulls donating good (freezable) and poor (non freezable) quality semen at the Germ Plasm Centre.

Semen Quality	*n*	MM (0–5)	IPM (%)	PTM (%)
Freezable	173	$3.23^{a} \pm 0.03$	$64.95^{a} \pm 0.68$	$44.10^{a} \pm 0.52$
Non Freezable	48	$1.94^{b} \pm 0.09$	$40.88^{b} \pm 1.34$	$28.02^{b} \pm 1.72$

n: Number of ejaculates; Means with dissimilar superscript within a column indicate the significance difference at $P < 0.01$.

ume of 10 μL. All reactions were performed in triplicate for accuracy and the mean C_t value was calculated. Samples without cDNA (no template control: NTC) as a negative control were also included in each PCR assay. Absence of amplification in NTC was indicative of specificity and complete lack of contamination. Samples were quantified using the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008). The gene expression values were normalized against values for the *B_2M* gene, an endogenous 'housekeeping' gene or internal control, allowing the comparison of samples independent of the amount of total cDNA that was used.

2.9. Statistical analyses

Generated data with respect to sperm motility variables were analyzed using SAS 9.3 statistical software (SAS Institute, Cary, NC, USA). Significance of differential expression patterns of genes under study between samples from bulls with semen classified as being of good and poor quality was estimated using the student *t*-test. The microarray data analysis was performed by using the GeneSpring GX 11.5 (Agilent Technologies, USA).

3. Results

3.1. Bull categorization and evaluation of semen variables

Ejaculates (221), obtained from 13 Vrindavani bulls were analyzed qualitatively for sperm motility (MM, IPM and PTM) by SAS 9.3 software. These were then grouped into good (normal/freezable) and poor (impaired motility/non freezable) quality samples based on MM, IPM and PTM scores (Table 1). The freezable and nonfreezable ejaculates were 173 and 48 respectively. The overall MM, IPM and PTM averaged $2.96 \pm 0.04\%$, $59.96 \pm 0.76\%$ and $40.61 \pm 0.71\%$ respectively. The MM, IPM and PTM in the group with greater freezing capacity were greater ($P < 0.01$) than from the semen samples from bulls with poor freezing capacity. Samples for the two most desirable bulls and two poorest bulls with regard to semen freezing capacity (Table 2) were selected for the microarray study. The analysis of variance revealed that all the motility variables from samples of bulls with good freezing capacity sperm were greater ($P < 0.01$) than those from bulls with poor sperm freezing capacity. The means of these sperm variables within groups were non-significantly different from each other. For real time gene expression analysis, another set of bulls comprising two bulls with sperm of good and two with sperm of poor freezing capacity were used (motility data not presented).

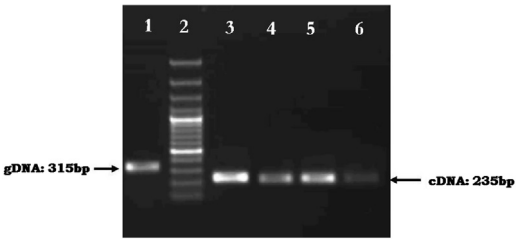

Fig. 1. Verification of RNA samples free from gDNA contamination by PCR using intron spanning primers specific to PRM1 gene; Lane 1 (Positive Control): An amplicon of 315 bp specific to genomic DNA, Lane 2: M, Molecular Ladder (100 bp) and Lane 3 through 6: 235 bp amplicon from cDNA with PRM1 primers.

3.2. RNA extraction and quality assessment

Total RNA from sperm of four selected bulls was extracted and quality was analyzed by gel electrophoresis, Nanodrop spectrophotometer (ND1000, NanoDrop Technologies, Inc. USA) and Bioanalyzer (Agilent 2100, USA). The RNA samples with an $OD_{260/280}$ ratio ranging from 1.60 to 2.00 and RNA integrity (RIN) values ranging from 6.7 to 9.2 were considered for downstream analysis. Samples with the desired OD ratios were analyzed for quality/purity to ensure utmost purity of the sample for qPCR. The purity of isolated sperm RNA samples was analyzed by PCR using intron-spanning primers specific to PRM1 gene. These primers produced an amplicon of 235 bp that was an estimate of the purity of isolated RNA sample (Fig. 1). A sample contaminated with genomic DNA would have also had an amplified 315 bp fragment of genomic DNA, along with the 215 bp fragment of cDNA. Sperm cDNA samples that were free from genomic DNA contamination were used for further downstream experimentation.

3.3. Microarray experiment and data analyses

Each array on the bovine microarray slide, in the $4 \times 44k$ format, contained probes specific to 21,535 genes. The total number of probes per array were 45,220 hence it provided $\geq$2 probes per gene. The pooled sperm RNA from bulls that had sperm that were classified as good or poor motility were subjected to hybridization with three technical replicates for each dye swap. The sperm RNA obtained from bulls that had sperm that were classified as good served as the control and samples from bulls with sperm that were classified as poor were used to make comparisons.

In the present study, microarray analysis revealed the expression of 19,454 genes (of 21,535 genes available) in the sperm of crossbred Vrindavani bulls. With comparison of the abundance of RNA between samples from bulls with good and poor sperm motility classifications, 305 genes were found to be differentially expressed ($P < 0.01$). Considering the two fold cut off, 160 genes were upregulated ($P < 0.01$) and 145 genes down-regulated ($P < 0.01$) in samples for bulls with poor sperm motility classifications compared with those with good sperm motility classifi-

Table 2
Least square means (±SE) of mass sperm motility (MM), initial progressive motility (IPM) and post thaw motility (PTM) in ejaculates of Vrindavani bulls donating good (freezable) and poor (non freezable) quality semen, selected for microarray.

Sl. No.	Category of Bull (Bull no.)	*n*	MM (0–5)	IPM (%)	PTM (%)
1.	Bull 1 (1034- Good Sperm Quality)	18	$3.94^{a} \pm 0.20$	$73.88^{a} \pm 2.27$	$48.88^{a} \pm 1.96$
2.	Bull 2 (402- Good Sperm Quality)	11	$2.36^{a} \pm 0.15$	$61.36^{a} \pm 2.53$	$39.27^{a} \pm 1.56$
3.	Bull 1 (969–Poor Sperm Quality)	14	$1.21^{b} \pm 0.11$	$27.14^{b} \pm 2.26$	$12.50^{b} \pm 1.36$
4.	Bull 2 (693 – Poor Sperm Quality)	10	$1.33^{b} \pm 0.16$	$23.33^{b} \pm 2.04$	$11.22^{b} \pm 2.22$

n: Number of ejaculates; Means with similar superscripts within a column do not differ ($P < 0.01$) from each other.

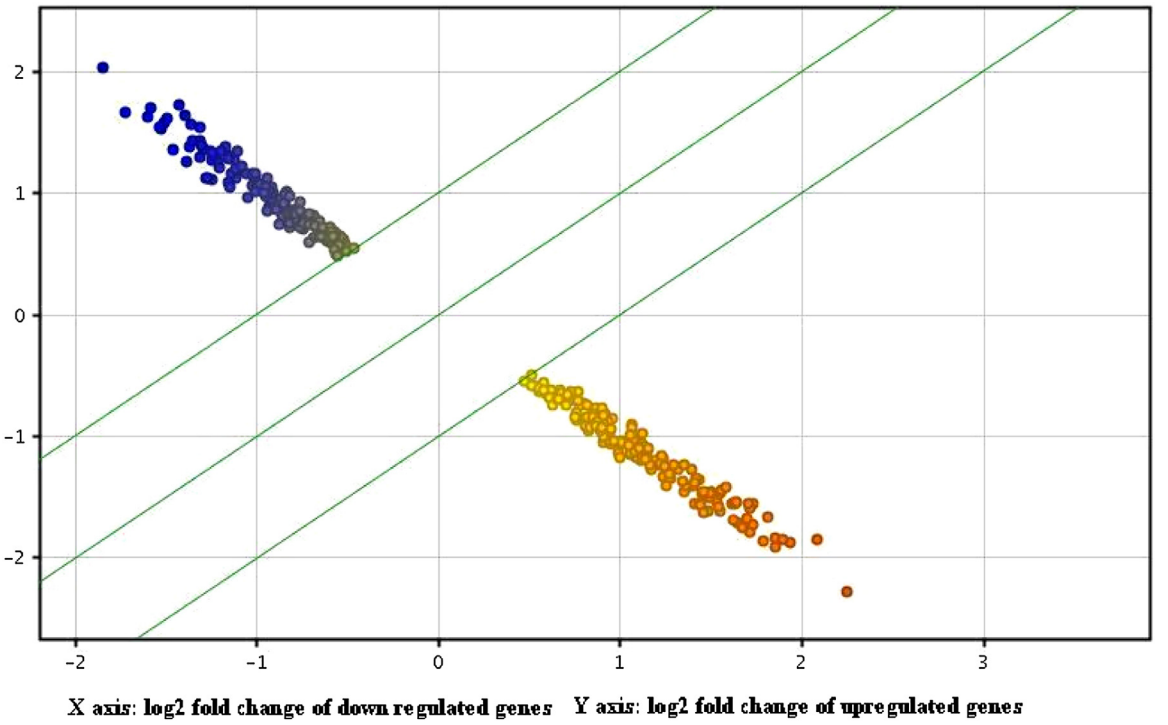

Fig. 2. Scatter plot diagram indicating the spots (genes) that are up (blue) or down (yellow) regulated with a cutoff of two fold ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Primers designed for RT-qPCR validation of top ten upregulated genes as well as the internal control gene (B2 M) along with their length and accession numbers.

Sl #	Primer Name	Sequence (5′-3′)	Bases	Accession Number
1.	FABP3-F	GGG CAA TAT GAC CAA GCC TAC CA	23	NM_174313.2
2.	FABP3-R	CGT CAA CCA TCT CCC GCA CAA GTG	24	
3.	CYB5R4-F	TGC CTT GGT CAT CCC CAG GAG AA	23	NM_001038159.1
4.	CYB5R4-R	TAG CCT GAG GTA AAC GTG TTG CCC A	25	
5.	CCT5-F	CAC AGG AGT GGT TGT GCT GGC T	22	NM_001034595.2
6.	CCT5-R	CCG TCT GGA TCA GGG GCT CAG T	22	
7.	GUK1-F	AGG ACC AAG GCC CGT TGT CCT G	22	NM_174078.3
8.	GUK1-R	GCA ATG TCG CGC TGC ATC ACC	21	
9.	PACSIN3-F	ATC GGC CTG TGC TGG GTG GCT T	22	NM_001191420
10.	PACSIN3-R	CGC TGT CTG CCT TTG CGT GGC TCT	24	
11.	CTRB1-F	CTG TGG GGT CAG GAA GGG TCA CCT C	25	NM_001105454
12.	CTRB1-R	TTC AGC AGG GCC ACG TCG TTG C	22	
13.	LAPTM4B-F	TCT GCC GTT CGC AAG CAG GAG C	22	XM_602449
14.	LAPTM4B-R	TGA GGT CGC TCG GGG TTG TTG C	22	
15.	SRMS-F	TTC CTC GTC CGG CCC AGT GAG A	22	NM_001192544
16	SRMS-R	AGT GGG CTC TGG ATC AGC TTC CAG	24	
17.	ISCU-F	CCG AAA CGT GGG GTC CCT TGA C	22	NM_001075683.1
18.	ISCU-R	ACC CAT TCG GTG GCT AAC GAG C	22	
19.	PJA1-F	AAG AGG AGC CGA TCA CCG TTT TCC	24	XM_002700048.2
20.	PJA1-R	TGA CAC TCG CTC AAC AGG CCC A	22	
21.	B2M-F	GCT GTC TGG ACT GGA CGC CAT CCA	24	XM_001251107.3
22.	B2M-R	TGC TGT TGG GAG TGA ACT CAG CGT G	25	

Table 4
Thermal profile employed for the amplification of cDNA samples from bull sperm in real time –qPCR for the ten candidate genes as well as the internal control gene (B2 M).

Segment	Temperature	Time	Number of cycles
Pre-Melt/RT	95 °C	30 s	1
Amplification	95 °C	10 s	40
	Annealing temperature (T_A) – *CTRB1, CYB5R4, PACSIN3* (60 °C) *SRMS, B2 M* (62 °C) *CCT5, FABP3, GUK1, ISCU, PJA1* (52 °C) *LAPTM4 B* (53 °C)	15 s	
	72 °C	20 s	
Dissociation/Melting	95 °C	60 s	1
	2 °C more than Annealing temperature (T_A) of respective gene	30 s	
	95 °C	30 s	

cations (Figs. 2 and 3). To confirm these results, a real time-qPCR assay on selected genes was performed. The present report pertains to genes that were upregulated in samples from bulls classified as having poor sperm motility as compared to those classified as having good motility that produced the strongest signal in the array.

The top ten upregulated genes were Fatty Acid Binding Protein 3 (*FABP3*), Cytochrome b5 reductase 4 (*CYB5R4*), Chaperonin containing T-complex polypeptide 1, subunit 5 (*CCT5*), Guanylate kinase 1 (*GUK1*), Protein kinase C and casein kinase substrate in neurons 3 (*PACSIN3*), Chymotrypsinogen B1 (*CTRB1*), Lysosomal-associated transmembrane protein 4B (*LAPTM4B*/LOC524129), SRC-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (*SRMS*), Iron-sulfur cluster scaffold, *E. coli*, homolog of (*ISCU*) and Praja Ring Finger 1 (*PJA1*). The primers for each gene for RT-qPCR were designed using the Primer Express software (Table 3). The amplification conditions and annealing temperature were optimized (Table 4). The gene expression was further validated by real time qPCR assay.

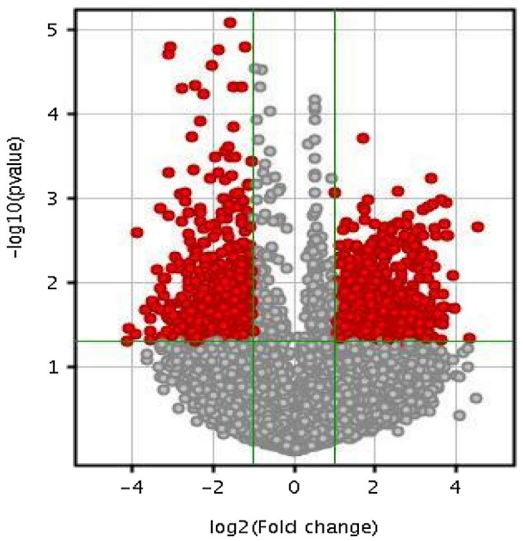

Fig. 3. Volcano Plot diagram indicating the spots (genes) that are up (right side, red spots) or down (left side, red spots) regulated with a cutoff of two fold ($P < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Real time expression profiling of selected genes

The short listed genes were real time expression profiled using the cDNA in samples from bulls classified to have poor sperm motility as compared to that of bulls with samples classified to have sperm with good motility keeping B_2M gene as the internal control in another set of bulls. Hence, the abundance of mRNA was expressed as relative abundance of transcripts using the B_2M as reference gene. The *FABP3, CYB5R4, CCT5, GUK1* and *PACSIN3* genes were upregulated ($P < 0.01$ and $P < 0.05$) with a 16–4 fold increase in samples from bulls with sperm classified as having poor as compared with samples of bulls classified as having good sperm motility. However, *CTRB1, LAPTM4 B (LOC524129), SRMS, ISCU* and *PJA1* genes exhibited <4 to 2 fold increase in samples of bulls characterized as having poor sperm motility as compared to those characterized with good sperm motility though statistically there was no significance (Fig. 4). Hence, the RT-qPCR results for all the selected candidate genes confirmed the microarray results.

4. Discussion

Fatty Acid Binding Protein 3 (*FABP3*) had the greatest (approx. 16 fold) up regulation ($P < 0.01$) in sperm of bulls with poor motility as compared to sperm with good motility (control). *FABP3* is a member of intracellular FABPs family that was reported to facilitate intracellular transport of long chain fatty acid and participate in cell growth by regulating the expression of specific genes during the synthesis and remodeling of membrane polar lipids in spermatogenesis (Haunerland and Spener, 2004; Furuhashi and Hotamisligil, 2008). This is essential for male fertility. Expression of *FABP3* gene was reported in testis (Watanabe et al., 1991) and it was detected only in small amounts in germ cells (Gerardo et al., 2013). This gene also exhibited a similar pattern of upregulation ($P < 0.01$) when a different reference gene, ACTB (beta actin), was used (Yathish, 2015).

Cytochrome b5 reductase 4 (CYB5R4) gene had the second greatest upregulation with over a 12 fold ($P < 0.01$) increase in transcripts levels. NADH-dependent enzyme

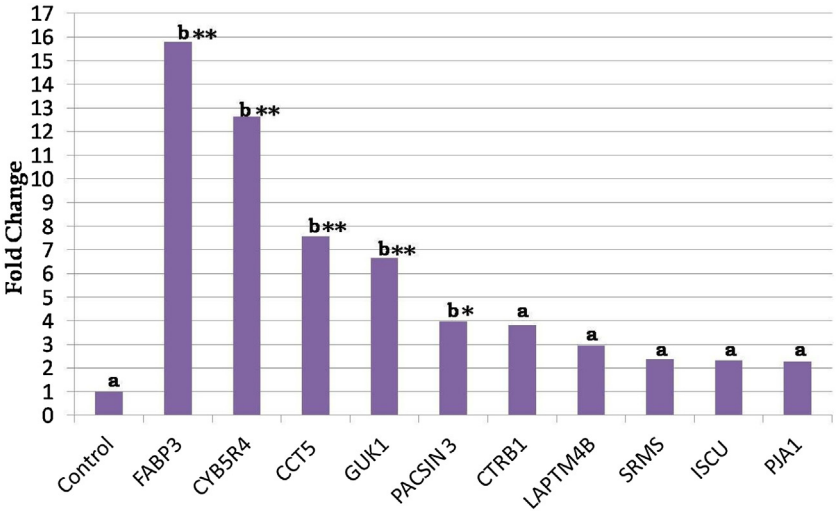

Fig. 4. Relative abundance of transcripts of different candidate genes in Vrindavani bull sperm with low motility as compared to those with high motility (control); Genes with different superscripts (a compared with b) differ with respect to control. **&* indicates significance at P < 0.01 and P < 0.05 respectively.

encoded by *CYB5R4* gene has a role in protecting cells from oxidative stress (OS), possibly by protecting the cell from excess buildup of reactive oxygen species (ROS). Therefore, *CYB5R4* is the crux of regulating stress induced ROS production in sperm. Sperm spontaneously produce a variety of ROS like superoxide (SO), anion, hydrogen peroxide (HP) and nitric oxide (Aitken et al., 2010). These ROS, when produced in greater amounts, causes ATP depletion leading to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability. Imbalance between ROS production and antioxidants i.e. oxidative stress in semen can lead to sperm damage, deformity and eventually male infertility. In addition, OS affects the integrity of sperm genome as a result of greater frequencies of single- and double-strand DNA breaks (Mostafa et al., 2001; Saleh and Agarwal, 2002; Wang et al., 2003; Smith et al., 2006) and is implicated as the major etiological factor leading to sperm DNA damage (Agarwal et al., 2008). In male reproduction, ROS are known mostly for the detrimental effects on sperm functions. Nitric Oxide (NO) clearly has a detrimental effect on both motility and the competence of sperm to attach with zona (Wu et al., 2004). Aziz et al. (2004) had found greater percent of motile sperm in semen with less ROS than in semen with greater ROS production. Also, they reported a negative correlation between sperm ROS production and percent sperm motility ($r = -0.5$, 95% CI = −0.67 to −0.26; $P < 0.0001$). This ROS induced reduction in sperm motility may be due to decreased ATP production (Armstrong et al., 1999), ATP utilization, or through actions on the contractile apparatus of flagellum (Guthrie and Welch, 2012). CYB5R4 gene also had a similar pattern of upregulation when a different reference gene, ACTB (beta actin) was used (Yathish, 2015).

Chaperonin containing T-complex polypeptide 1, subunit 5 (*CCT5*) is also known as CCT-Epsilon (*CCTE*). CCT5 protein is a molecular chaperone that is a member/subunit of 950-kD Chaperonin Containing T-complex polypeptide 1 (TCP1) complex (CCT), which is involved in proper folding of cytoskeletal proteins in an ATP dependent manner. Presence of three CCT subunits, including *CCT5* has been reported in ejaculated sperm from fertile humans (De Mateo et al., 2007). The *CCT5* protein was found to be located on sertoli cells while comparing the testicular proteomes from newborn, young adult and aged men and it was proposed that *CCT5* has a role on germ cell maturation in humans (Liu et al., 2015). In the present study, the expression of the *CCT5* gene is seven fold upregulated ($P < 0.01$) in the sperm of bulls characterized to have poor motility. Similar to the present study, *CCT5* gene was expressed in sperm from Holstein Friesian bulls characterized to have poor fertility as compared to sperm from highly fertile bulls (Olivier et al., 2010). Because CCT subunits are transported from sperm cells during typical spermiation processes, greater *CCT5* gene expression in sperm samples from bulls with lesser motile sperm and with poor fertility indicates the incomplete occurrence of processes involving these proteins during spermatogenesis (Olivier et al., 2010).

Guanylate kinase 1 (*GUK1*) catalyzes the reversible phosphoryl transfer from adenosine triphosphate (ATP) to guanosine monophosphate (GMP) to yield adenosine diphosphate (ADP) and guanosine diphosphate (GDP). Guanylate kinase catalyzes the phosphorylation of either GMP to GDP or dGMP to dGDP and is an essential enzyme in nucleotide metabolism pathways (Brady et al., 1996). In the present study, expression of the *GUK1* gene was six fold upregulated ($P < 0.01$) in bulls characterized to have poorly motile sperm.

Protein kinase C and casein kinase substrate in neurons (*PACSIN3*) is a member of PACSIN protein family involved in synaptic vesicular membrane trafficking and regulation of dynamin-mediated endocytosis, ultimately controlling the cell surface expression of transmembrane proteins (Qualmann et al., 1999; Modregger et al., 2000; Qualmann and Kelly, 2000; Anggono et al., 2006). *PACSIN3* protein precisely affects endocytosis of *TRPV4* protein, thereby modulates the sub-cellular localization of ion channel (Cuajungco et al., 2006). Therefore, *PACSIN3* has a role in

endocytosis and regulates the internalization of plasma membrane proteins. Over expression of *PACSIN3* impairs internalization of *TRPV4* protein and thereby increases the amounts of *TRPV4* protein in cell membrane. This *PACSIN3* protein inhibits the *TRPV4* calcium channel activity. Expression of *PACSIN3* was detected in many cell types and tissues of all vertebrates (Sumoy et al., 2001) and in testes also. In the present study, the expression of *PACSIN3* gene was four fold upregulated ($P < 0.05$) in sperm of bulls that were characterized to have very little cell motility.

Chymotrypsinogen B1 (*CTRB1*) encodes a protein which is a member of serine proteases family. *CTRB1* protein is secreted as an inactive precursor and is activated by proteolytic cleavage with trypsin. This protein has a molecular function of serine-type endopeptidase activity. *CTRB1* protein was greatly methylated in 24 cell lines across 13 tissue types examined by mass spectrometry (Lu et al., 2014). The consistent methylation of *CTRB1* in humans occurs in many tissues and cell lines (Lu et al., 2014). Therefore, *CTRB1* has a possible role as an internal controlling molecule in methylation studies and is also a marker for the efficiency of bisulfite conversion (Lu et al., 2014). Expression of *CTRB1* gene is reported in reproductive organs like testes, placenta, uterus, ovary, prostate and more specifically in seminiferous tubule, testis germ, testis interstitial and leydig cells. In the present study, expression of the *CTRB1* gene was four fold upregulated (not significant; $P > 0.05$) in sperm from bulls characterized to have poorly motile cells.

Lysosomal-associated transmembrane protein 4B (*LAPTM4B*) is a type III transmembrane protein with four transmembrane regions. *LAPTM4B* is a novel protein associated with the proliferation and differentiation of cells (Hogue et al., 2002; Hogue et al., 1996). This protein has biological functions of forming membranous, membrane-bound or membrane-associated, structures for directional transportation of substances such as macromolecules, small molecules, ions, electrons across into, out of or within a cell, or between cells, or within a multicellular organism at some cell site such as a transporter or pore. Regulation of *LAPTM4B* gene expression, in response to hormones, ion fluxes, growth factors and stress signals, is under the control of cAMP Responsive Element Binding Protein-1 (Zhang et al., 2013). cAMP molecule, independent of protein kinase A (PKA), activates the cation (calcium) channels present in the cell membranes. These calcium channels present in sperm membranes and calcium are known to regulate both activated and hyperactivated motility of sperm during capacitation (Turner, 2006; Ho et al., 2002). Analysis using RT-PCR revealed the greatest expression of *LAPTM4B* gene in the uterus, followed by testis and skeletal muscle. Northern blot analysis for LAPTM4B mRNA in several cattle tissues revealed itsgreatest levels in the fetal ovary, testis, adrenal gland, liver and uterus (Ndiaye et al., 2015). Also, the expression of *LAPTM4B* gene during ovarian follicle development and ovulation was studied using semi-quantitative RT-PCR and it was found to be differentially expressed ($P < 0.003$) in the dominant ovarian follicle as compared with the small follicle, ovulatory follicle and corpora lutea (Ndiaye et al., 2015) which suggests a potential role of *LAPTM4B* in the growth of ovarian follicles. In individuals affected with prostate cancer, the expression of *LAPTM4B* gene was found to be greater than in the control group (Zhang et al., 2014). In the present study, expression analysis by RT-qPCR revealed a three fold up-regulation of *LAPTM4B* gene in sperm cells from bulls characterized to have poorly motile sperm. The present study is apparently the first to find the expression of *LAPTM4B* gene in sperm of bovines.

SRC-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (*SRMS*) belongs to a family of nonreceptor tyrosine kinases. The *SRMS* protein lacks a C-terminal regulatory tail but possesses an extended N-terminal region. This N-terminal region regulates the enzymatic activity of the protein (Goel et al., 2013). In the present study, sperm from bulls characterized to have poorly motile sperm, have an expression of *SRMS* gene that was two fold upregulated.

Iron sulfur cluster assembly enzyme/iron-sulfur cluster scaffold which is an *E. coli*, homolog of *ISCU* encodes the iron-sulfur (Fe-S) cluster scaffold protein involved in iron-sulfur assembly (Foster et al., 2000; Hoff et al., 2002). Fe-S clusters are major components of respiratory chain that define electron transport pathways in numerous membrane bound and redox enzymes (mitochondrial and cytosolic enzymes), and constitute the redox-active centers of ferredoxins (Johnson et al., 2005; Lill and Muhlenhoff, 2008). Assembly of Fe-S clusters requires scaffold proteins, such as *ISCU*, as well as cysteine desulfurases, iron donors, and chaperones (Li et al., 2006). Iron-sulfur (Fe-S) clusters are necessary for several mitochondrial enzymes and other subcellular compartment proteins. ISCU gene encodes two isomeric forms, *ISCU*1 and *ISCU*2, of Fe-S cluster scaffold protein. The probable role of this gene lies in energy production and conversion. Expression of *ISCU* gene was different ($P < 0.01$) among sperm with X and Y chromosomes and is greater in sperm with the X chromosome which implies a greater requirement of energy in these gametes (Xiaoli et al., 2014). The present study is the first to compare expression of the *ISCU* gene in differentially motile sperm. In the investigation, expression of the *ISCU* gene was found to be two fold upregulated in sperm from bulls that were characterized to be poorly motile.

Praja Ring Finger 1 (*PJA1*) gene encodes an enzyme that has E2-dependent E3 ubiquitin-protein ligase activity. This enzyme belongs to a class of ubiquitin ligases that include a RING finger motif and it can interact with the ubiquitin-conjugating enzyme UBCH5B (Yu et al., 2002). Sasaki et al. (2002) found that mouse *PJA1* binds to the C-terminal necdin (NDN) homology domain of DLXIN1 (MAGED1) *in vitro* and *in vivo*. Overexpression of the *PJA1* gene caused a decrease in DLXIN1 protein, which was reversed by including a proteasome inhibitor. Northern blot analysis by Ping et al. (2002) has revealed a 2.7-kb transcript of *PJA1* in all brain regions that were evaluated. In the present study, the expression of the *PJA1* gene was two fold upregulated in sperm from bulls that were characterized to have poor cell motility though differences were non-significant ($P > 0.05$).

Researchers have observed the polymorphism and real time expression in candidate genes such as cation channel of sperm 1 and 2 (CatSper 1 and 2), Protamine 1 and 2 (PRM 1 and 2), Transient nuclear protein 1 and 2 (*TNP 1 and*

2) and Sperm Adhesion Molecule 1 (*SPAM 1*) in Vrindavani bulls as well as various other crossbred bulls. *CatSper 1*, *PRM 1*, *PRM 2*, *TNP 1* and *TNP 2* were upregulated in bulls with poor sperm motility (Geetha et al., 2011, 2013; Geetha and Kumar, 2014; Sivakumar, 2014; Yathish, 2015) whereas *CatSper 2* and *SPAM 1* were down regulated in sperm of bulls with poor cell motility (Sivakumar, 2014; Yathish et al., 2014). However these genes were not observed in top ranking genes, upregulated in bulls characterized to have poor sperm motility in the present study.

5. Conclusion

In the present study, microarray analysis revealed the differential expression of 19,454 genes in Vrindavani crossbred bull sperm that were characterized to have sperm with poor motility as compared to those characterized to have sperm with high motility. At a cutoff of two fold, 305 (160 up and 145 down regulating) genes had a differential expression ($P < 0.01$). The top ten upregulated genes, were subjected to realtime qPCR assay, wherein these genes had the same pattern of expression as resulted from microarray analysis. The FABP3, CYB5R4, CCT5, GUK1 and PACSIN3 genes were upregulated ($P < 0.01$ and $P < 0.05$) with a 16 to four fold increase in sperm from bulls characterized to have poor motility as compared with those characterized to have sperm with greater motility.

Conflict of interest

There is no conflict of interest.

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