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Chhaya Rani
Ph.D. Scholar, Animal
Genetics Division, ICAR-
Indian Veterinary Research
Institute, Izatnagar, Bareilly,
Uttar Pradesh, India

Umesh Singh
Dean, S. G. Institute of Dairy
Technology, BASU, Patna,
India

TV Raja
Principal Scientist, ICAR-
NDRI, Karnal, Haryana,
India

Sushil Kumar
Principal Scientist, Cattle
Genetics & Breeding, ICAR-
CIRC, Meerut Cantt, Meerut,
Uttar Pradesh, India

Achintya Kumar Das
Principal Scientist, Cattle
Genetics & Breeding, ICAR-
CIRC, Meerut Cantt, Meerut,
Uttar Pradesh, India

Siddhartha Saha
Principal Scientist, Cattle
Physiology & Reproduction,
ICAR-CIRC, Meerut Cantt,
Meerut, Uttar Pradesh, India

Megha Pandey
Scientist, Cattle Physiology &
Reproduction, ICAR-CIRC,
Meerut Cantt, Meerut, Uttar
Pradesh, India

AK Mohanty
Director, ICAR-CIRC, Meerut
Cantt, Meerut, Uttar Pradesh,
India

Corresponding Author:
Chhaya Rani
Ph.D. Scholar, Animal
Genetics Division, ICAR-
Indian Veterinary Research
Institute, Izatnagar, Bareilly,
Uttar Pradesh, India

Identification of significantly expressed up and down regulated genes in categorised crossbred bulls

Chhaya Rani, Umesh Singh, TV Raja, Sushil Kumar, Achintya Kumar Das, Siddhartha Saha, Megha Pandey and AK Mohanty

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Abstract

Fertility issues and sperm abnormalities can be associated with RNAs found in bovine ejaculates. Currently, bull fertility cannot be fully predicted by the quality parameters that are regularly utilized for semen evaluation. Therefore, the uncertain and questionable predictability of the currently available semen diagnostic procedures encouraged us to search for a sperm transcript-based method for crossbred bull fertility prediction. Keeping in view the importance of transcriptome profiling of bull spermatozoa between high and low fertile Frieswal bulls and their correlation with fertility, the proposed study was designed to generate the spermatozoal transcriptomics profiles of Up and down regulated genes categorised crossbred bulls. The study was carried out at ICAR - CIRC, Meerut Cantt (UP), India; a unit of ICAR-AICRP on Cattle. The RNA-Seq profiling results revealed transcripts for 23,987 genes; of which 498 transcripts were significantly differed ($\log_2 FC \pm 2$, p -value < 0.05) between high and low fertile crossbred bull spermatozoa. Out of these 498 genes, 227 transcripts were significantly upregulated (fold change > 2) and 271 were significantly downregulated (fold change < 2) between these groups. Furthermore, identifying the fertility related genes that are specific to these groups can help to discover the potential marker for selection of superior bulls for semen quality traits. Since the study is limited by the small sample sizes, larger sample sizes can be preferred for future validation investigations.

Keywords: Crossbred, gene ontology, RNA sequencing, semen

1. Introduction

Around the world, 150 million rural households (representing 750 million people) are involved in milk production, primarily in developing nations (FAO, 2018) ^[1]. Due to heterosis, where the crossbred animals are more robust and economically efficient in comparison to the parental breeds (Pedersen and Christensen, 1989) ^[2], interest in crossbreeding has grown over the past few decades in many countries. Since the selection and upgrading of a large number of non-descript cattle could not bring about the anticipated increase at the production level in previous decades, crossbreeding of local/Indigenous cattle with exotic dairy breeds was adopted as an alternative to meet the milk requirement of India's expanding population.

Globally, a significant proportion of reproductive failure is attributable to bull subfertility due to poor semen quality (DeJarnette *et al.*, 2004) ^[3]. Generally, fertility is more vital in the case of bulls compared with cows, because one bull might be used for breeding nearly up to 40 cows through natural service or possibly hundreds of thousands using artificial insemination (Khan *et al.*, 2018) ^[4].

Researchers investigated the physiological and molecular variations between crossbred and purebred males to comprehend the causes of the increased frequency of infertility/subfertility in crossbred males. The exact causes of the greater prevalence of infertility/subfertility in crossbred males, however, are still unknown. Analysis and thorough documentation have been carried out on the differences between crossbred and purebred males in terms of testicular histology, endocrinology, and molecular structure. To evaluate the vitality, motility, concentration, acrosomal integrity, and morphology of semen, many assays have been developed (Amann and Hammerstedt, 2004) ^[5]. However, bull fertility cannot be accurately predicted using the quality indicators that are frequently employed for semen examination.

Since this characteristic poses a significant economic barrier to the livestock enterprise, an improved method to evaluate male reproductive potential would be very beneficial.

In light of this, this study presents a thorough examination of variations in the gene expression between spermatozoa from high and low-fertile crossbred bulls.

2. Materials and Methods

Cryopreserved semen straws of Frieswal bulls ($n = 04$) categorized into good quality bulls (with conception rate $> 45\%$) and poor quality (with rejection rate $> 65\%$) bulls with their satisfactory semen quality phenotypes were obtained from Institutional Semen Freezing Laboratory, ICAR-Central Institute for Research on Cattle, Meerut, Uttar Pradesh, India. All the bulls were maintained using similar management practices. Vaccination, deworming, regular check-up for communicable diseases, and other herd-health programs were imposed as per the farm schedule, and the bulls were free from major infectious diseases. Spermatozoa from high- (HF) and low-fertile (LF) bulls ($n = 02$ samples) were individually subjected to high-throughput RNA-seq analysis to catalogue differentially expressed transcripts as detailed below. RNA isolation from the categorized bull spermatozoa samples was done using TRIzol (Invitrogen, USA) as per the manufacturer's instructions with minor modifications (Parthipan *et al.*, 2015) [6]. Quality and quantity of the purified RNA samples were determined using Spectrophotometer and fluorometer Qubit quotation system (Life Technologies, USA), respectively. Final libraries were quantified using a Qubit 4.0 fluorometer using a DNA HS assay kit and were sent for RNA sequencing. Paired-end sequencing protocol from the sperm samples of four bulls was carried out to generate around 50 million reads of length 150 bp per sample using NOVASEq 6000 (Neuberg Supratech Research Laboratories (NSRL), Ahmedabad, Gujarat, India).

2.1. Bioinformatics analysis

The raw data generated from the twelve samples were evaluated for the read quality using FastQC (Babraham Bioinformatics, UK) and the reads were processed with Cutadapt tool. All the processed reads were then pooled and aligned to the bovine genome (ARS-UCD2.0/Btau9) using HISAT2 and the samples were sorted with aligned sequences using Samtools (Galaxy Version 2.0.2). The mapped and properly paired sequence to the reference genome was calculated based on tabular descriptive statistics dataset tool Flagstat. The number of reads corresponding to each transcript were obtained using the FeatureCounts software. For obtaining the significantly differentially expressed genes (DEGs) along with their Log2 fold change values, DESeq2 package of R software was used. Based on the Log2 fold change values, the transcripts with absolute log2 fold change ± 2 and p -value < 0.05 were considered as differentially expressed and subjected to gene ontology (GO) analysis, and pathway enrichment analysis.

2.2. Gene ontology and KEGG pathway analysis

Gene ontology (GO) classification of sperm transcripts was done using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources v6.8 (<https://david.ncifcrf.gov/>) and categorized as molecular function (MF), biological process (BP), cellular component (CC), and Kyoto Encyclopedia of Genes and

Genomes (KEGG) pathway. The top BPs, CCs, and MFs based for both up and downregulated genes are given in table 1 & 2. All the analysis were performed using *Bos taurus genome* as background.

3. Results

With the use of Illumina NOVASEq 6000 RNA sequencing, the total reads identified for the good and poor quality bulls were 26,139 and 25,194, respectively. A total of 23,987 transcripts were differentially expressed in the categorized crossbred bull spermatozoa, of which 498 genes significantly differed ($\log_2 FC \pm 2$, p -value < 0.05) between the groups. Of these 498 genes, 227 were significantly upregulated ($\log_2 FC > 2$) and 271 were significantly downregulated ($\log_2 FC < 2$) between the high and low fertile groups. The volcano plot showing significantly up and downregulated transcripts with p -value < 0.05 and $\log_2$ fold change ± 2 is shown in Figure 1. The top 5 upregulated transcripts were OR3A4, LOC100847415, NSMCE3, LOC132346647, and CLDN4. The top 5 down regulated transcripts were TMEM92, FLCN, LOC785907, GSC2, and PARS2.

3.1 Gene Ontology Analysis

Additionally, the total transcripts of interest showing differential expression (the set of 498), including known (identified transcripts) and unknown genes (hypothetical transcripts). There were a total of 421 known transcripts and the rest were unknown. Gene ontology (GO) classification of sperm transcripts using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources v6.8 identified 600 biological processes (BP), 212 cellular components (CC), and 239 molecular pathways (MF) for all the significantly differentially expressed transcripts.

For significantly upregulated transcripts, Gene ontology analysis revealed 18 biological processes (BP), 3 cellular components (CC), and 6 molecular pathways (MF) whereas for significantly downregulated transcripts, revealed 15 biological processes (BP), 9 cellular components (CC), and 3 molecular pathways.

3.2 KEGG Pathway analysis

KEGG pathway of identified upregulated transcripts was related to Olfactory transduction, Chemical carcinogenesis - receptor activation, Chemical carcinogenesis - DNA adducts, and Viral life cycle - HIV-1. KEGG pathways related to downregulated transcripts were Phagosome, and Herpes simplex virus 1 infection. The details of the KEGG pathway of Up and down regulated transcripts are given in Table 3 and 4. Pathway enrichment of genes specific to Up and down regulated sperm transcripts are given in Table 4.7 and 4.8.

4. Discussion

Profiling of spermatozoal transcriptomes is an accurate tool to assess male fertility and the success of assisted reproductive technologies. Spermatozoal transcriptome profiling is a reliable method for evaluating male fertility and the efficacy of assisted reproductive technologies. Therefore, spermatozoal transcript profiling has been done in a variety of species, including humans (Sendler *et al.*, 2013) [7] to evaluate the function of spermatozoal RNA in fertility. The quantity of differentially expressed

spermatozoa transcripts in semen from crossbreds bulls was investigated in the present study. A total of 23,987 transcripts were differentially expressed in the categorized crossbred bull spermatozoa, of which 498 genes significantly differed ($\log_2FC \pm 2$, p -value < 0.05) between the groups. Of these 498 genes, 227 were significantly upregulated ($\log_2FC > 2$) and 271 were significantly downregulated ($\log_2FC < 2$) between the high and low fertile groups. The number of genes differentially expressed in the categorized crossbred bull spermatozoa is comparatively higher than those received by Selvaraju *et al.* (2017) [8] and Raval *et al.* (2019) [9]. In contrast, Card *et al.* (2013) [11] only found 6,166 transcripts in bovine sperm. The contributing

factors to these variations could be the season of sample collection (Godia *et al.*, 2019) [12], spermatozoa state (fresh or frozen) (Chen *et al.*, 2015; Card *et al.*, 2017; Singh *et al.*, 2019) [13, 10, 14], RNA isolation technique (Parthipan *et al.*, 2015; Fraser *et al.*, 2017) [6, 15], integrity of sperm RNA, RNA sequencing device (Selvaraju *et al.*, 2017) [8], and library preparation techniques (Mao *et al.*, 2014) [16].

Our analysis is limited to only gene ontology and KEGG pathways. However, identifying the relevant genes and finding their association with fertility or male reproduction can contribute to the development of molecular markers and strategies for improving sperm quality, managing sperm-related diseases, and increasing bovine fertility.

Table 1: Gene ontology analysis of upregulated transcripts

	Term	Count	%	P Value
BP	GO:0007186~G protein-coupled receptor signaling pathway	18	8.490566	0.007999
	GO:0001525~angiogenesis	5	2.358491	0.00904
	GO:0007420~brain development	5	2.358491	0.011228
	GO:0060021~roof of mouth development	3	1.415094	0.02031
	GO:0030154~cell differentiation	7	3.301887	0.039665
	GO:0030238~male sex determination	2	0.943396	0.040808
	GO:0009303~rRNA transcription	2	0.943396	0.040808
	GO:0006935~chemotaxis	3	1.415094	0.040894
	GO:0031115~negative regulation of microtubule polymerization	2	0.943396	0.046501
	GO:0000122~negative regulation of transcription by RNA polymerase II	9	4.245283	0.049994
	GO:2000353~positive regulation of endothelial cell apoptotic process	2	0.943396	0.052161
	GO:0060122~inner ear receptor cell stereocilium organization	2	0.943396	0.068941
	GO:0007218~neuropeptide signaling pathway	3	1.415094	0.074939
	GO:0008104~protein localization	3	1.415094	0.076649
	GO:0045944~positive regulation of transcription by RNA polymerase II	8	3.773585	0.078268
	GO:0071353~cellular response to interleukin-4	2	0.943396	0.079963
	GO:0051607~defense response to virus	4	1.886792	0.089558
	GO:0048469~cell maturation	2	0.943396	0.096255
CC	Term	Count	%	PValue
	GO:0005789~endoplasmic reticulum membrane	10	4.716981	0.030531
	GO:0016020~membrane	37	17.45283	0.045965
MF	GO:0005743~mitochondrial inner membrane	6	2.830189	0.073576
	Term	Count	%	PValue
	GO:0004930~G protein-coupled receptor activity	16	7.54717	0.011222
	GO:0004984~olfactory receptor activity	14	6.603774	0.015232
	GO:0000978~RNA polymerase II cis-regulatory region sequence-specific DNA binding	13	6.132075	0.0248
	GO:0008083~growth factor activity	4	1.886792	0.049006
	GO:0000981~DNA-binding transcription factor activity, RNA polymerase II-specific	12	5.660377	0.050447
	GO:0000062~fatty-acyl-CoA binding	2	0.943396	0.071938

Table 2: Gene ontology analysis of downregulated transcripts

BP	Term	Count	%
	GO:0007339~binding of sperm to zona pellucida	4	1.509434
	GO:0002682~regulation of immune system process	5	1.886792
	GO:0005975~carbohydrate metabolic process	6	2.264151
	GO:0006607~NLS-bearing protein import into nucleus	3	1.132075
	GO:0045184~establishment of protein localization	3	1.132075
	GO:0009267~cellular response to starvation	4	1.509434
	GO:0043065~positive regulation of apoptotic process	6	2.264151
	GO:0070979~protein K11-linked ubiquitination	3	1.132075
	GO:0032008~positive regulation of TOR signaling	3	1.132075
	GO:0050821~protein stabilization	5	1.886792
	GO:0032715~negative regulation of interleukin-6 production	3	1.132075
	GO:0016567~protein ubiquitination	8	3.018868
	GO:0090307~mitotic spindle assembly	3	1.132075
	GO:2000510~positive regulation of dendritic cell chemotaxis	2	0.754717
	GO:0043568~positive regulation of insulin-like growth factor receptor signaling pathway	2	0.754717
CC	Term	Count	%
	GO:0005634~nucleus	66	24.90566
	GO:0005765~lysosomal membrane	7	2.641509
	GO:0000781~chromosome, telomeric region	4	1.509434
	GO:0042564~NLS-dependent protein nuclear import complex	2	0.754717
	GO:0005730~nucleolus	11	4.150943
	GO:0000775~chromosome, centromeric region	3	1.132075
	GO:0030670~phagocytic vesicle membrane	3	1.132075
	GO:0000138~Golgi trans cisterna	2	0.754717
	GO:0005737~cytoplasm	50	18.86792
MF	Term	Count	%
	GO:0051724~NAD transmembrane transporter activity	2	0.754717
	GO:0038023~signaling receptor activity	4	1.509434
	GO:0061630~ubiquitin protein ligase activity	7	2.641509

Table 3: KEGG Pathways of the up regulated transcripts

Category	Term	Count	%	P Value
KEGG_PATHWAY	bta04740:Olfactory transduction	14	6.603774	0.071876
KEGG_PATHWAY	bta05207:Chemical carcinogenesis - receptor activation	5	2.358491	0.074051
KEGG_PATHWAY	bta05204:Chemical carcinogenesis - DNA adducts	3	1.415094	0.08318
KEGG_PATHWAY	bta03250:Viral life cycle - HIV-1	3	1.415094	0.098712

Table 4: KEGG Pathways of the down regulated transcripts

Category	Term	Count	%	P Value
KEGG_PATHWAY	bta04145:Phagosome	6	2.264151	0.027715
KEGG_PATHWAY	bta05168:Herpes simplex virus 1 infection	9	3.396226	0.069713

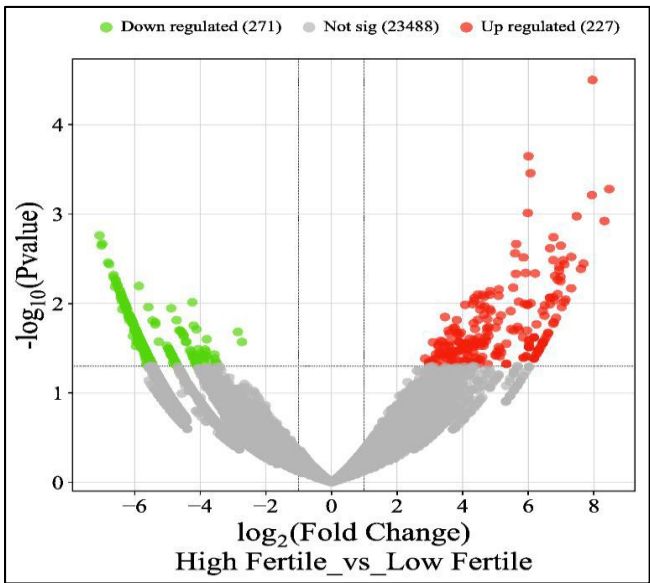


Fig 1: The volcano plot showing significantly up and down regulated genes with p-value <0.05 and log2 fold change±2

5. Conclusion

The proposed study was designed to generate the gene ontology profiles of the categorised bulls and to perform the comparative evaluation of the up and down regulated sperm transcripts between the groups. Identifying the significant genes that are up or down regulated between the groups can help us to identify the marker genes in the bulls which can help in the selection of superior bulls for semen quality traits. Identifying the significant UP/Down pathways can also help to determine the potential cause of reproductive problems in crossbred bulls. Therefore, they can contribute to the development of molecular markers and strategies for improving sperm quality, managing sperm-related diseases, and increasing bovine fertility in addition to providing new insights into the genetic basis of sperm cryoresistance. For more accurate results validation of the identified relevant genes can be performed using qRT-PCR. Since, the study is limited by the small sample sizes, larger sample sizes can be preferred for future validation investigations.

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