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Genomic and transcriptomic evaluations of infertile or subfertile Arunachali yak sperm

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Abstract

Sperm infertility or subfertility is detrimental to the precious highland germplasm like yak whose population has been gradually declining in India. Understanding the 'omic' landscape of infertile or subfertile yak sperm can reveal some interesting insights. In an attempt to do the same, this study considered the semen of infertile or subfertile yak bulls for whole-genome and transcriptome evaluations. DNA sequencing revealed that the yak sperm genome contains the necessary genes to carry out all the important biological processes related to the growth, development, survival and multiplication of an organism. Interestingly, RNA Seq results highlighted that genes like *VAMP7*, *MYLK*, *ARAP2* and *MARCH6* showed increased expression, while biological processes related to immune response (GO:0043308, GO:0002447, GO:0002278, GO:0043307, GO:0043312, GO:0002283, GO:0043299 and GO:0002446) were significantly overrepresented. These findings hint at a possible role played by immune system in regulating infertility or subfertility in yaks. Further, in-depth studies can validate these findings and help in improving our biological understanding in this area.

Introduction

Bull infertility or subfertility critically regulates the herd reproductive performance and consequently, the economics of a dairy farm (Amann and DeJarnette, 2012, Taylor *et al.*, 2018, Butler *et al.*, 2020). It has a significant effect on the major performance traits including days open (Hagiya *et al.*, 2018), daughter pregnancy rate (Raheja *et al.*, 1989), average daily gain (Raidan *et al.*, 2017), *in vitro* seminal parameters (Oliveira *et al.*, 2012, Mapel *et al.*, 2022) and sperm defects (Leite *et al.*, 2022). The '*omics*' revolution in animal breeding has brought new insights into the genetic make-up of sperm and its variability in individuals (Taylor *et al.*, 2018), thus bringing more clarity into the cases of bull infertility or subfertility (Han and Peñagaricano, 2016, Rezende *et al.*, 2018, Das *et al.*, 2020, Kumaresan *et al.*, 2021). It has been found that sperm infertility is modulated by the immunological milieu in the male reproductive tract.

Spermatogenesis (and the consequent sperm fertility) is crucially regulated by the inflammatory and non-inflammatory responses induced by the immune cells (Ye *et al.*, 2021). Physiological and pathological injuries activate the immune regulatory molecules which, though, offer protection to the sperm against these attacks but end up having a detrimental effect on its fertility (Archana *et al.*, 2019). Oxidative stress produced as a result of microbial and viral attack on the sperm can also cause anatomical changes in the male reproductive tract including testicular damage, reduction in Leydig cell mass and atrophy of seminiferous tubules and Sertoli cells (Akhigbe *et al.*, 2022, Das *et al.*, 2022a). Immune response-related genes like *IL6*, *IL8*, *IL1A* (Robertson and Sharkey, 2016), *IFN* (Hansen, 2007), *HLA-DRA*, *HLA-DRB1*, *TNFRSF14* and *VRK1* (Salvi *et al.*, 2022, Cerván-Martín *et al.*, 2022) have been usually implicated in sperm infertility or subfertility resulting from immunological causes.

Yak (*Bos grunniens*) is a seasonal breeder owing to the pastoral management system that offers rich forages and nutrition for breeding in the high altitudes during summers, whereas scarcity of vegetation renders them reproductively anestrous during winters (Prakash *et al.*, 2005, Das *et al.*, 2022b). This production system renders the species highly vulnerable to the dangers of extinction (Das *et al.*, 2020, Kour *et al.*, 2022). In this scenario, culling of infertile or subfertile bulls is crucial for continued economic viability and conservation of this unique germplasm. Infertility studies in yaks have been majorly directed at improving the understanding of hybrid male sterility seen in cattle yak (Tumennasan *et al.*, 1997; Wang *et al.*, 2012). Most of these studies have been conducted by comparing mRNA expression between hybrids and their parents (Wang *et al.*, 2012; Cai *et al.*, 2017; Wu *et al.*, 2019; Zhao *et al.*, 2022), while others have focused on DNA methylome of the male hybrids to reveal the probable epigenetic roles (Liu *et al.*, 2011; Luo *et al.*, 2022). Some of the workers have pursued a candidate

gene approach targeting Y-chromosome linked genes, namely, *MSY, TSPY, TSPY2, PRAMEY, UTY, OFD1Y, USP9Y* and *SCYP3* to identify their roles in regulating yak bull fertility (Wang *et al.*, 2012; Zhang *et al.*, 2019; Wu *et al.*, 2019). Das and co-workers (2020) deduced the involvement of small non-coding RNAs like *miRNA19a*, *miRNA142* and *miRNA143* in determining subfertility in *Arunachali* yak bulls.

This study particularly aimed at understanding the *omic* backgrounds of infertile or subfertile yak bulls. Therefore, infertile or subfertile yak sperm was subjected to genomic and transcriptomic evaluations to unveil the genes and biological processes regulating infertility or subfertility in yak bulls. This study provided initial leads for further detailed investigations in the area.

Materials and methods

Sample collection

Three adult healthy *Arunachali* yak bulls with true to the breed characters (Das *et al.*, 2022b) and showing infertility or subfertility were considered for the study. Animals were kept in the bull shed of the experimental yak farm of the Indian Council of Agricultural Research (ICAR)-National Research Centre on Yak at Nyukmadung, Arunachal Pradesh, at an elevation of 9000 ft above mean sea level (Figure 1). Animals were housed in open shelters (CGI roof) with concrete floors and were fed concentrate feed at 2–3% of their body weight. This was supplemented with green grass and paddy straw (roughages) and an *ad libitum* supply of drinking water. All the bulls were apparently healthy and were regularly vaccinated against major diseases. Semen was generally collected at fortnightly intervals using a teaser bull and was microscopically evaluated for its quality before being used for insemination. A bull was considered infertile when its conception rate was zero after being successively used for breeding for 2 years, and a bull was considered subfertile when its conception rate was less than 5% after being used successively for 5 years in the herd.

Fresh ejaculates were collected from three infertile or subfertile yak bulls by artificial vagina method using the Missouri model (Das *et al.* 2013). Semen samples were collected in accordance with the approval of the Institute Animal Ethics Committee of the ICAR-National Research Centre on Yak, Dirang, India, and the approved animal use protocol number was 4(17)/NRCY/IAEC-02. Subsequently, semen samples were processed and purified (Das *et al.* 2013) and stored at –80°C until further use. DNA was isolated using a protocol developed by Wu *et al.* (2015) with slight modification. RNA from sperm was isolated using a published protocol with the help of a 27 gauge needle and Trizol (Das *et al.* 2010).

Whole-genome and transcriptome sequencing

1 ng of sperm DNA concentration was used to prepare libraries using Illumina's Nextera XT DNA (Catalog no: FC-131-1024) and Nextera DNA Flex (Catalog no: 20018704) library preparation kits. Each pool of libraries with raw cluster densities of 202 and 189 for Nextera XT and Nextera DNA Flex, respectively, were loaded and sequenced separately on a NextSeq 500 System. Finally, paired-end reads of 2 × 151 bp were generated for further analysis.

Total RNA from the sperm of three infertile or subfertile yak bulls was pooled together and used for next-generation sequencing on the Roche platform. RNA concentration was measured using Nanodrop and the integrity of RNA was checked by Bioanalyzer. A cDNA library was prepared from the sperm RNA involving the following steps: fragmentation of nucleic acids to 300–400 bp lengths followed by end repair, ligation of adapters to ends of target sequences, library amplification and quantification, selection of appropriate fragments and removal of adapter dimers. Roche single-end RNA sequencing was performed using library fragments constructed according to the Roche protocol.

Data analysis

DNA sequencing data

The generated data were demultiplexed using bcl2fastq (version v2.17.1.14; Illumina), and the final sequence reads were trimmed using trimmomatic (version 0.38) (Bolger *et al.*, 2014) using default parameters (illuminaclip :2:30:10). The sequenced contigs data were processed in mpiBLAST (Darling *et al.*, 2003) to generate an xml file. This file was subsequently used to search for similarity in BLAST2GO software (Götz *et al.*, 2008), and contigs with similarity mean >80% were considered for further mapping and annotation. Gene ontology (GO) IDs associated with the data were analysed in PANTHER (Mi *et al.*, 2010) to find out the statistical overrepresentation of particular biological processes in yak sperm DNA at False Discovery Rate (FDR) ≤ 0.01.

RNA-sequencing data

The raw reads obtained by RNA sequencing were converted to fastq format, and the quality check was performed. The quality of the reads was evaluated using FASTQC (Andrews, 2010), and PRINSEQ lite v0.20.4 (Schmieder and Edwards, 2011) was used to trim the adaptor tags from the single-end reads. Thereafter, *Bos taurus* (assembly ARS-UCD1.2), as well as *Bos grunniens* (GCA_005887515.2 BosGru v3.0) reference genome, was downloaded and indexed, and reads were aligned to both the genomes using Hisat2 (Kim *et al.*, 2015). The .sam file containing the mapped reads was sorted with the help of SAMtools (Li et al, 2009) and, subsequently assembled using StringTie (Pertea *et al.*, 2015). StringTie was also used to estimate the gene abundance of the transcripts assembled from both *Bos taurus* and *Bos grunniens* reference genomes. The transcripts with gene coverage ≥1 were selected for further gene annotation and ontology. The retrieved gene list was fed into PANTHER (Mi *et al.*, 2010) to identify statistically overrepresented GO terms (FDR ≤ 0.01).

Results

The total 36,542 contigs obtained through sperm genome sequencing were blasted, mapped and annotated to reveal GO IDs corresponding to >80% similarity mean. These GO IDs were fed into Ensembl Biomart to retrieve a list of 8,337 protein-coding genes (Supplementary Table 1). Gene ontology analysis highlighted 1,089 statistically overrepresented biological processes (Figure 2). The complete list of statistically significant GO terms has been presented in Supplementary Table 2. The significant biological processes could be majorly categorized into parent groups, namely, biological regulation (GO: 0044848), cellular process (GO: 0009987), localization (GO: 0051179), metabolic process (GO: 0008152), response to stimulus (GO: 0050896) and signalling (GO: 0023052). Significant biological processes related to immunity included immune system development (GO:0002520), immune system process (GO:0002376), regulation of immune response (GO:0050776), regulation of T cell (GO:0050863) and lymphocyte activation (GO:0051249), natural killer cell activation involved in immune response (GO:0002323),

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Figure 1. Arunachali yak bull housed at the ICAR-National Research Centre on Yak.

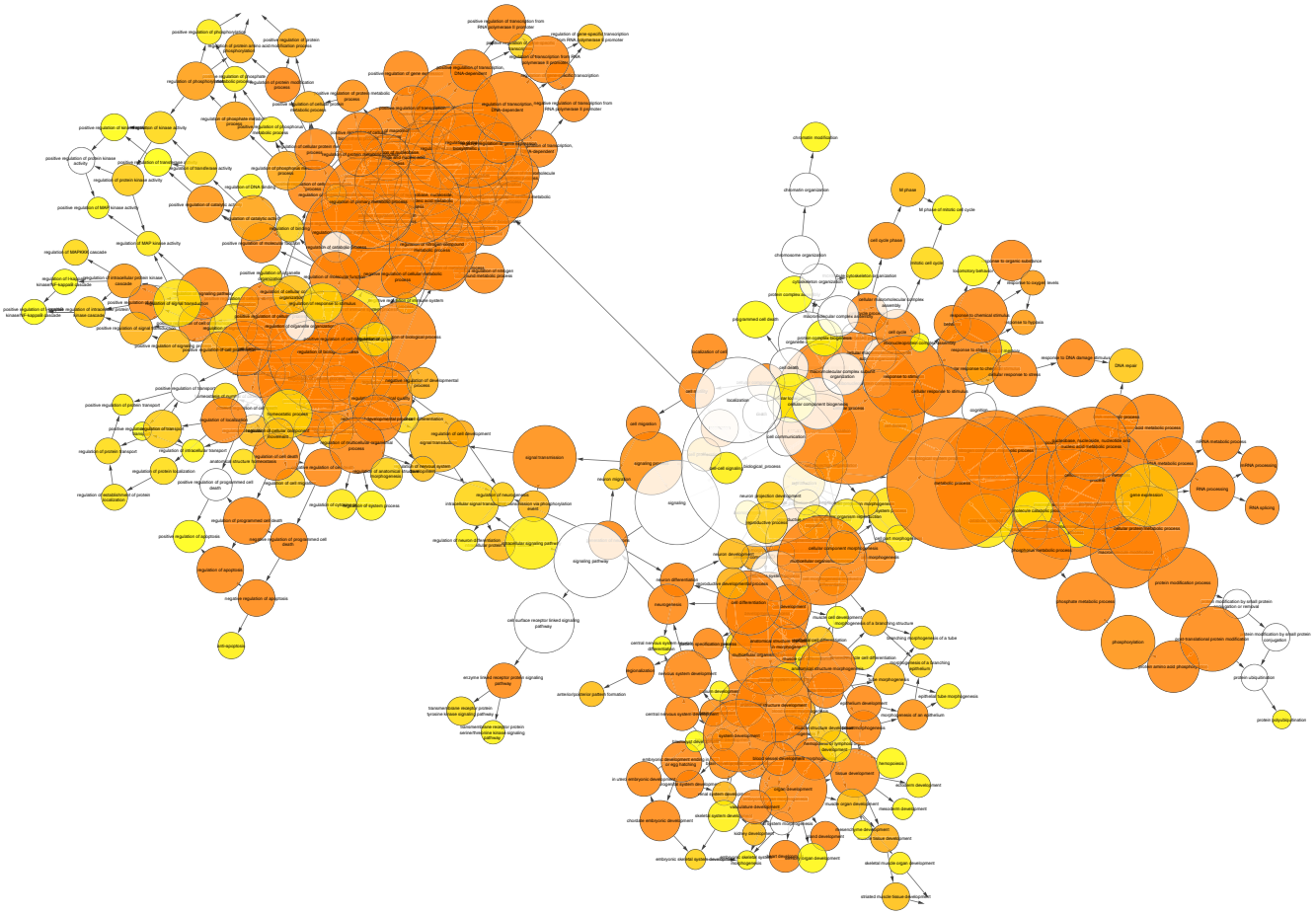

Figure 2. Network map of gene ontology terms related to biological processes in sperm DNA.

regulation of cytokine production involved in immune response (GO:0002718) etc.

A cDNA library was prepared from the sperm samples of *Arunachali* yak and raw reads were generated. After alignment of the reads with *Bos grunniens* and *Bos taurus* genomes, assembled transcripts from both genomes were evaluated for gene abundance, and those with gene coverage ≥1 were highlighted. However, transcripts assembled from cattle genome corresponded to a number of protein-coded genes including *VAMP7, MYLK, ARAP2* and *MARCH6.* All the protein-coding genes were subjected to gene

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Table 1. Significant gene ontology (GO) terms for biological processes in yak sperm cDNA

S. no.	Biological processes	GO term	FDR
1.	Eosinophil degranulation	GO:0043308	0.002
2.	Eosinophil mediated immunity	GO:0002447	0.002
3.	Eosinophil activation involved in immune response	GO:0002278	0.005
4.	Eosinophil activation	GO:0043307	0.001
5.	Neutrophil degranulation	GO:0043312	0.002
6.	Neutrophil activation involved in immune response	GO:0002283	0.008
7.	Leukocyte degranulation	GO:0043299	0.01
8.	Neutrophil mediated immunity	GO:0002446	0.01

ontology analysis for reflecting statistically overrepresented biological processes (FDR ≤ 0.01). The GO terms for significant biological processes were all related to immune responses and have been presented in Table 1. Since we did not perform a comparative transcriptomic study in fertile bulls, it may be possible that bulls considered in this study may be suffering from some underlying infection or inflammation at the time of sampling due to which immune response was overrepresented. Although there was no clinical history of any disease or infection at the time of sampling, we cannot ignore this reason. This is also possible since biological processes related to immune responses were only found to be significantly overrepresented.

Discussion

The infertile yak sperm genome is comprised of all the cellular components, molecular functions and biological processes necessary for the growth, development, survival, immunity and multiplication of an organism. This is quite obvious given the fact that sperm, being a gamete, possesses one complete set of genes that combines with the other set from the ovum to produce a diploid individual with normal bodily functions and development.

The transcriptome of infertile or subfertile yak sperm highlighted that the GO terms related to immune-related processes were highly significant. Though this study suffered from a limitation that differential expression and transcriptome comparison with fertile yak sperm was not carried out, nonetheless, it provided insights into the genomic and transcriptomic landscape of infertile or subfertile yak sperm. These findings will definitely help in designing further detailed downstream studies in the future.

Based on RNA Seq results, granulocytes-mediated immune response was found to be significant in infertile yak sperm granulocytes or polymorphonuclear leukocytes (PMN), namely, eosinophils, neutrophils and basophils, constituting the most prevalent white blood cells in semen followed by macrophages and T-lymphocytes (Wolff, 1995). Additionally, granulocytes in semen are negatively correlated with normal sperm morphology and positively with mid-piece abnormalities, thus highlighting their role in male infertility or subfertility (Thomas *et al.*, 1997). Specifically, neutrophil activation results in the production of reactive oxygen species, which severely dents the sperm motility (Kovalski *et al.*, 1992). Being the first-line innate immune defense system, PMNs release bactericidal enzymes by degranulation to form neutrophil extracellular traps (NETs) and, hence, phagocytosis (Brinkmann *et al.*, 2004; Borregaard, 2010). When the blood-testis barrier is breached, antigens on the sperm surface induce PMN activation, thus extruding their DNA and resulting in the trapping of sperm in NETs, thus hindering sperm motility. However, neutrophil activation does not necessarily indicate deterioration of sperm function as DNAse activity of seminal plasma proteins helps in the digestion of extruded DNA and frees the entangled spermatozoa, greatly boosting the chances of conception in the female reproductive tract (Alghamdi and Foster, 2005). Also, recent evidence suggests that the phagocytic activity executed by PMNs is crucial for the therapeutic activity of the sperm head in the female reproductive tract (Pakravan *et al.*, 2021).

Eosinophils also play an important role in the bodily immune response by protecting against allergens and parasitic infestation (Shamri *et al.*, 2011). Eosinophilic degranulation is exacerbated in response to infections and inflammation, and an RNAse-mediated positive feedback loop is established between eosinophils and mast cells in which the former induces mast cell activation by secretion of RNase and mast cells, in turn, secrete cytokines like IL-5, which further stimulate RNase release from eosinophils (Bystrom *et al.*, 2011). Subsequently, eosinophilic activation is mediated by these RNases through the toll-like receptor signalling pathway and results in the release of cytokines and various immune regulatory molecules (Rudd *et al.*, 2005; Phipps *et al.*, 2007). It has been reported that eosinophilic degranulation is observed in female cervical mucosa on exposure to IgA antibodies, thus evoking an allergenic reaction and disrupting immune tolerance (Brazdova *et al.*, 2016). This may be an important mechanism underpinning sperm infertility or subfertility in the female reproductive tract.

Another probable proposition for the increased expression of immune response in infertile or subfertile yak spermatozoa could be the presence of antisperm antibodies (ASAs). Autoimmunity to spermatozoa or the presence of ASAs is increasingly being implicated as a major cause underlying human male infertility or subfertility (Archana *et al.*, 2019). Though the testis is an immune-privileged site and protects the spermatozoa against autoimmune attack, the blood-testis barrier provided by the organ is not invincible (Wang and Holstein, 1983) as ASAs have been reported in around 18% of the infertile males (Bozhedomov and Teodorovich, 2005). Antigenic interaction between microorganisms and sperm (Bozhedomov and Teodorovich, 2005), inflammatory conditions (Marconi *et al.*, 2009), tumors (Bronson *et al.*, 1992) and reduced levels of cellular and humoral immunomodulatory factors in seminal plasma (Cooley *et al.*, 2016) pose a grave challenge to the immunosuppressive environment provided by the testis. These insults lead to the production of cytokines, leukocytes and T-cell activators, all of which subsequently impact the fertilizing ability of sperm (Bohring and Krause, 2003). This has been further validated by improvements seen in sperm motility, sperm concentration and overall conception rate on the administration of immune-suppressive corticosteroid therapy in infertile human males (Skau and Folstad, 2005). Although there is a paucity of research in this area concerning farm animals, a significant association has been reported between ASAs in serum or seminal plasma and fertility in cattle bulls (Zodinsanga *et al.*, 2015; Ferrer *et al.*, 2015). Sperm-bound ASAs were reported to be associated with poor post-thaw motility and breeding soundness in stallions (Ferrer and Miller, 2018; Ferrer *et al.*, 2021). Though we did not test for the presence of ASAs in the sperm of infertile or subfertile bulls considered in the study, this cause cannot be ruled out completely.

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Genes like *VAMP7, MYLK, ARAP2* and *MARCH6* showed increased expression in infertile or subfertile yak bulls. Overexpression of *VAMP7* has been associated with increased transcription of oestrogen receptors resulting in reduced sperm motility and spermatogenic failure (Tannour-Louet *et al.*, 2014). *MYLK* gene plays a crucial role in upregulation of *AGBL4* gene which subsequently, results in teratozoospermia in males (Wu *et al.*, 2015, Han *et al.*, 2021). *CENTD1* gene (analogue of human *ARAP2* gene) is significantly downregulated in the presence of miR-10a which causes acute myeloid leukaemia in humans (Bryant *et al.*, 2012). *MARCH6* was identified as a differentially methylated gene in infertile sperm samples and could be postulated as a novel biomarker gene for male infertility (Cassuto *et al.*, 2021). This indicates that the reduction in DNA methylation in immune-related genes can lead to increased transcript expression of these genes in infertile males (Schütte *et al.*, 2013). Though this study suffered from some limitations, it explored the '*omic*' landscape of infertile or subfertile yak sperm. Further functional studies can be carried out to validate our findings and to bring out interesting insights.

Conclusions

'*Omic*' analysis of infertility or subfertility in yak sperm highlighted that genes including *VAMP7, MYLK, ARAP2* and *MARCH6* were regulating the phenotype. Furthermore, immune-related biological processes (GO:0043308, GO:0002447, GO:0002278, GO:0043307, GO:0043312, GO:0002283, GO:0043299 and GO:0002446) were significantly overrepresented ($FDR \leq 0.01$). These findings may be indicative of a crucial role being played by genotype-environment interactions in determining infertility or subfertility in male yaks. However, further downstream studies in this direction can validate our findings and propositions.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0967199424000194.

Data availability. The data generated as a part of this study have been successfully submitted to Sequence Read Archive with Bioproject ID PRJNA931839.

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Author contributions. PJD, AK and MS conceptualized and designed the study; AK, JB and DCM carried out the data analysis; AK and PJD wrote the paper; and DCM, JB and MS critically revised the manuscript.

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Competing interests. All the authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical Standards. This study was approved by the Institute Animal Ethics Committee of the ICAR-National Research Centre on Yak, Dirang, India, vide approval number 4(17)/NRCY/IAEC-02.

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