TRANSCRIPTOME ANALYSIS OF SPERMATOZOA OF CLONED BUFFALO BULLS AND THEIR RESPECTIVE SOMATIC CELL DONOR BULLS

THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE
IN
ANIMAL BIOTECHNOLOGY

BY

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B.Sc. (Biotechnology)

ANIMAL BIOTECHNOLOGY CENTRE
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KARNAL-132 001 (HARYANA), INDIA

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Approved by:

21/09/2021

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This is to certify that the thesis entitled **"Transcriptome analysis of spermatozoa of cloned buffalo bulls and their respective somatic cell donor bulls"** submitted by **Ms. KANIKA GANDHI** towards the partial fulfilment of the award of the degree of **Master of Science in Animal Biotechnology** of the **ICAR-National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: September 08, 2021

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Dedicated

to

Almighty

&

My Family.....

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At last we should always remember thatfailure and setbacks are ingredients for a success story but it's our consistency and perseverance that help us convert our destiny into destination.

KanikaGandhi

Date: September 08, 2021 **(KANIKA GANDHI)**

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ABBREVIATIONS AND SYMBOLS

%	=	Percent
μg	=	Microgram
μl	=	Microlitre
AI	=	Artificial insemination
ART	=	Assisted reproductive technologies
BME	=	β-mercaptoethanol
Cm	=	Centimetre
DAHD	=	Department of Animal Husbandry and Dairying
DEPC	=	Diethyl Pyrocarbonate
DIC	=	Differential Interface Contrast
FC	=	Fold Change
GO	=	Gene Ontology
GSR	=	Gross Swirl Rating
HOST	=	Hypo-Osmotic Swelling Test
IVF	=	*In vitro* fertilization
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
mg	=	Milligram
mGSCs	=	Male Germline Stem Cells
miRNAs	=	MicroRNAs
ml	=	Millilitre
mm	=	Millimetre
ng	=	Nanogram
NGS	=	Next Generation Sequencing
PCA	=	Principal Component Analysis
PCR	=	Polymerase chain reaction
PRM	=	Protamines
QC	=	Quality check
qPCR	=	Quantitative- Polymerase chain reaction
RIN	=	RNA Integrity Number
RT-PCR	=	Reverse transcriptase- Polymérase chain reaction
SCLB	=	Somatic cell lysis buffer
SCNT	=	Somatic cell nuclear transfer

*****

Abstract

सारांश

सांडों की प्रजनन क्षमता की भविष्यवाणी करने के लिए पारंपरिक तरीके से वीर्य का संपूर्ण मूल्यांकन ही पर्याप्त नहीं है। अत: नए जीनोमिक्स, प्रोटिओमिक्स, मेटाबॉलिकमिक्स, ट्रांसक्रिपटॉमिक्स और एपिजेनोमिक्स का अध्ययन प्रजनन कार्यक्रमों के उपयोग को नियोजित करने से पहले क्लोन सांडों द्वारा उत्पादित वीर्य की प्रजनन क्षमता को निर्धारित करने के लिए किया जा सकता है। इस प्रकार वर्तमान अध्ययन में अगली पीढ़ी के अनुक्रमण का उपयोग करते हुए ग्लोबल ट्रांसक्रिप्ट्स प्रोफ़ाइल और क्लोन किए गए सांडों और उनसे संबंधित दैहिक कोशिका दाता सांडों के शुक्राणुओं के विभेदित रूप से व्यक्त ट्रांसक्रिप्ट्स की पहचान करने के लिए किया गया था। वीर्य से दैहिक कोशिकाओं को सफलतापूर्वक हटाने के बाद प्राप्त शुद्ध शुक्राणुओं की आबादी से RNA को अलग किया गया, जिसमे अच्छे RNA की मात्रा 116ng/µl से 204ng/µl के बीच थी, और उसका RIN मान 7.0 से 7.5 के बीच पाया गया। cDNA libraries को एकल अंत प्रारूप में प्रत्येक बायोलॉजिकल रेप्लिकेट्स के लिए तैयार किया गया जिसमे इलुमिना सीक्वेंसर का उपयोग करके अनुक्रमित किया गया था। सभी नमूनों का FastQC से Phred स्कोर 30 के आसपास था। बॉस टॉरस के संदर्भ जीनोम, में UMD 3.1.1 के खिलाफ संरेखित कुल रीड 76.76 से 88.25% के बीच थी जिसमे कुल 27,481 ट्रांसक्रिप्ट्स की पहचान की गई थी। जिनमें से 18,703 ट्रांसक्रिप्ट्स आमतौर पर क्लोन और सोमैटिक सेल डोनर सांडों के स्पर्मेटोजोआ दोनों ने व्यक्त किया तथा 4120 ट्रांसक्रिप्ट्स क्लोन किए गए सांडों के स्पर्मेटोजोआ समूह के लिए अद्वितीय थे और 4658 ट्रांसक्रिप्ट्स पैरेंट सांडों के स्पर्मेटोजोआ समूह के लिए अद्वितीय थे। (FC≥2; P<0.05) पर 2349 ट्रांसक्रिप्ट्स क्लोन सांडों में पेरेंट्स सांडों के शुक्राणुओं के सापेक्ष में अंतर था, जिनमें से 51 ट्रांसक्रिप्ट्स FC≥10 पर, 559 ट्रांसक्रिप्ट्स FC≥5 पर और 1739 ट्रांसक्रिप्ट्स पर FC≥2 पर व्यक्त थे। इससे यह मूल्यांकन किया गया कि दैहिक कोशिका दाता सांडों की शुक्राणुओं के सापेक्ष क्लोन सांडों के स्पर्मेटोजोआ में 566 ट्रांसक्रिप्ट्स में अप-रेगुलेशन पाया गया और 410 ट्रांसक्रिप्ट्स को महत्वपूर्ण रूप से FC≥2 P<0.05) में डाउन-रेगुलेशन पाया गया। GO विश्लेषण से पता चला कि जैविक प्रक्रिया, आणविक कार्य और सेलुलर घटक प्रभावित थे जो कि 331 पाथवे को प्रभावित किये। KEGG संवर्धन विश्लेषण से पता चला कि क्लोन सांडों के शुक्राणुओं में अप-रेगुलेशन ट्रांसक्रिप्ट्स से प्रभावित पाथवे बेस एक्सिशन रिपेयर और ऑटोफैगी पाथवे थे। जो इसी तरह के डाउन-रेगुलेटेड ट्रांसक्रिप्ट्स में एडिपोसाइट्स का वसापघटन, AMPK में सिग्नलिंग पाथवे और इंसुलिन सिग्नलिंग पाथवे इत्यादि प्रभावित थे, जबकि दीर्घायु पाथवे का क्लोन सांडों के शुक्राणुओं पर महत्वपूर्ण प्रभाव पाया गया था। प्रजनन क्षमता, शुक्राणु गतिशीलता, शुक्राणु क्षमता, सांडों प्रजनन क्षमता और प्रारंभिक भ्रूण विकास से जुड़े कुछ महत्वपूर्ण ट्रांसक्रिप्ट्स की तुलना करने पर दोनों समूहों में लगभग समान पाए गए। निष्कर्ष रूप में, हम कह सकते हैं कि क्लोन किए गए सांडों के वीर्य और गैर-क्लोन किए गए सांडों के वीर्य के ताजा वीर्य मापदंडों के साथ-साथ क्लोन और सोमैटिक सेल डोनर सांडों के स्पर्मेटोजोआ के NGS डेटा इस तथ्य का समर्थन करते हैं कि क्लोन किए गए सांडों के वीर्य और सोमैटिक सेल डोनर के वीर्य में कोई ज्यदा अंतर नहीं है। जब इन क्लोन सांडो के वीर्य का उपयोग कुछ मादा भँसो में AI के द्वारा किया गया तो पाया गया की गर्भाधान की दर बिना क्लोन किये सांडो के वीर्य के ही सामान थी। अत: इस सीमित अध्ययन के साथ यह सुझाव दिया जा सकता है कि क्लोन किए गए सांडों के वीर्य का उपयोग विभिन्न सहायक प्रजनन तकनीकों (एआरटी) में किया जा सकता है।

ABSTRACT

Conventional methods to predict bull fertility are not sufficient for a thorough evaluation of semen, thus the new genomics, proteomics, metabolomics, transcriptomics, and epigenomics study can be used to determine the fertility potential of semen produced by cloned bulls before employing them in breeding programs. Thus the present study was carried to generate the global transcriptome profile and identification of differentially expressed transcripts of spermatozoa of cloned bulls and their respective somatic cell donor bulls using next generation sequencing. The RNA was isolated from pure spermatozoa population obtained after successful removal of somatic cells from semen. The quality RNA yield varied between 116 ng/µl to 204 ng/µl with RIN values between 7.0 to 7.5.cDNA libraries were prepared for each biological replicate in single end format and were sequenced using Illumina sequencer. The FastQC reported the Phred scores around 30 indicating that the sequence read was almost correct. The total reads that got aligned against *Bos Taurus* reference genome*,* UMD 3.1.1 for all the samples were between 76.76 to 88.25%. Total 27,481 transcripts were identified out of which 18,703 transcripts were expressed commonly in both cloned and somatic cell donor bulls spermatozoa, 4120 transcripts were unique to cloned bull spermatozoa group, and 4658 transcripts were unique to parent bull spermatozoa group. At (FC≥2; P<0.05) 2349 transcripts were found to express differentially in cloned bulls spermatozoa relative to parent bulls spermatozoa. Out of which 51 transcripts were expressed at FC≥10, 559 at FC≥5 and 1739 at FC≥2. From this it was evaluated that 566 transcripts were up-regulated and 410 transcripts were down-regulated significantly FC≥2 (P<0.05) in clone bull spermatozoa relative to somatic cell donor bull spermatozoa. The GO analysis revealed that biological processes, molecular function and cellular components were affected with and the pathways affected by these transcripts were 331. KEGG enrichment analysis revealed the pathways affected by 566 significantly up-regulated transcripts at FC>2 in cloned bulls spermatozoa were majorly base excision repair and autophagy pathway. Similarly, the pathways affected by 410 down-regulated transcripts, affected the regulation of lipolysis in adipocytes, AMPK signaling pathway, insulin signaling pathway etc. non-significantly (*P<0.05*) while longevity pathway was found to have significant effect on cloned bulls spermatozoa. Furthermore, on comparing some of the important transcripts associated with regulating reproduction, sperm motility, sperm capacitation, bull fertility, and early embryonic development were almost similar in both the groups. In conclusion, we can say that fresh semen parameters of cloned bulls semen and non-cloned bulls semen as well as NGS data of cloned and somatic cell donor bulls spermatozoa support the fact that there is no difference between semen of cloned bulls and somatic cell donor bulls. Hence, with this limited study it can be suggested that the semen from cloned bulls may be used in various assisted reproductive technologies (ART).

CHAPTER – 1

Introduction

1. INTRODUCTION

Domestic buffalo (*Bubalus bubalis*), a multipurpose livestock species in India, provides milk, meat and a source of employment to the millions of farmers. In 2018-2019, buffalo contributed 49% towards the milk production. Due to ever increasing demand of milk and hence the demand for frozen semen to pursue artificial insemination, there is an urgent need to multiply genetically elite male buffalo. Contribution of bulls to breeding programs for genetic improvement of milk production between generations is about 64% which is much higher than that of females i.e. only 36%. Conventionally, the genetic improvement in domestic animals has been achieved by inseminating females with the semen of desirable genotype bulls to produce higher productive calves. Therefore, availability of best genotype bulls is a fundamental requirement to boost genetic gains to achieve higher production.

According to 20th livestock census total buffalo population in the country is 109.85 million and it is reported that female buffalo population increased by 8.61% whereas male buffalo has been declined by 42.35% over previous census. In the animal production industry selection of males for breeding is not only important for the genetic benefits of progeny, but also upgrading the genetics of future generations to maximize future fertility/ production as the semen from a single bull is used to inseminate thousands of females. In India, due to a severe shortage of elite bulls, semen available from progeny-tested bulls is not sufficient to cover even 1% of the breedable population of livestock. This is because progeny-testing is time consuming, needs large number of animals and infrastructure facilities. With the conventional progeny-testing programs, by the time a bull is declared to be progeny-tested, it is either dead or culled from the herd. It has been estimated that there will be a need of around 3000 young bulls every year to cover the 60 million breedable populations of buffalo in India. Thus, the resulting population of elite bull from progeny testing programme is not enough to breed the current buffalo population.

Presently several ART are available i.e. AI, IVF, ETT, OPU but buffalo bull cloning by somatic cell nuclear transfer (SCNT) is considered the best available option to reproduce elite bulls in shortest time and their semen would contribute to achieve the demand of ever-growing frozen semen, which is the prime requirement of conventional breeding programme (Selokar *et al.,* 2019). ICAR-NDRI has produced several cloned bull calves out of which three cloned bulls are currently producing semen namely namely Shresth (2010, produced from fetal fibroblast cells), Swarn (2013, produced from somatic cell isolated from seminal plasma) and Rajat (2014, produced from somatic cells isolated from frozen semen). Birth of Rajat proved that cloning technique can be used to multiply progeny-tested bulls even if they have died years before.

Over the last few years, conventional tests, such as sperm concentration, morphology and motility, were used along with other tests of sperm functional parameters, including plasmalemma integrity, functional membrane integrity, chromatin distribution and mitochondrial membrane potential, (Selvaraju *et al.,* 2008) to ascertain the intrinsic fertility of semen samples but it meant for minimal value as the decline in fertility was constantly observed in livestock (Walsh *et al.,* 2011; Lonergan *et al.,* 2016). However, to recognize and predict precise semen deficiencies that could affect the fertility of a bull, these conventional parameters cannot be totally relied upon. Recently a number of in vitro semen evaluation tests have been developed to understand the subtle molecular differences between spermatozoa which would help in the identification of candidate molecules for fertility prediction. It was indicated by these studies that transcriptomic, genomics and proteomic approaches have the possibility to be used in the efficient assessment of bull fertility (Somashekar *et al.,* 2015; Selvaraju *et al.,* 2017).

Sperm transcriptome, the complete set of transcripts is considered as one of the most significant component because it contains the coding RNAs that specify the composition of the proteome and hence determine the biochemical capacity of the cell. The studies include sperm genetic material (Silva and Gadella, 2006), sperm RNAs (e.g. mRNA, miRNA, snRNA, piRNA) and seminal proteins (e.g. clustrin (CLU), osteopontin (OPN), binder

of sperm (BSP) that are totally or partially transmitted to the oocyte during fertilization and embryo development (Lalancette *et al.* 2008; Liu *et al.* 2019; Jodar *et al.* 2013; Somashekar *et al.* 2015).

Several studies describing global transcriptome profile of bull spermatozoa and the differential expression of genes in high and low fertility bull are already available. Also, there are reports mentioning specific genes/RNAs that have role in determining bull's fertility and in early embryonic development. However, till date no literature is available on the global transcriptome profile of spermatozoa of cloned bulls. Cloned buffalo bulls have same genotypes as of their somatic cell donor bulls so we believe that the global transcriptome profile of cloned buffalo bulls and their somatic cell donor bulls could be similar. Keeping this view, the present study is proposed with the following objectives:

1. To generate transcriptome profile of spermatozoa of cloned and their respective somatic cell donor bulls using next generation sequencing.

2. To identify differentially expressed transcripts in spermatozoa of cloned bulls and their respective somatic cell donor.

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CHAPTER – 2

Review of Literature

2. REVIEW OF LITERATURE

2.1 Introduction

Buffaloes play an essential role in south-east Asian countries by their contributions in milk, meat, hides and draft power for agricultural operations. The total buffalo population in country constitutes 109.85 million, according to 20th livestock census. Over the previous census, female buffalo population has increased by 8.61% whereas male buffalo population has declined by 42.35%. Contribution of bulls to breeding programs for genetic improvement has been underestimated for long time, which is about 64%, much higher than that of females (only 36%). In India, due to a severe shortage of elite bulls, semen available from progeny-tested bulls is not sufficient to cover even 1% of the breedable population of livestock. This is because progeny-testing is time consuming, needs large number of animals and infrastructure facilities. With the conventional progeny-testing programs, by the time a bull is declared to be progeny-tested, it is either dead or culled from the herd. It has been estimated that there will be a need of around 3000 young bulls every year to cover the 60 million breedable populations of buffalo in India. Thus, the resulting population of elite bull from progeny testing is insufficient to breed the current buffalo population.

Although several Artificial reproduction techniques such as AI, IVF, ETT, OPU are available yet buffalo bull cloning by somatic cell nuclear transfer (SCNT) is considered the best available option to reproduce elite bulls in shortest time and their semen would contribute to achieve the demand of ever-growing frozen semen, which is the prime requirement of conventional breeding programme (Solekar *et al.,* 2019). Cloning, the process of having offspring's with similar DNA, is the quickest means of multiplying the selected bulls in the possible time period and the individuals produced are exact replica of selected individuals (Sharma *et al.,* 2011). Till date ICAR-National Dairy Research Institute, Karnal has produced six cloned male calves by SCNT, out of which three cloned bulls are currently producing semen namely Shresth (MU-6253; produced in 2010 from frozen thawed cloned embryo (fibroblast cells), Swarn (MU-6708);

produced in 2013 from somatic cell isolated from seminal plasma) and Rajat (MU-6923); produced in 2014 from somatic cells isolated from frozen semen straws. Birth of Rajat proved that cloning technique can be used to multiply progeny-tested bulls even if they have died years before.

Fertility is related to the capacity of a bull to produce sperm that are able to fertilize oocytes and produce new offspring (Liu *et al.,* 2016). The studies related to bull's fertility have been carried out through different approaches including transcriptomics, genomics and proteomics (Jodar *et al.,* 2013; Vijayalakshmy *et al.,* 2018; Sood *et al.,* 2019). Selection of males for breeding is not only important for the genetic benefits of progeny, but also upgrading the genetics of future generations to maximize future fertility/ production.

2.2 Traditional vs omics approach for determining semen quality

Conventionally semen evaluation parameters, such as ejaculated volume (5-8 ml), sperm concentration (800-2000 million per ml), motility (more than 75%), ph (6.4-8) were employed to select semen samples (Alves *et al.,* 2020). However, these parameters are not always effective because some of the cases of subfertility cannot be weaken by modifying sperm concentration in a semen dose (Saacke *et al.,* 2000). Thus, there was a need to improve semen evaluation. Recent advances in genomics transcriptomics and proteomics have helped to elucidate the molecular mechanisms associated with spermatogenesis, fertilization and embryogenesis (Selvaraju *et al.,* 2017). The mammalian spermatozoa deliver not only the paternal haploid genome but also coding, non-coding transcripts and other molecules to the oocyte during fertilization. These transcripts influence the phenotype of the offspring and it might help in understanding the fertility potential and early embryonic developmental efficiency of spermatozoa of cloned bull (Selvaraju *et al.,* 2017; Fair and Lonergan, 2018; Carrell *et al.,* 2019).These molecular biomarkers offer a promising solution to the current challenges with male fertility prediction as they have direct ties to the etiology behind fertility failure. Several biological biomarkers which affect the fertilizing capacity of sperm include DNA markers (Abdollahi-Arpanahi *et al.,* 2017; Nani *et al.*, 2019; Rezende *et al.,* 2019), DNA fragmentation (Kasimanickam *et al.,* 2006), proteins (Harayama *et al.*, 2017),

RNAs (Card *et al.,* 2017), microRNAs (Govindaraju *et al.,* 2012; Fagerlind *et al.,* 2015) and DNA methylation (Fang *et al.,* 2019). Also spermatogenesis is associated with various growth factors and apoptotic markers (Saeednia *et al.,* 2015). The presence of these markers in seminal plasma could be used to assess the success of the spermatogenic process and the fertilizing ability of the ejaculate.

2.3 Importance of sperm transcript

In the last two decade Spermatozoa has gained a lot of attention, from being considered as a mere vehicle for DNA delivery to the female, to having a pivotal role in fertility and offspring well-being. Sperm transcripts are considered to carry remnants of RNA from spermatogenesis. Transcriptome is considered as one of the most significant component because it contains the coding RNAs that specify the composition of the proteome and hence determine the biochemical capacity of the cell. Generally, the goal of transcriptome analysis is to identify genes differentially expressed among different conditions, leading to a new understanding of the genes or pathways associated with the conditions (Liu *et al.,* 2016). Understanding the make-up of sperm RNA transcripts may prove to be important in understanding the events surrounding capacitation, motility, fertilization and ultimately in explaining the male fertility. The phenotype of the offsprings which might prove helpful in diagnosing and managing male infertility are influenced by spermatozoal transcripts. These analyses have revealed molecular defects as well as provided clues about how to overcome the defects. A new focus into the role of sperm transcriptome and how this may affect the long-term health of his offspring has emerged (Presicce *et al.,* 2020).

At time of fertilization only chromosomes are being contributed to the embryo by the sperm, which is why, their role and the relative input during embryo formation has not been fully commended, and underestimated only to the contribution of the paternal DNA (Raval *et al.,* 2019). The various events occurring during the last steps of the spermatogenesis, such as the progressive replacement of histones by transition proteins and then protamines, and the removal of most of the sperm cytoplasm are thought to be responsible for transcriptionally and translationally inactive mature

mammalian spermatozoa at the nuclear level (Kropp *et al.*, 2017). During cytoplasmic extrusion transcriptional and translational processes are obstructed and most of the RNAs are lost, still a small but complex population of RNAs is non-randomly preserved in mature sperm (Godia *et al.,* 2018). Therefore, for the anticipation of animal fertility and selection of the best male for future breeding, sperm RNA is budding as a valuable tool with an aim to improve the cost-effectiveness of the livestock production (Suliman *et al.,* 2018). The functional importance of sperm-derived RNAs during embryonic development remains largely unknown. Therefore, the sperm transcripts have a defined role to play during embryo formation.

2.4 Tools used for global gene expression profiling of sperms

The development of new investigative method such as analysis of mRNA profile in sperm and understanding the significance of the transcripts would be helpful as additional diagnostic tool and be of prognostic value to fertilization and establishment of pregnancy (Kumar *et al.,* 2020). The spermatozoa transcripts composition and expression levels are associated with spermatogenesis, functional parameters of spermatozoa, early embryonic development, and pregnancy outcomes. Studies inculcating gene expression analysis in bovine sperm cell for the identification of genes expressed included the use of RT-PCR, microarray. This method identifies only a few of the genes. Presently, techniques like next generation sequencing are used to analyze the entire expressed genome of cell.

2.4.1 Microarrays

Microarray technology allows studying the expression of many genes at once. It involves placing thousands of gene sequences in known locations on a glass slide called a gene chip. The sample of DNA or RNA is placed in contact with the gene chip. The intensity of light produced by binding between the complementary base pairing of the sample and the gene sequences on the chip is measured and quantified to identify differentially expressed transcripts. It had relatively low throughput and is not considered ideal for quantifying transcripts.

2.4.2 Next Generation Sequencing

The development of high throughput Next Generation Sequencing has revolutionized transcriptomics by enabling RNA analysis through sequencing of cDNA. RNA sequencing makes use of high throughput sequencing method to provide insight into transcriptome of cell. Its efficiency over microarray includesi) it allows identification of novel transcripts which was not possible via microarray, ii) it does not require prior sequence knowledge, iii) it identifies single nucleotide polymorphism and iv) it also quantify gene expression and detects allele specific expression etc. Different NGS platforms available now are illumina (solexa) sequencing, roche 454 sequencing, and ion torrent: proton/pgm sequencing.

2.5 Transcriptome related to spermatogenesis

There are several reports on regulation of spermatogenesis, fertility and embryonic development by transcriptome of sperm. Legare *et al.* (2017) reported that the gene expression along the bovine epididymis is extremely regulated in the caput, corpus and cauda segments which have their own transcriptomic signs in fertile and sub fertile bulls. When the bulls with a high non-return rate (NRR) were compared with sub-fertile bulls, gene expression profile was found to be affected in subfertile bulls and considering the bull's fertility, caput emerge out to be the most affected region. The transcriptional profiles between fertile vs sub-fertile conditions clustered most closely in the corpus and cauda segments, whereas the profiles in the caput segment were different between fertile and sub-fertile bulls. 2029 genes were found to be differentially expressed across the diverse segments of epididymis. Cauda had more number of up-regulated genes than those expressed during propagation from caput to cauda. Towards the cauda, number of genes up regulated was higher than the number of genes with down-regulated (Legar *et al.,* 2017).

Sub-fertile group expressed differentially regulated 365 genes in the caput compared to the fertile group. Of these 365 genes, 13 showed common differential regulation with the other two epididymal regions, whereas 352 showed highly differential regulation. 84 genes in the corpus segment were

differentially regulated in sub-fertile against fertile group analysis, of which 15 and 69 genes showed common and exclusive differential expression with other analyzed groups, respectively. There were 77 genes differentially regulated in the cauda section between the sub-fertile against fertile groups, with 14 genes showing common differential expression. Only two genes were commonly regulated all along the epididymis. Of the differently expressed genes *ADAM28, AKAP4, SMCP, TCP11, SPATA3, ODF1, CTCFL, SPATA18, SORD* and *FAM161A* were found to exert functions associated to reproductive systems and *DEAD, CYST11, DEFB119, DEFB124* and *MX1* genes were found to be associated with the defense response, which may help in elimination of defective spermatozoa (D'Amours *et al.,* 2010). It was also reported that in contrast to genes up-regulated in fertile bulls, low-expressed genes in fertile bulls were less dependent on the anatomical epididymal segment, which show more dependency on epididymal segment (Legare *et al.,* 2017).

Li *et al.* (2020) stated the downward trend in gene expression during spermiogenesis. The decrease in expression was correlated with replacement of histones by transition proteins and protamines during spermiogenesis which indicated gradual condensation of chromosomes. They analyzed 7652 differentially expressed genes during spermiogenesis process in the bull. Out of these DEGs, 264 genes were up-regulated and 253 genes were down-regulated from round spermatids to elongated spermatids. The number of up-regulated genes was slightly higher than that of down-regulated genes. There were 241 up-regulated and 7038 down-regulated genes from elongated spermatids to epididymal sperm. The number of up-regulated genes was far less than the number of down-regulated genes. From the perspective of the entire spermiogenesis, the number of up-regulated genes slowly decreased, and the down-regulated genes progressively increased. In particular, at the later stage from the elongated spermatid to epididymal sperm, down-regulated genes significantly increased.

A slow decrease in gene expression was the main feature of this stage expression. They observed the expression level of *DNAL1* was significantly reduced from round spermatid to elongated spermatid; *ART3*, *SDHA, HIP1* and

YBX2 showed significant reduction in relative expression levels from elongated spermatid to epididymal spermatid. The localization of ART3 (a member of ADP-ribosyltransferase family) was also inspected via immunofluorescent analysis and it revealed its position on the outer periphery of the spermatid in the lumen. ART3 involvement in deformation and elongation of sperm cell, the formation of tails, or in shedding of cytoplasm was also observed. IZUMO1 protein which is considered as one of the core protein involved in fusion of sperm with ovum was also observed. SPACA3 protein which is involved in interaction with the ovum plasma membrane was also found. SPCA1 played a major role in formation of acrosome and sperm-oocyte fusion was also present (Li *et al.,* 2020).

Sperm associated antigen 17 (*SPAG17*) gene was highly expressed in testis at different development stages, which revealed that *SPAG17* played an indispensable role in male reproduction, being involved in spermatogenesis and in sperm migration and movement (Kazarian *et al.,* 2018; Xu *et al.,* 2018). It also has an important role in regulating litter size in Shaanbei White Cashmere (SBWC) goats (Zhang *et al.,* 2020). *RAD21* is expressed in spermatogonia and is involved in sister chromatid cohesion and segregation during meiosis (Lee *et al.,* 2002).

2.6 Transcripts associated with spermatozoa

Recent development in the field of comparative genomics and global gene expression analysis have provided new molecular detection tools that allow the heterogeneous RNA content of a spermatozoon to be used as a parameter for genomic analysis of semen, both in terms of spermatogenesis and fertility. The presence of RNA in spermatozoa is well established, yet little is known regarding its function and purpose (Selvaraju *et al.,* 2017). A decade back, RNA in mammalian spermatozoa was believed to be non-functional, but now it is known that spermatozoa deliver more than the paternal genome into the oocyte , that are remnant messenger RNA from spermatogenesis. The spermatozoon transfers coding as well as non-coding transcripts to the oocyte during fertilization. A rich population of RNAs including mRNA, ribosomal RNA (rRNA), mitochondrial RNA (mt-RNA), long non-coding RNA (lncRNA), small non-

coding RNA (sncRNA), small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA) are present in bovine spermatozoa. Spermatozoal transcript composition and expression levels are associated with spermatogenesis, functional parameters of spermatozoa, early embryonic development and pregnancy outcomes. Spermatozoal transcripts also influence the phenotype of the offspring and might help in diagnosing and managing male infertility (Jodar *et al.,* 2015). The spermatozoal transcripts are significantly associated with germ cell development. They are associated with processes such as DNA packaging, chromatin assembly, chromatin silencing and DNA metabolic processes essential for sperm nuclear formation (Selvaraju *et al.,* 2017). Transcripts associated with biological processes such as regulation of amide and peptide biosynthesis, energy homeostasis and generation of precursor metabolites, motility and acrosomal reactions are significantly enriched in spermatozoa (Sevaraju *et al.,* 2018).

The potential for DNA methylation to serve as a predictor of fertility in cattle has already been shown (Kropp *et al.,* 2017; Fang *et al.,* 2019). A similar work was carried by (Gross *et al.,* 2020) where they compared the DNA methylation data with previously published RNA sequencing results in order to identify candidate markers for sire fertility. The DNA methylation marks on sperm are found to be associated with the fertility of sires and RNA content of blastocysts produced from high and low-fertility sires (Kropp *et al*., 2017). The team found out 814 sites with hypermethylation and 951 sites with hypomethylation in high fertile sires in comparison with low-fertility sires. The genes with the highest number of differentially methylated cytosines included FER tyrosine kinase (FER), LPS responsive beige like anchor protein (LRBA), syntaxin binding protein 4 (STXBP4), F-box and leucine-rich repeat protein 17 (FBXL17), sperm tail PG-rich repeat containing 2 (STPG2), and phosphodiesterase 10A (PDE10A). The gene TM9SF2 had a DMC that was hypomethylated in high-fertility sires, and also showed increased RNA expression in sperm from high-fertility sires (Card *et al*., 2017). Another gene, SPATA6 had a hypermethylated DMC and also had decreased RNA expression in sperm of high fertility sires (Card *et al*., 2017). SPATA6 is required for the assembly of segmented columns and capitulum, (Yuan *et al*., 2015) and also for the fusion of the flagellum to the sperm head in

late spermiogenesis (Chemes *et al.,* 1999; Chemes and Rawe, 2006). *AFF4* gene was hypermethylated in intronic region in high fertile sires. The gene AFF4 played a central role in embryonic development and subsequent fertility.

Paul *et al.* (2020) reported the differences in sperm functional attributes and transcriptomic profile of high- and low-fertile buffalo bulls. During their study they found low (*P<0.05*), sperm membrane and acrosomal integrity and high (*P<0.05*) protamine deficiency and lipid peroxidation in low fertile bulls when compared to high-fertile bulls. A total of 51,282 transcripts were detected in buffalo spermatozoa using mRNA microarray technology, of which 4,050 transcripts were differentially expressed, and 709 transcripts were found to be significantly dysregulated (*P*<0.05 and fold change >1) between high- and low-fertile bulls. Among these, in low-fertile buffalo bull spermatozoa 113 transcripts were up-regulated and 596 transcripts were down-regulated. The genes mainly involved in cellular processes, binding activities, metabolic processes, and catalytic activities related to sperm function (sperm motility, capacitation, and acrosome reaction), fertilization (sperm–oocyte binding and zygote formation), and early embryonic development (maternal zygotic transition and early embryonic growth) were dysregulated genes.

During the pathway enrichment analysis of dysregulated genes it was indicated that in low-fertile buffalo bulls, MAPK signaling, ribosome pathway, and oxidative phosphorylation were dysregulated. It was observed that 28 genes were downregulated and were associated with MAPK signaling pathway in low-fertile buffalo bull spermatozoa. The function that MAPKs are involved in are regulation of transcription in the testis, regulation of sperm motility, hyperactivation, and acrosome reaction whereas its inhibition is required for resumption of meiosis in the oocyte, which triggers the formation of pronuclei in the process of fertilization. 24 genes associated with the oxidative phosphorylation pathway were down-regulated in low-fertile bull spermatozoa. 27 down-regulated transcripts were associated with ribosome pathway. Several genes associated to sperm functional attributes were observed to be significantly down-regulated in low-fertile bull spermatozoa. 10 genes were identified as having a role in sperm

functional attributes (*YBX1, RPL39, PGAM1, CASP4, TFAP2C, H3F3B, ZAR1, CHRNA3, MAP2K6* and *ORAI3*) and found that their expression was significantly altered in low-fertile bulls (Paul *et al*., 2020).

Bovine (*Bos indicus*) spermatozoal whole transcriptome profile recognized 14,306 genes expressed with FPKM>0, while 405 genes were expressed when threshold increased to FPKM>5. Amongst top 20 transcribed genes, most of the genes were identified as non-coding RNA (ten were lncRNA, one was SRP RNA, one was tRNA and one pseudo gene) (Raval *et al.,* 2019) the remaining eight were protein coding genes. Genes associated with biological processes such as translation, ribosomal small subunit assembly, cytoplasmic translation, translational elongation ribosomal large subunit assembly, maturation of SSU-rRNA from tricistronic rRNA transcript (SSUrRNA, 5.8S rRNA, LSU-rRNA), RNA processing, endonucleolytic cleavage to generate mature 30-end of SSU-rRNA from (SSU-rRNA, 5.8S rRNA, LSU-rRNA), ATP synthesis coupled proton transport, cell differentiation, ribosome biogenesis, were significantly (*P>0.05*) enriched in the spermatozoa. Proteins involved in the top clusters included ribosomal protein, rRNA binding, nucleotide binding protein, ubiquitin conserved site, ferritin conserved site, ATP synthesis coupled proton transport, cadherin binding involved in cell-cell adhesion were found in spermatozoa. They also compared highly abundant transcriptome from fresh semen sample from *Bos indicus* and cryopreserved sample from *Bos taurus* and concluded that only two transcripts were common viz. *PRM1* and *RPL37*. When compared the highly abundant transcripts between fresh semen sample of *Bos indicus* and *Bos taurus RPL23, RPL27A, RPS18, VGLL3, RPL6* were common whereas, when compared highly abundant transcripts with cryopreserved semen sample of *Bos indicus* and *Bos taurus C3H1orf189, CCDC91, COX5B, CYLC2, FTH1, MGC148328, RPL14, RPL38, RPS28, TMSB10* were common (Raval *et al.,* 2019).

Selvaraju *et al.* (2017) worked to identify the amount and composition of spermatozoal transcripts in fresh bull semen. To determine the possible role of transcripts associated with spermatogenesis, fertilization, and embryonic development, they used two platforms that were Ion Proton and Illumina

platform. 13,833 spermatozoal transcripts in fresh bovine (*Bos taurus*) semen were identified and upon the analysis of spermatozoal transcripts it was revealed that about 36% of the top 100 transcripts were full length with intact 5' and 3' ends. Various stages of spermatogenesis, spermatozoal function, fertilization and embryo development were found to be associated with many of these intact transcripts. The presence of intact transcripts of PAGs in the spermatozoa suggested their possible role in early embryonic development, during the study.

The transcripts *PRM1, CHMP5* and *YWHAZ* were the most abundant and were involved in male gamete generation. *YWHAZ* transcripts regulated the spermatogenesis and acrosomal reactions, whereas, *KIF5C* and *KCNJ6* was involved in microtubule function (Puri *et al.,* 2008). The *TNP1*, *RAD21*, *UBE2B* and *RAN* transcripts were highly abundant in spermatozoa and were involved in the crucial stage of spermatid development, wherein high-order chromosomal organization takes place(Lee *et al.,*2002). *TEKT1* protein located in the apical region of the acrosome and on flagella is important for spermatozoal cytoskeleton development and could play a key role during fertilization (Oiki *et al.,* 2014). Thymosin β10 and CAPZA3 involved in spermatozoa capacitation and spermatozoa egg fusion. Testis-specific protein kinase (*TSSK6*) and a metalloproteinase non-coding RNA (*ADAM5P*) was associated with high-motility of spermatozoa (Bissonnette *et al.,* 2009). It was also observed that spermatozoa consistently retained specific regions of particular transcripts in all animals with high read coverage. These retained regions could be either exonic or intronic. i.e., *GPS2* transcript retained its third exon with the high read coverage, whereas the *CHCHD2* transcript retained its 3′ intron sequence.

2.7 Transcriptome related to seminal plasma

Shilpa *et al.* (2017) reported the effectiveness of seminal plasma mRNAs as markers to evaluate the reproductive performance of bulls. They found that expression of *PRM1* was positively associated with the mitochondrial membrane potential of raw semen, whereas expression of Fas Ligand (*FASLG*) was negatively linked with sperm velocity, membrane integrity and chromatin

distribution in post-thaw semen samples. The percentage of Type-A spermatozoa in raw semen was positively associated with *BMP2*, ubiquitin conjugating enzyme E2D3 (*UBE2D3*), tumour-associated necrotic factor-associated death domain (*TRADD*) and caspase-3 (*CASP3*) expression. Nerve growth factor (*NGF*) expression was positively associated with the maintenance of post-thaw functional membrane integrity in spermatozoa and could be used to assess the cryotolerance of bull semen.

Prakash *et al.* (2021) performed a broad analysis of global differential gene expression between spermatozoa from high- and low-fertile crossbred bulls. They aimed at identifying the most relevant molecules for fertility prediction and to follow the reason behind crossbred bull infertility, using high-throughput RNA sequencing technique. Crossbred bull sperm contained transcripts for 13,563 genes, in which 2,093 were exclusive to high-fertile and 5,454 were exclusive to low-fertile bulls. A total of 176 transcripts were up-regulated (FC>1) and 209 were down-regulated (*P<0.05*) and they were associated with conception rate. The highly up-regulated sperm transcripts were *TPT1, RPL14, PFN1, DDX39B, RPL3, PABPC1, RPS8,* and *CD7 and* the highly down-regulated sperm transcripts in low-fertile crossbred bull were *ZNF706, PICK1, LUZP1, ANKRD9, RUNDC3A, LYRM4, FAM71F1*, and *EPOP,* both in low-fertile crossbred bulls. Proteins such as ribosomal protein L14 (*RPL14*) was found to be in abundance in the testis of *Bos taurus* and *Bos indicus* bulls (Selvaraju *et al.,* 2018; Raval *et al.,* 2019), Profilin 1 (*PFN1*) modulated actin showed involvement in oocyte maturation, fertilization, embryo development (Rawe *et al.,* 2006), and also spermatogenesis (Selvaraju *et al.,* 2018).

Protein interacting with PRKCA 1 (PICK1) showed involvement in cytoskeletal organization (Selvaraju *et al.,* 2017) and acrosome formation (Chen *et al.,* 2016), and its deletion resulted in globozoospermia (Liu *et al.,* 2019). Singh *et al*. (2019) also reported downregulation of PRKCA1. Wang *et al.* (2009) reported that ankyrin repeat domain 9 (ANKRD9) is involved in lipid metabolism and in astheno-zoospermic humans, it was down-regulated (Jodar *et al.,* 2013). FAM71F1 protiens were also detected in Leydig cells and was down-regulated in azoospermic males (Malcher *et al.,* 2013). They also found some novel genes in bull spermatozoa it included elongin BC and polycomb repressive

complex 2-associated protein (EPOP). They identified some of the important transcripts related to sperm development (*QKI, ODF1, TNP1, PRM2, CFDP1, TNP2, ODF2, SPEM1* and *MEA1*), spermatogenesis (*ODF1, BCL2L11, PRM2, TNP2, ODF2, SPEM1* and *MEA1*), cell differentiation (*QKI, ODF1, TNP2, ODF2, SPEM1*and *MEA1*), in utero embryonic development (*YBX1, UBE2B, BCL2L11, MYH10*and *RBBP6*), and oxidative phosphorylation pathway (*MT-ATP6, ND1, MT-ND2, MTND4, ND5, MT-CYB, COX1, MT-CO2*and*COX3*), which are important for sperm development, fertility and sperm cell interaction but their abundance was downregulated in low-fertile crossbred bull sperm. But on validating the NGS result via real time PCR they found that *ZNF706, CRISP2, TNP2* and *TNP1* genes was lower in low-fertile bulls than high-fertile bulls. It was presumed that impaired oxidative phosphorylation could be one of the main reasons for low fertility in crossbred bulls and that transcriptional abundance of *ZNF706, CRISP2, TNP2,* and *TNP1* genes could serve as potential biomarkers for fertility in crossbred bulls males (Malcher *et al.,* 2013).

Further Prakash *et al.* (2020, 2021) studied the profile and analyzed sperm transcripts using Next Generation Ribonucleic acid sequencing technology in Holstein Friesian x Tharparkar crossbred bulls. The results from global transcriptomic profiling discovered transcripts for 13,814 genes; of which 431 transcripts were expressed with >1 FPKM and 13,383 transcripts were expressed with >0 or <1 FPKM. In their study, the top ten abundant sperm transcripts were consisted of non-coding RNAs (rRNA, miRNA, miscRNA). The top ten transcript (FPKM>1) in their study coded for protein coding transcripts *PRM1, HMGB4, CCDC181, CHMP5, TNP2, RPS28, RPL37, TPT1, ENSBTAG00000047411* and *ANKRD9* genes. Protamine-1 (*PRM1*) was involved in cell differentiation, chromosome condensation, stabilizing sperm DNA and spermatogenesis (Oliva, 2006; Jodar and Oliva, 2014). It is one the most abundant transcript present in bull sperm and it has been confirmed by (Card *et al.,* 2013, 2017; Selvaraju *et al.,* 2017; Raval *et al.,* 2019; Singh *et al.,* 2019), High mobility group protein B4 (*HMGB4*) was the second most abundant mRNA transcript present in crossbred bull spermatozoa. The protein encoded by this gene is a potential transcriptional repressor with functional properties of chromatin binding (Catena *et al.,* 2009; Card *et al.,* 2013).

2.8 Transcriptome related to bull's fertility

A large number of transcriptomic studies on bull sperm using different techniques has been reported till date. In one of the reports by (Wang *et al*., 2019), where they used strand-specific RNA sequencing to ascertain the semen transcriptome including both long non-coding and mRNAs and to determine the functions these long non coding RNAs and mRNA in bull sperm motility. 20,875 transcripts of protein-encoding genes was detected by them in semen and 19 different mRNAs were found between high- and low-motility sperm. They also identified five differentially expressed genes, such *AS EFNA1, RBMX, MLPH, RPL30* and *AQP2,* which took part in extracellular exosome functions. They found that the ephrin A1 (*EFNA1*) protein was localized on cell surfaces and participated in membrane integrity and sperm morphology. High abundance of Efna1 has also been reported in both seminal plasma and sperm (Rego *et al.,* 2016; Kasimanickam *et al,* 2019) and also its role in sperm motility has been reported by (Wang *et al.,* 2019). Heterogeneous nuclear ribonucleoprotein G (Rbmx) is considered for its role in splicing that might further affect spermatogenesis (Delbridge *et al.,* 1999). Through immune-histochemical methods, it was found that aquaporin 2 (Aqp2) was expressed in male germ cells, seminiferous epithelium, Leydig cells and in the male reproductive tract (Yeste *et al.,* 2017), suggestive of that Aqp2 directly or indirectly involvement in male fertility.

Selvaraju *et al.* (2021) in his study identified sperm transcriptome to detect important transcripts regulated to male fertility. They collected semen samples from 47 bulls having varied fertility rate. Sperm RNA was isolated and subjected to transcriptome sequencing. On the basis of the expression pattern obtained, the bulls were classified into high-fertile and sub-fertile, and important transcripts controlling sperm functions and fertility were identified. After validation by real time PCR they reported the presence of 1100 to 1700 intact transcripts, of which *BCL2L11* and *CAPZA3* were found to be the most abundant and both of them were associated ($P<0.05$) with spermatogenesis and post-embryonic organ morphogenesis. They also observed that the up-regulated genes in the functional membrane and acrosome integrity groups were closely linked with the fertility rate. Through Gene ontology they found that the biological functions of these up-

regulated transcripts (*P<0.05*) in the high-fertile bulls were associated with spermatogenesis (*AFF4* and *BRIP1*), sperm motility (*AK6* and *ATP6V1G3*), capacitation and zona binding (*AGFG1*), embryo development (*TCF7* and *AKIRIN2*) and placental development (*KRT19*).

Li *et al.* (2021) used RNA SEQ technology to sequence high-throughput transcriptome of the Xiangxi and Simmental cattle's sperms by and screened the expression genes related to the development and reproduction of the sperms. A total of 4890 new genes were discovered, and out of 4890 new gens 2451 were annotated. One hundred fifty-five differentially expressed genes were selected, including 88 up-regulated genes and 67 down-regulated genes. The differentially expressed genes involved in biological processes accounted for 40.9%, cell components accounted for 42.5%, and molecular functions accounted for 16.7% KEGG pathway analysis, according to the GO analysis. It showed that the differentially expressed genes were more distributed in the three pathways of signal transduction, cancer-related and neurological diseases. Ten genes amongst the up-regulated genes were directly involved in signal pathway regulation, cell cycle regulation and cell proliferation and differentiation, which were *ETNK1, VDAC2, RICTOR TRIM36, SRSF5, SET, JMJD1C, RIOK3, HDAC1* and *ZNF280D*.

2.9 Sperm specific transcriptome and their role in early embryonic development

Di-methylated H3K4 (H3K4me2) and tri-methylated H3K27 (H3K27me3) histone modification profiles in spermatozoa of buffalo bulls are important for fertility. A total of 84 genes for H3K4me2 and 80 genes for H3K27me3 were found differentially enriched in sperm of high and sub-fertile buffalo bulls, respectively (Verma *et al.,* 2015). These differentially enriched genes were found to be involved in the processes of germ cell development, spermatogenesis and embryonic development. H3K4me2 modifications impact on transcriptional activation of the associated genes like *CDC45, DMC1* and *MLH1* implicated for imparting better fertilizing ability to the sperm of HF bulls, while other set of genes found in HF bulls involved in embryonic and fetal development viz. *PAX3, PRDM14, SOX4, SOX14* and *TBX15*.

Sperm delivers transcription factors and epigenetic components that are required for fertilization and proper embryonic development. Kropp *et al.* (2017) worked on sire's fertility in association with sperm and embryonic transcriptome profiles. IVF embryos were generated with high and low fertility Holstein bull and embryos derived from them were evaluated for morphology, development and transcriptome analysis. They observed that embryo morphology and developmental capacity did not differ however; RNA-sequencing revealed that 98 genes were differentially expressed. A total of 65 genes were up-regulated in high fertility bull derived embryos, and 33 genes were up-regulated in low fertility derived embryos. Assessment of the epigenetic signature of spermatozoa between high and low fertility bulls revealed 76 differentially methylated regions. The *CYCS, TFB2M,* and *MEPCE* all of which were highly expressed in embryos derived from high fertility sires. The genes *EEA1* and *SLC16A7*, which were highly expressed in embryos of low fertility sires.

Sperm is a better cell for mRNA transcription and embryonic gene regulation in mice (Vassena *et al.,* 2007; Ihara *et al.,* 2014). On comparing the development of sperm and spermatid derived embryos, it was observed that during spermatid maturation into sperm certain genes lose H3K4me2/3 and certain retain H3K27me3 epigenetic marks (Teperek *et al.,* 2016). Experimental removal of these epigenetic marks at fertilization de-regulates gene expression in the resulting embryos in a paternal chromatin-dependent manner. This demonstrated that epigenetic instructions delivered by the sperm at fertilization were required for correct regulation of gene expression in the future embryos. Embryos generated through spermatids showed a reduced developmental potential compared to sperm. Out of 18,340 expressed genes, 255 were differentially expressed in spermatid-derived embryos compared to sperm-derived embryos. The majority were up-regulated in spermatid-derived embryos, and they include transcriptional regulators *GATA2, GATA3, HES1*, and *FOS* as well as morphogens*BMP2, BMP7* and *DHH* essential for embryonic development.

Singh *et al.* (2019) provided a use fulax non-invasive tool to understand the causes as well as an effective way to predict male infertility in crossbred bull Frieswal (Holstein-Friesian X Sahiwal) through RNA deep sequencing. A total 15,46,561 and 10,19,308 numbers of reads were identified among good and poor quality bull spermatozoa based on their conception rate. Post mapping with *Bos taurus* reference genome they identified 1,321,236 and 8,42,022 number of transcripts among good and poor quality RNA libraries, respectively. However, a total number of 3,510 and 67,59 functional transcripts were identified among good and poor quality bull spermatozoa, respectively. Most of the identified transcripts were related to spermatozoa functions, embryonic development and other functional aspects of fertilization. They observed that the transcripts such as *CHMP5, GPX4, PPP1R42* had a direct relation to the sperm's function. They validated the NGS result with qPCR the top five selected transcripts (*AKAP4, PRM1, ATP2B4, TRIM71* and *SLC9B2*). Validation of the selected top five transcripts and revealed that *AKAP4, PRM1, ATP2B4, TRIM71* and *SLC9B2* were expressed significantly (*P<0.01*) in the good quality crossbred bull semen than the poor quality samples.

The blastocyst proteins derived from sperm RNAs include transcript factors, such as the *BBX*, the HMG-box containing (BBX) and the zinc finger protein 646 (*ZNF646*), as well as histone modifiers, such as the Ankyrin repeat domain 12 (*ANKRD12*), which might be able to regulate gene expression of the activated zygote genome. The GTPases activity essential for the cytoskeleton dynamics during early embryo development is regulated by some of these sperm RNAs encoding for proteins. It includes the Rho GTPase-activating protein 26 (ARHGAP26), the FYVE, RhoGEF and PH domain containing 4 (FGD4) and the signal induced proliferation associated-1 like-3 (SIPA1L3) (Duquette and Vane, 2014). Also the depletion of blastocyst centrosomal protein KIAA0586, in mice leading to embryos with abnormal left-right axis patterning has been predicted as paternally derived(Bangs *et al.,* 2011).

Proteins such as WNT4 which are crucial for murine early embryogenesis were predicted to be paternally derived as a result of sperm RNA translation in the zygote. DEAD-box helicase 3 Y-linked (*DDX3Y*) is yet another example of a functional sperm RNA. The sperm-borne *DDX3Y* transcript was found in freshly fertilized mouse zygotes, but not in oocytes. The number of male cleavage-stage embryos produced was reduced by microinjection of an antisense RNA which produced embryos with a lower cleavage rate. These studies revealed that during early embryonic development few sperm-borne RNAs may be are essential. *PKP2* and *CTTNBP2NL* N-terminal genes present in sperm, were associated with conception rates in Holstein cows. Knockdown of *PKP2* and over-expression of *CTTNBP2NL* each resulted in reduced embryo implantation rates in mice, demonstrating their paternal control over the presence of these RNAs in pre-embryonic genomic expression. Also, the cleavage of sperm RNAs by treatment with a RNAse drastically decreased the rate of blastocyst formation and the live birth rate of embryos produced by intra-cytoplasmic sperm injection (ICSI) compared to those obtained with untreated spermatozoa.

The comparative analysis on human sperm RNA profiles has helped to present the possible abnormal mechanisms responsible basically for male infertility. It revealed sperm molecular disturbances occurring during either spermatogenesis or sperm maturation. The reduction in the developmental potential of embryos generated with sperm containing disturbed sncRNAs profiles from testicular conditional knockouts of DICER and DROSHA, found that testicular conditional DICER knockout resulted in 15% miRNA up regulation, 32%, miRNAs down regulation, 2% endo-siRNAs up regulation and 6% endo-siRNAs down regulation. The embryos generated by this profile showed significantly reduced developmental potential at 2-cell, 4-cell, morula and blastocyst stage. Percentage of embryos reaching blastocyst stage also reduced and a lower ratio of live births was observed when mature oocytes were injected with sperm containing 90% less RNA than control. These negative outcomes were partially rescued when total RNA from the wild-type

sperm was injected into embryos derived from in vitro ART with RNA-deficient sperm (Guo *et al*., 2017). Apart from this, seminal plasma also have population of RNAs, whose expression in female reproductive tract, contributes in regulation of implantation or immune processes. The current studies on sperms reveals the presence of remarkable groups of transcripts, that may prove to be key players during fertilization and post-fertilization processes, occurring during the pre-implantation embryo development.

Given the emerging role of sperm transcriptome in various sperm functions and embryonic development, their use as biomarkers of male fertility, it is important to carry out exhaustive studies on cloned bulls with the sole purpose to identify sperm transcripts which are (i) expressed in spermatozoa of cloned bulls, (ii) expressed differentially between the cloned bull and its somatic cell donor parent. Till date, there is no report available on global transcriptome profile of spermatozoa of cloned animal with its parent in any farm animal species. The present work will be carried out to analyze the sperm transcriptome expression profile of semen of cloned buffalo bulls and its somatic cell donor bulls.

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CHAPTER – 3

Materials and Methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plasticware and Glassware

All the glasswares used in present investigation were made up of high-grade pyrex glass. They were thoroughly cleaned and rinsed with ultrapure water and then heated to sterilization at 250°C for 4h.The plasticware used, were pre-sterilized from the manufacturer itself. Centrifuge tubes (15 ml and 50 ml) were from Corning Incorporated, USA. Disposable micropipette tips (10 µl, 200 µl, 1000 µl) and 0.2 to 2 ml tubes were from Thermo Fisher Scientific (USA). Disposable plastic syringes, which were non-toxic and non-pyrogenic, were from Becton, Dickinson and Co. USA. The 0.22 µm filters, were from Millipore Corp., Bedford, USA. Plasticware such as microtips, eppendorf tubes etc., were made RNase free by soaking them in 0.1% diethyl pyrocarbonate (DEPC) overnight followed by autoclaving.

3.1.2 Chemicals

The chemicals accustomed for the present study were purchased from Sigma Chemical Co. (USA) unless otherwise stated. The washing of semen was performed using Bovipure and Bovidilute from Nidacon International AB**,** Sweden. RNA Later was procured from Invitrogen, USA. Total RNA extraction was carried out using TRI Reagent Sigma Chemical Co. (USA) and RNAqueous Micro Kit, Ambion, USA, while cDNA was synthesized using Revertaid first strand cDNA synthesis kit from Thermo Fisher Scientific (USA). Dream Taq green PCR master mix 2X for RT-PCR from Thermo Scientific (USA).

3.1.3 Equipments

3.1.3.1 Microscope

Inverted microscope (NIKON, Japan, Model TMD) equipped with 4x, 10x, 20x and 40x variable numerical aperture was used for examination of physical parameters of spermatozoa and for checking somatic cell contamination among the spermatozoa population. The microscope having differential interface

contrast (DIC) attachment and UV fluorescence, with light source and a long working distance condenser allowed spermatozoa to be viewed and photographed whenever needed. It was also equipped with digital photography.

3.1.3.2 Biosafety cabinet/Laminar air flow

Experiments including washing of sperm processing were performed in biosafety cabinet (ESCO, USA), which minimizing the error caused by any contamination. RNA extraction was carried out in separate biosafety cabinets. To assure the RNA quality laminar air flow (CLEANAIR Laminar Flow Systems, India) used for RNA extraction were wiped with RNase Zap to avoid any foreign RNA contamination.

3.1.3.3 Refrigerated centrifuge

For washing of semen and centrifugation of different samples etc. as and when needed refrigerated centrifuge (Hermle, Germany) with adjustable centrifugation speed, time and temperature was used.

3.1.3.4 Microcentrifuge

Micro centrifuge (Spinwnin, India) was used for centrifugation of small samples such as mixing of primers, used for PCR or real time PCR reactions mixtures.

3.1.3.5 Vortex shaker

To enhance homogenization of sperm pellet for proper mixing of reagent for sperm lysis, vortex shaker (Bio-Rad, USA) was used.

3.1.3.6 Dry bath

Heating of TRI reagent and incubation of homogenized spermatozoa with TRl reagent to enhance sperm lysis was performed using Dry bath (ELITE Major Science, USA) and also used in RNA isolation steps.

3.1.3.7 Thermal cycler

Complementary DNA (cDNA) synthesis from mRNA of cloned bull and somatic cell donor bull spermatozoa was performed through reverse transcription in

presence of reverse transcriptase enzyme required for amplification of genes of interest via a thermal cycler (C1000 Touch Thermal Cycler, Bio-Rad, USA). It was also used for performing all other PCRs such as gradient PCR for standardization of annealing temperature of primers, touchdown PCR. All PCR reactions gave 2^n number of DNA strands, where n=number of cycles.

3.1.3.8 Electrophoresis unit

The electrophoretic unit (Bio-Rad, USA) comprised of the buffer chamber, UV transparent tray, casting gates, comb set, safety lid with cables and power supply. Agarose gel electrophoresis was performed for the resolution of PCR products.

3.1.3.9 Gel documentation

Images of PCR products under bright light and UV light were captured using gel documentation system (Vilber Gel Imaging System, France).

3.1.3.10 Nanodrop

RNA qualification and quantification was performed using nanodrop (Thermo Scientific, USA). The sample loading stand of nanodrop was cleaned gently with nuclease-free water before and after use to avoid any chances of contamination.

3.1.4 Biologicals

Fresh semen of cloned bulls (MU-6253: Shresth; MU-6708: Swarn; MU-6923: Rajat) and non-cloned bulls (MU-6409, MU-6646 and MU-6942) were used for assessment of preliminary semen parameters. Cryopreserved semen straws (in liquid nitrogen at -196°C) of cloned buffalo bulls (MU-6253: Shresth; MU-6708: Swarn; MU-6923: Rajat) as well as their somatic cell donor buffalo bulls (MU-7263 from population because Shresth was produced from slaughterhouse based fetal cells, whose pedigree was not known, MU-5926: donor of Swarn, MU-4393: donor of Rajat) were used for isolation of RNA from spermatozoa and next generation sequencing work.

3.2 METHODS

3.2.1 Assessment of semen preliminary parameters

The fresh semen ejaculates of cloned bulls and non-cloned bulls were collected aseptically for comparison of semen preliminary parameters. Cloned and non-cloned bulls with similar age were grouped together as mentioned in the table-1 below:

Table 3.1: Cloned bull and non-cloned bull groups.

Group	Bull ID	Cloned/Non-cloned	D.O.B
1.	MU-6253 (Shresth)	Cloned	26.08.2010
	MU-6409	Non-cloned	09.02.2011
2.	MU-6708 (Swarn)	Cloned	18.03.2013
	MU-6646	Non-cloned	07.02.2013
3.	MU-6923 (Rajat)	Cloned	23.07.2014
	MU-6942	Non-cloned	23.08.2014

A total of six ejaculates from each animal were collected to perform the comparative analysis of following semen parameters of cloned versus non-cloned animals:

3.2.1.1 Volume

Semen collected aseptically by using artificial vagina method. Then semen volume of each sample was recorded and samples with semen volume equal or more than 2ml were further evaluated and processed.

3.2.1.2 Sperm concentration

Automated photometer sperm cell counter (IMV, L'Aigle, France), was used to resolve the sperm concentration for each sample. Semen samples with concentration less than 500 million sperms/ml were discarded. *The total sperm count = sperm concentration x volume (ml) of ejaculate.*

3.2.1.3 Mass motility

In order to determine the mass motility, a drop of undiluted semen was placed on grease free glass slide and covered with cover-slip. The slide was placed on thermal stage and was observed under 10x DIC phase contrast microscope. Gross Swirl Rating (GSR) was made use to rate the semen sample of each animal on scale of 0-5. The samples with a score above 2.5 were selected for further assessment.

Sr. No.	Semen activity characteristics	Motility score
1.	Rapid waves and swirls	+5
2.	Less rapid swirls and eddies	+4
3.	Swirls are slowly scattered in the field	+3
4.	Swirls are absent. Individual movement of spermatozoa are more evident from the field	+2
5.	No wave motion observed	+1
6.	Spermatozoa are immobile	0

3.2.1.4 Progressive motility

For progressive motility, the semen samples were diluted in ratio of 1:1 with semen extender. A 10 µl of diluted semen was placed on a warm (37°C) slide and observed under 20xDIC phase contrast microscope. The samples with progressive motility more than 75% were selected for the study.

3.2.1.5 Sperm viability

For determining sperm viability 5 µl of diluted semen was mixed with 15 µl of eosin-nigrosine staining solution. A small amount of this mixture was taken on the edge of a slide and then a thin smear was prepared by spreading this on another clean slide. On complete drying, the slide was observed under bright field at 100x and 200 spermatozoa were counted. Unstained sperms appeared white as they did not take the eosin nigrosine stain and were classified as non-eosinophilic (live sperms) while those that took the stain showed a slight pink

or reddish appearance and were classified as eosinophilic (dead). *Viability (% live spermatozoa) = (Number of live spermatozoa x 100)/ Total number of spermatozoa (200).* The semen samples with minimum 65% viability were considered for further assessment.

3.2.1.6 Sperm membrane integrity

Sperm membrane integrity was determined by hypo-osmotic swelling test (HOST) test. 1 ml control solution and 1 ml HOST solution (Annexure-I) were mixed with 0.1 ml of semen and incubated at 37°C for 1 h. Following incubation, a drop of well mixed solution was taken from both control and HOST mixtures on a clean dry glass slide and covered it with a cover slip. The fluid influx causes the sperm tail to swell. Sperm tail curling was recorded by counting total of about 200 spermatozoa in different fields at 40x magnification. Proportion of coiled sperm in both control and HOST fraction were calculated as % coiling = (Number of reacted spermatozoa x 100)/ Total number of spermatozoa (200).Spermatozoa were classified as per cent HOS responsive according to the presence of the tail swelling patterns (Prasad *et al*., 1999) as mentioned below:

Pattern-A	:	No swelling-complete loss of membrane integrity.
Pattern-B	:	Swelling at the tip of the tail.
Pattern-C	:	Different types of hair-pin like swelling
Pattern.-D	:	Complete tail swelling.

The sperm cells displaying the swelling pattern B, C and D were considered positive for HOST. Actual proportion of Hypo-osmotic swelling responsive spermatozoa was obtained as follows: *HOS responsive spermatozoa (%) or (%membrane integrity) = % Coiling in HOST solution - % Coiling in control solution.* The semen samples with minimum 40% (frozen semen) membrane integrity were considered in the study.

3.2.1.7 Acrosome membrane integrity

Spread a thin smear of extended semen on glass slide. Dried slide kept in Hancock's solution for fixation for about 15 min. After fixation the slide was

washed gently under running tap water for 5 minutes and dried. The slide was then buffered in Giemsa solution for 90 min and rinsed and dried. The smears were observed under 100x magnification. About 200 spermatozoa were counted for estimating acrosomal status after staining. *The proportion of spermatozoa with integrated acrosome were calculated as % Acrosome integrity = (Number of spermatozoa with integrated acrosome x 100) / Total number of spermatozoa (200).*

3.2.2 Semen processing

RNA extraction was done from pure spermatozoa population of somatic cell donor buffalo bull and its cloned bull for which semen samples were processed as below:

3.2.2.1 Sperm washing

For semen washing bovipure and bovidilute containing tubes were prepared and they were incubated for least for 30 minutes at 38°C in dry bath. 500 µl of 80% bovipure in bovidilute was transferred to the micro centrifuge tube. Then 500 µl of 40% bovipure in bovidilute was transferred on the top of it. Two semen straws were thawed at 37°C and the contents were slowly placed on top of the prepared gradient. An aliquot from each semen straw was visualized under inverted microscope to monitor parameters such as spermatozoa motility and concentration. The tubes were centrifuged at 300xg for 15 minutes at room temperature. The supernatant containing all the debris was discarded and the formed pellet was transferred to a new tube containing 1 ml of filtered DPBS. The tube was again centrifuged at 300xg for 5 minutes, the supernatant was discarded and the pellet obtained was used further.

3.2.2.2 Somatic cell lysis

The washed sperm was treated with 500 µl somatic cell lysis buffer (SCLB), vortexed for 2 minutes and kept in ice for 30 minutes for somatic cells lysis present in the spermatozoa population. 1 ml of DPBS was added and an aliquot from SCLB treated semen was observed under inverted microscope to check somatic cell contamination. In order to remove lysed somatic cell debris,

mixture was washed with DPBS by centrifuging at 4000 rpm for 5 minutes at room temperature. The supernatant containing lysed somatic cell debris were discarded and the sperm pellet was collected in 1.5 ml tube.

3.2.3 Extraction of total RNA from spermatozoa

3.2.3.1 RNase-free plasticware

RNase-free plasticware was prepared by immersing the tubes and tips in 0.1% DEPC (Diethyl pyrocarbonate) overnight at 37˚C over a magnetic stirrer. The solution was prepared by adding 1.0 ml of DEPC/ liters of distilled water and then mixing vigorously over a magnetic stirrer. The solution was prepared fresh every time. DEPC has been reported to destroy the enzymatic activity of ribonuclease by modifying -NH, -SH, -OH group in RNase (Ambion, USA). DEPC is also a known carcinogen, therefore, extra precaution was taken and gloves were worn every time it was handled. The DEPC solution with immersed materials was stirred 3-4 times so that all tips and tubes were soaked completely in the solution. Next day, the solution was drained off and the treated material was dried after wrapping in aluminum foil in hot air oven. Tips and tubes were not touched with anything and gloves were used while handling. After drying, tips were carefully filled in the tip boxes and tubes were filled in jars and then the materials were double autoclaved to remove the remaining traces of DEPC which can otherwise interfere with the RT reaction during cDNA synthesis. Prior to using the laboratory surfaces e.g., work table, bench tops, laminar hoods centrifuge and electrophoresis tanks, the surfaces were decontaminated by wiping it down with RNase inhibitor solution, RNAZap (Ambion, USA).

3.2.3.2 Spermatozoa lysis

Spermatozoa was lysed as per Darbandi *et al*. (2018) with slight modifications. Briefly, for spermatozoa lysis 1.5 ml tubes containing spermatozoa pellet were placed in liquid nitrogen for 5 min. The tubes were carefully taken out from the liquid nitrogen and spermatozoa pellet was crushed vigorously with the help of micropestle to homogenize sperm pellet. After that 250 µl of TRI reagent, 3 µl of β-mercaptoethanol and 250 µl of lysis solution (RNAqueous Micro Kit,

Ambion, USA) was added and the solution was repeatedly passed through 26 gauze syringe, vortexed for 2 minutes and incubated at 65°C for 30 min. During this incubation period, the spermatozoa were vortexed thrice for 3 min. At the end of this incubation period the tube content was observed under inverted microscope to check for spermatozoa lysis.

3.2.3.3 RNA isolation

RNA binding: RNA isolation was done using RNAqueous Micro Kit, Ambion, USA. Briefly, sperm lysate mixture was prepared by mixing 500 µl of above spermatozoa lysis solution with 250 µl of 100% ethanol. 150 µl of the lysate mixture was loaded onto micro-filter cartridge assembly and was centrifuged at 14000 rpm for 10 s at room temperature. The lysate mixture was loaded repeatedly into the same micro-filter cartridge assembly until the entire lysate had passed through the filter. RNA was bound to filter in micro-filter cartridge. The flow through was discarded.

RNA elution:The micro-filter cartridge bound with RNA was washed with 180 µl of wash solution-1 bycentrifugation at 14000 rpm for 10 s at room temp. Again the micro-filter cartridge was washed twice with wash solution 2/3 by centrifuging it at 14000 rpm for 10 s at room temp. The flow through obtained in the collection tube was discarded. Again the micro-filter cartridge was centrifuged at 14000 rpm for 1 min. at room temperature to remove the residual fluid. The micro-filter cartridge was then placed in hot air oven for drying of the filter. After drying the micro filter cartridge was fitted with new collection tube. Pre heated (75°C) elution solution 10 µl was placed at the center of filter. The assembly was kept for 1 min. at room temperature and then centrifuged for 30 s to elute RNA from the filter. The elusion process was again repeated with 10 µl of elution solution.

DNase treatment: DNase inactivation was carried out to remove trace amount of contaminating genomic DNA. For that 2 µl of 10x DNase buffer and 1 µl of DNase-1 was added to the eluted RNA. The reaction sample formed was incubated for 20 min. Meanwhile DNase inactivation reagent was thawed and vortexed vigorously to complete resuspend the slurry. 2.3 µl of DNase

inactivation reagent was added to reaction sample and kept at room temperature for 2 min. The reaction mixture, then was centrifuged at 14000 rpm for 90 s at room temperature to pellet the DNase inactivation Reagent. The supernatant containing the RNA was collected in a fresh RNase free tube and was stored at -20°C for further use.

3.2.3.4 RNA quantitation

RNA was quantified using nanodrop (Thermo Scientific, USA). For this purpose, first the nanodrop was washed with 2 µl of nuclease-free water. The device was blanked by 2 µl of elution buffer followed by loading 2 µl of isolated RNA sample. RNA concentration in per microliter and 260/280 ratio was noted down.

3.2.4 Synthesis of complimentary DNA (cDNA)

cDNA was prepared by using Revertid First-Strand Kit (Invitrogen, USA). The reaction mixture is prepared as follows:

RT-PCR reaction mixture-I

Components		**Volume**
Oligo (dT)	:	1.0 µl
RNA	:	8.0 µl
Water	:	3.0 µl
Total	**:**	**12.0 µl**

This above reaction mixture was incubated at 65°C for 5 min. in thermal cycler then transferred in ice for 1 min. Then the following components were added:

RT-PCR reaction mixture-II

Components		**Volume**
DNTPs	:	2.0 µl
Buffer	:	4.0 µl
Ribolock	:	1.0 µl
MMLVRT	:	1.0 µl
Total	**:**	**8.0 µl**

This reaction mixture-II was mixed with reaction mixture-I and incubated again at 42°C for 60 min and 70°C for 5 min in a thermal cycler. The synthesized cDNA was stored at -20°C till further use.

3.2.4.1 Confirmation of cDNA synthesis

cDNA formation was confirmed by PCR amplification of GAPDH gene (housekeeping gene). The reaction mixture was prepared as follows:

Preparation of GAPDH PCR reaction mixture

Components		**Volume**
Dream Taq Green PCR Master Mix (2X)	:	12.5 µl
Forward primer	:	0.3 µl
Reverse primer	:	0.3 µl
Template cDNA	:	1.0 µl
Nuclease free water	:	10.9 µl
Total	**:**	**25.0 µl**

The PCR cycling conditions for GAPDH gene were 95°C for 5 min, followed by a cycling programme of 95°C for 30s, 60°C for 30s and 72°C for 30s for 40 cycles. The reaction ended with a final extension at 72°C for 10 min. PCR negative controls were set for all the PCR reactions. PCR products were loaded in 2% agarose gel containing 0.5 µg/ml of ethidium bromide and were run at 100 V for 30 min. and gel was visualized by gel documentation system.

Preparation of agarose gel: For electrophoresis, 0.80 gagarose powder was mixed with 40 ml 1x TAE buffer in conical flask. The contents were boiled and the flask was swirled from time to time till agarose was completely dissolved. Ethidium bromide (0.5 µg/ml) was added to it when temperature reached down to around 50°C. Gel tray was placed in casting tray holder. Comb (5.2 cm long, and 4 mm width) was inserted in such a way that at least 0.5 mm (in length) of the comb remained above the gel tray. The warm (~50°C) agarose solution was carefully poured in tray containing a comb and bubble formation was avoided to obtain gel thickness of about 3-5 mm. The gel was allowed to solidify resulting into a matrix. The gel solidified within 20-30 minutes after pouring and was ready to use. A little amount of buffer (1x TAE) was layered over the solidified gel and then comb was gently removed. The gel was placed at appropriate place in electrophoresis tank containing 1x TAE buffer to completely submerge the gel.

Preparation of samples and electrophoresis: 10 µl amplified PCR products were loaded into the agarose gel wells. Separate tip was used for each sample. A 100 bp DNA ladder (Thermo Scientific, USA) was also made to run along with PCR products for the confirmation of the desired product size. After loading the sample, electrophoresis was carried out at constant voltage (100 V) at room temperature for 20-30 min. The progress of electrophoresis was judged by visualizing the migration of dyes present in loading buffer (Bromophenol blue and Xylene cyanol). The gel was handled after wearing gloves and then photographed by Gel Doc imaging system (Vilber, France).

3.2.4.2 Sperm cDNA purity confirmation

To check the purity of sperm cDNA and to assure the absence of genomic DNA and contamination of other cell types, RT-PCR was set up using cell specific primers. i.e. *SRY* gene (136 bp) and *DZL* genes (148 bp) (sperm specific), *V-kit* (somatic cell specific), *CD4* (Leucocyte cell specific), *CDH1* (Epithelial cell specific), Table-1.The reaction mixture was as follows:

Preparation of PCR reaction mixture

Components		**Volume**
DreamTaq Green PCR Master Mix (2X)	:	12.5 µl
Forward primer	:	0.3 µl
Reverse primer	:	0.3 µl
Template cDNA	:	3.0 µl
Nuclease free water	:	8.9 µl
Total	**:**	**25 µl**

3.2.5 Next Generation Sequencing (NGS)

Global transcriptome analysis of sperm cell of cloned bulls and their somatic cell donor bulls was done using NGS by a commercial service provider, DNA Xpert, New Delhi, India.

3.2.5.1 Transportation of RNA samples

The isolated RNA samples of cloned bulls and their somatic cell donor bulls were transported to sequencing company on dry ice to minimize RNA degradation.

Table-3.2: List of primers to check sperm purity.

Gene	Primer sequence	Product size (bp)	Annealing temp. (°C)	Accession No.
SRY	F- TGTAGAGACATTGCACCCCT	136	56	DQ119747.1
	R- GCTGCTGTGATGCTCCTTTT			
DAZL	F- AAGGCAAAATCATGCCAAAC	148	57	EU106872.1
	R- CCATAGCCTTTGGACACACC			
V-KIT	F- GACCTGGAGGACTTGCTGAG	316	60	XM_612028
	R- AGGGGCTGCTTCCTAAAGAG			
CD4	F- CAATGGCAAAGTCCTGTTGG	184	58	AJ535319
	R- GATCTGAGACATCCGTTCTGC			
CDH1	F- CCGTGAGAGTTTTCCCACAT	296	58	NM_001002763.1
	R- CATTGGTGACTGGGTCTGTG			
GAPDH	F- GGGTCATCATCTCTGCACCT	176	60	NM_001034034.2
	R- GGTCATAAGTCCCTCCACGA			

3.2.5.2 RNA quality check (QC)

RNA quality control analysis was carried out using Agilent 2100 Bioanalyzer (Agilent technologies, CA, USA) using Agilent Total RNA 6000 pico series kit. RNA samples with RIN value ranging from 6 to 9 were used further for library preparation.

3.2.5.3 Library preparation and sequencing

Equimolar amounts of each RNA sample of cloned bulls and their somatic cell donor bulls were taken for construction of cDNA libraries using the SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio USA, Inc.) according to the manufacturer's instructions. The library was prepared in paired end 2x100bp format with average fragment length of approx. 380bp. The quality control of the library was done using Agilent DNA High Sensitivity 1000 kit. The sequencing run was carried out on Illumina HiSeq 2500 instrument. Quality check of raw reads was carried out using FastQC. Based upon Phred score value, all the reads were found to be of good quality, therefore, so no filtering was required for any of the samples. The QC-passed high-quality reads generated (Phred Score Cut off of Q 20) were used for further analysis.

3.2.5.4 RNA-seq data analysis

HISAT2 and StringTie software was used for analysis of raw data. The RNA-seq data analysis workflow employed in the present study consisted of following major steps viz., loading of raw data into DESeq2 software, normalization of data, quality check of data, identification of differentially expressed genes based upon fold change criteria, classification/clustering of genes. The reads generated were aligned to *Bos Taurus* reference genome, UMD 3.1.1.The gene ontology enrichment analysis and pathway analysis was done by using Panther Classification System and Web based Gene set analysis Toolkit software. Gene Ontology (GO) provides descriptions of the molecular functions, biological processes and sub-cellular locations attributed to gene products from all organisms. The ontology covers three domains: **Molecular function:** The elemental activities of a gene product at the molecular level, such as binding or catalysis. **Biological process:** operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of integrated living units: cells, tissues, organs, and organisms. **Cellular component:** The parts of a cell or its extracellular environment.

Pathway analysis: To determine pathways associated with differentially expressed genes, pathway analysis was performed by Web based Gene set analysis Toolkit software. Pathway analysis is a promising tool to identify the mechanisms that underlie adaptive physiological compensatory responses and new avenues for investigation. Pathways are collections of genes and proteins that perform a well-defined biological task.

3.2.6 Statistical analysis

Statistical analysis was performed using SPSS version 9.0 (SPSS Inc. USA) and differences between mean percentages were analysed by students -'t' test. A value of $P<0.05$ was considered to be statistically significant.

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CHAPTER – 4

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Comparative analysis of semen preliminary parameters of cloned and non-cloned bulls

The fresh semen of cloned and non-cloned bulls was used for preliminary semen analysis and a number of parameters were evaluated (Table 4.1). The data obtained showed that there was no significant difference (*P<0.05*) in semen parameters of cloned and non-cloned bulls semen.

Table 4.1: Comparison of physical parameters of fresh semen of cloned bulls and non-cloned bulls.

Semen	Cloned bulls	Non-cloned bulls	P-value
Volume (ml)	2.82±0.33[a]	2.44±0.23[a]	0.35
Spermatozoa conc. (million/ml)	1607.56±161.90[a]	1589.44±211.76[a]	0.95
Total spermatozoa count (million)	4306.00±463.94[a]	3638.47±328.66[a]	0.26
Mass motility	3.17±0.08[a]	3.11±0.20[a]	0.80
Progressive motility (%)	85.56±1.00[a]	85.56±1.76[a]	1.00
Viability (%)	90.39±1.79[a]	90.87±2.50[a]	0.88
Acrosome integrity (%)	85.39±1.66[a]	87.61±1.80[a]	0.38
HOST (%)	79.61±1.76[a]	83.22±1.82[a]	0.17

Values quoted as Mean±SEM. Values with different superscripts within the same row differ significantly (P<0.05).

4.2 Processing of semen

4.2.1 Semen washing and collection of pure spermatozoa

The spermatozoa pellet obtained from both cloned bull (Mu-6253, Mu-6708 and Mu-6923) as well as parent bull (Mu-7263 from population, Mu-5926 and Mu-4393) after bovipure density gradient centrifugation and washing with DPBS was treated with somatic cell lysis buffer (SCLB). The somatic cell population

in semen was highly reduced by SCLB which was composed of 0.5% Triton X-100 and 0.1% SDS. The reason for somatic cell lysis was increased permeability of cell membrane by Triton X-100 and denaturation of proteins by SDS. The observation of samples under inverted microscope before (Figure 4.1) and after somatic cell lysis, there were no somatic cell contamination in spermatozoa population (Figure 4.2).

4.2.2 Spermatozoa lysis

For spermatozoa lysis a number steps were followed. These steps involved initial exposure of spermatozoa to a very low temperature of -196°C of liquid nitrogen, thus causing cracks and injuries to sperm membrane structure. Then the sperm pellet was homogenized by vigorous crushing with the help of micropestle. After that spermatozoa were lysed by adding lysis buffer, pre-heated Trizol and β-mercaptoethanol (BME) and incubating the content for minimum 30 min at 65°C. An upsurge in sperm lysis was observed on vortexing the contents during the incubation period. The addition of BME played an important role in lysis of spermatozoa as it reduces the disulfide bonds present in sperm membrane proteins thus enhancing the process of spermatozoa lysis.

4.2.3 Total RNA isolation from buffalo bull spermatozoa

Total RNA Isolation from spermatozoa of cloned bulls and their somatic cell donor bulls was done using RNAqueous Total RNA Isolation Kit (Invitrogen, USA). 20 µl elution buffer was used to elute the RNA bound to microfilter cartridge. The quality and quantity of RNA isolated from spermatozoa of cloned bulls and their somatic cell donor bulls is given in the Table 4.2.

Table 4.2: Concentration of sperm RNA.

Source	RNA (ng/µl)	O.D.
Cloned buffalo bulls	102.19±4.49	1.59±0.03
Somatic cell donor bulls	109.73±8.05	1.70±0.08

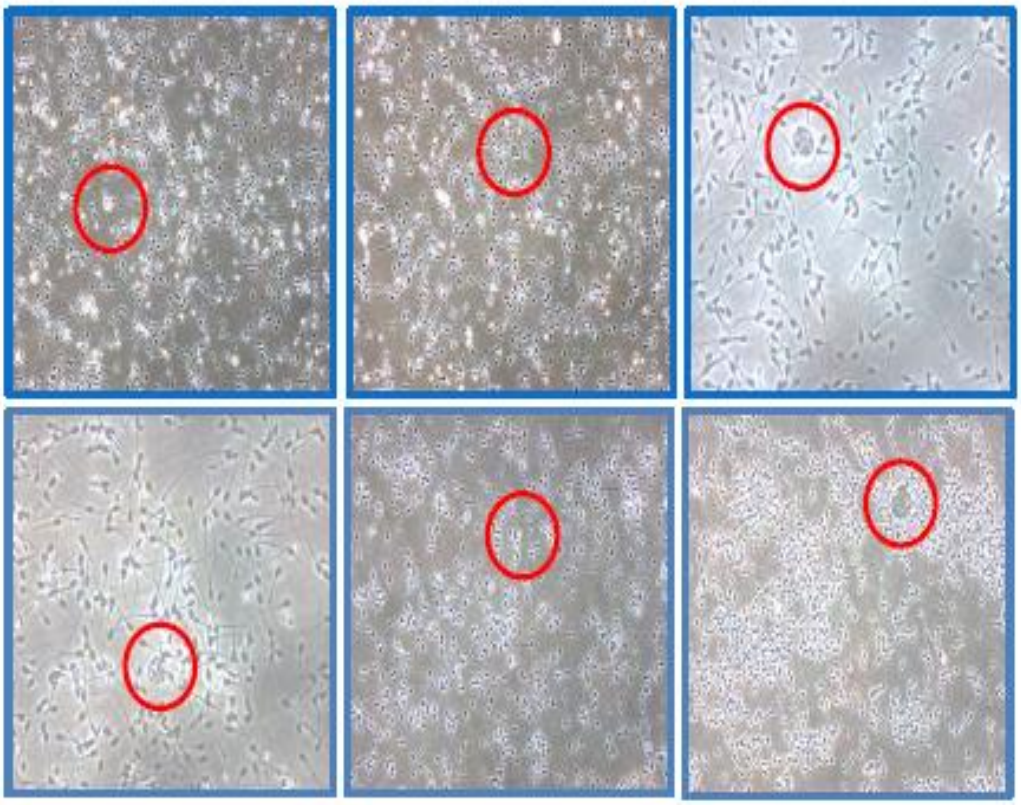
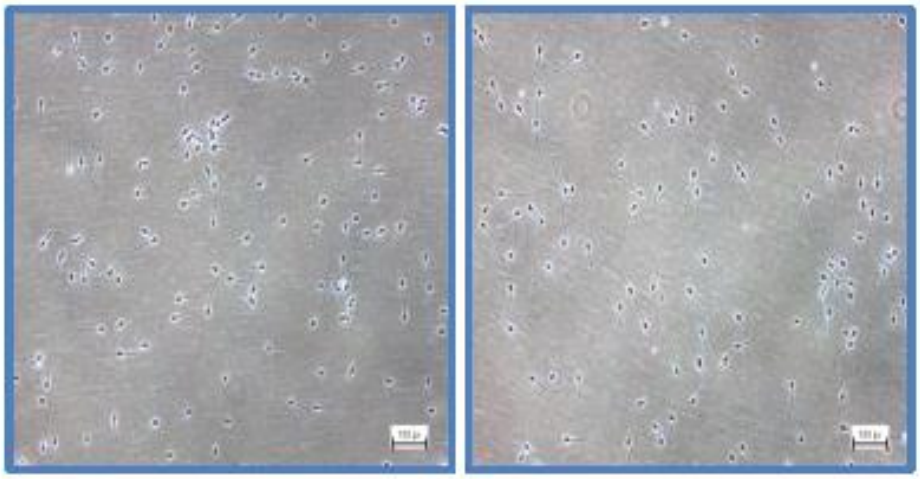


Figure 4.1: Spermatozoa population in semen sample before treatment with somatic cell lysis buffer. Somatic cell is shown enclosed in red color circle.



Cloned buffalo bull | Somatic cell donor bull

Figure 4.2: Spermatozoa population in semen sample after treatment with somatic cell lysis buffer.

4.2.4 cDNA synthesis

cDNA synthesis was done using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). cDNA synthesis was confirmed by the amplification of housekeeping *GAPDH* gene. 1.5% agarose gel was used to the amplified *GAPDH* gene. A clear band of 176 bp (Figure 4.3) for the *GAPDH* gene in the agarose gel confirmed the synthesis of cDNA from RNA obtained from the spermatozoa of cloned bulls and parent bulls.

4.2.5 Confirmation of somatic cell removal

To confirm the removal of somatic cells from sperm population amplification of sperm specific gene and somatic cell specific *V-KIT* gene was performed. The products were visualized on 1.5% agrose gel. A single band each for *SRY* gene and *DAZL* and no band obtained for *V-KIT*, *CD4* and *CDH1* gene clearly showed that the sperm population was free of somatic cells (Figure 4.4 and 4.5).

4.3: NGS of spermatozoal transcripts of cloned and somatic cell donor bulls

4.3.1 Total RNA isolation from spermatozoa for NGS

For transcriptome analysis of spermatozoa two main groups were made consisting of cloned bulls and parent bulls (somatic cell donor bulls). Each group consisted of three biological replicates MU-6253, MU-6708 and MU-6923 forming the cloned animal group and the parent group comprised of MU-7263 from population, MU-5926 and MU-4393 which were the somatic cell donor group. For each replicate RNA isolation from spermatozoa was done by pooling $30x10^6$ spermatozoal cells. RNeasy Plus Universal Mini Kit was used to isolate RNA from spermatozoal cells of each replicate. The yield of RNA varied from 116 ng/µl to 204 ng/µl. The quality of RNA was confirmed by the commercial RNA-seq service provider using Bioanalyzer. The RNA Integrity Number (RIN) values for all the samples was between 7.0 to 7.5 (Table 4.3) and electropherograms showing bands of 28S rRNA and 18S rRNA (Figure 4.6) clearly reflected that the RNA isolated was intact and of good quality, and these RNAs were further used for NGS.

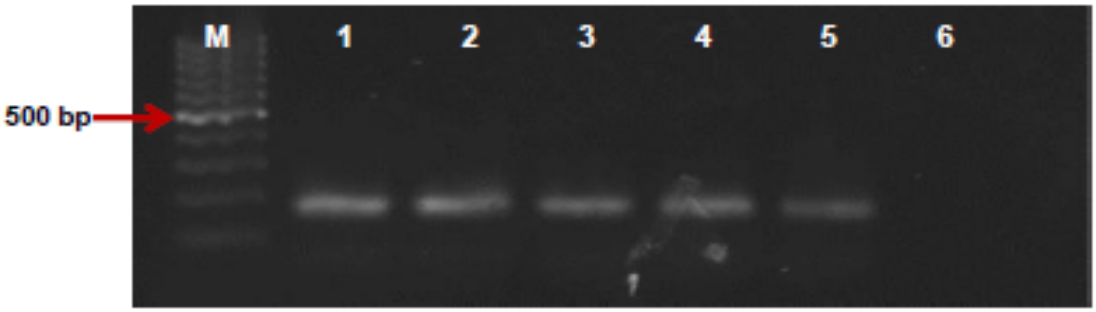


Figure 4.3: Amplification of *GAPDH* gene (176 bp) in RNA of cloned buffalo bulls (Lane- 1 to 5), Lnae-6: NTC, and Lane-M: 1000 bp ladder.

Figure 4.4: Amplification of spermatozoa specific genes *SRY* (Lane- 1; 136 bp) *DAZL* (Lane- 2; 148 bp) and no amplification of somatic cell specific *V-KIT* gene (Lane- 3; 316 bp) leukocyte specific cell CD4 (Lane- 4; 184 bp), epithelial cell CDH1 (Lane-5; 296 bp), NTC (Lane- 6), GAPDH (Lane- 7; 176 bp) in the purified spermatozoa of cloned bulls; Lane M: 1000 bp ladder.

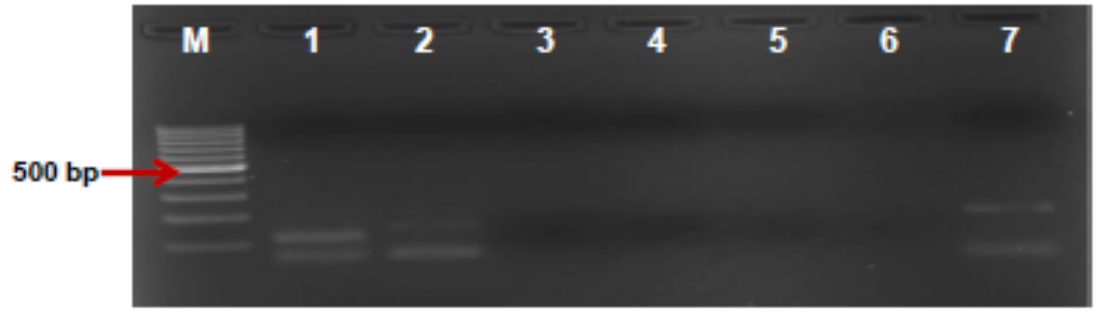


Figure 4.5: Amplification of spermatozoa specific genes *SRY* (Lane- 1; 136 bp) *DAZL* (Lane- 2; 148 bp) and no amplification of somatic cell specific *V-KIT* gene (Lane- 3; 316 bp) leukocyte specific cell CD4 (Lane- 4; 184 bp), epithelial cell CDH1 (Lane-5; 296 bp), NTC (Lane- 6), GAPDH (Lane- 7; 176 bp) in the purified spermatozoa of somatic cell donor bulls; Lane M: 1000 bp ladder.

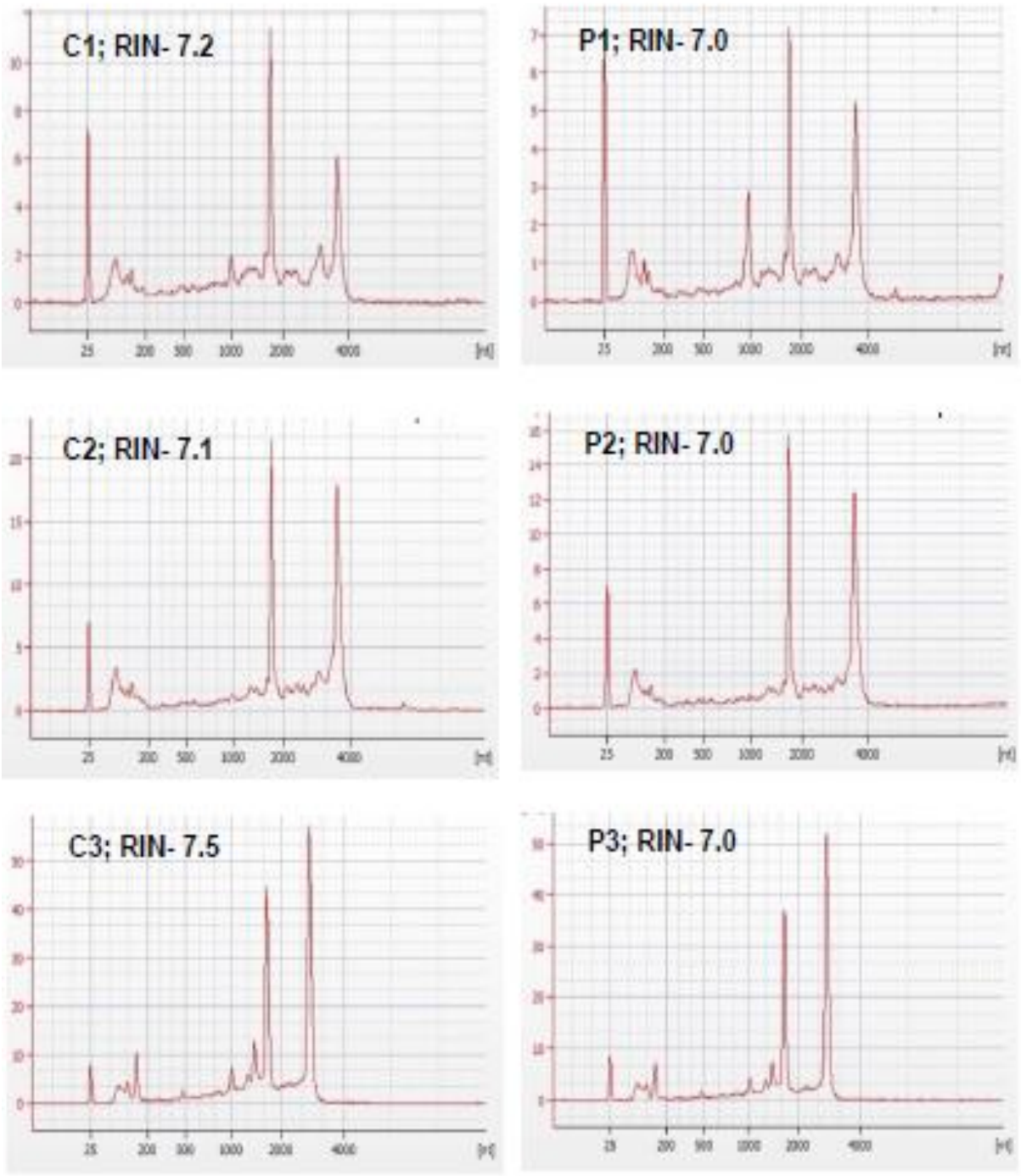


Figure 4.6: Electropherogram images depicting the quality of RNA isolated from spermatozoa of cloned bulls and parent bulls. The RIN values and the 18S rRNA and 28S rRNA peaks determine the intactness of the RNA isolated from replicates C1 (MU-6253); C2 (MU-6708); C3 (MU-6923) of cloned bulls spermatozoa and replicates P1 (MU-7263); P2 (MU-5926); P3 (MU-4393) of parent bulls spermatozoa, respectively.

Table 4.3: The quantity and quality of RNA obtained from spermatozoa of cloned bulls and somatic cell donor bulls.

Semen	Replicate	Concentration ng/µl	RIN Value
Cloned bulls	MU-6253	194	7.2
	MU-6708	134	7.1
	MU-6923	190	7.5
Somatic cell donor bulls	MU-7263	147	7.0
	MU-5926	204	7.0
	MU-4393	116	7.0

4.3.2 cDNA library preparation, quality check and sequencing

After examining the quality of RNA, cDNA libraries were prepared for each biological replicate using the Tru Seq Small RNA Library Preparation Kits in single end format. Then sequencing of these cDNA libraries was done using Illumina sequencer. Quality control of raw reads obtained was done using FastQC tool. The FastQC reports give Phred scores on Y axis and tells about the probability of occurance of a particular base at a specific position in the reads on X-axis. The Phred score below 30 shows bad quality data and requires filtering. The Phred scores for all the reads in our study were above 30 and hence required no filtering (Figure 4.7). The good quality reads obtained after QC were then aligned to *Bos taurus* reference genome UMD3.1.1 for further analysis.

4.3.3 Spermatozoal transcriptome analysis of cloned versus parent bulls

4.3.3.1 Read alignment statistics

Patman software was used to align the reads obtained against *Bos taurus* reference genome UMD 3.1.1. The read alignment statistics for all replicate of cloned as well as parent bull spermatozoa groups is provided in the Table 4.4. For cloned bulls MU-6253, MU-6708 and MU-6923 the total reads that got aligned were 83.68%, 87.39% and 76.76% respectively. Simultaneously, forpaents bulls the MU-7263, MU-5926 and MU-4393 the total reads that got aligned were 77.3%, 88.25%, 80.14% respectively (Figure 4.8).

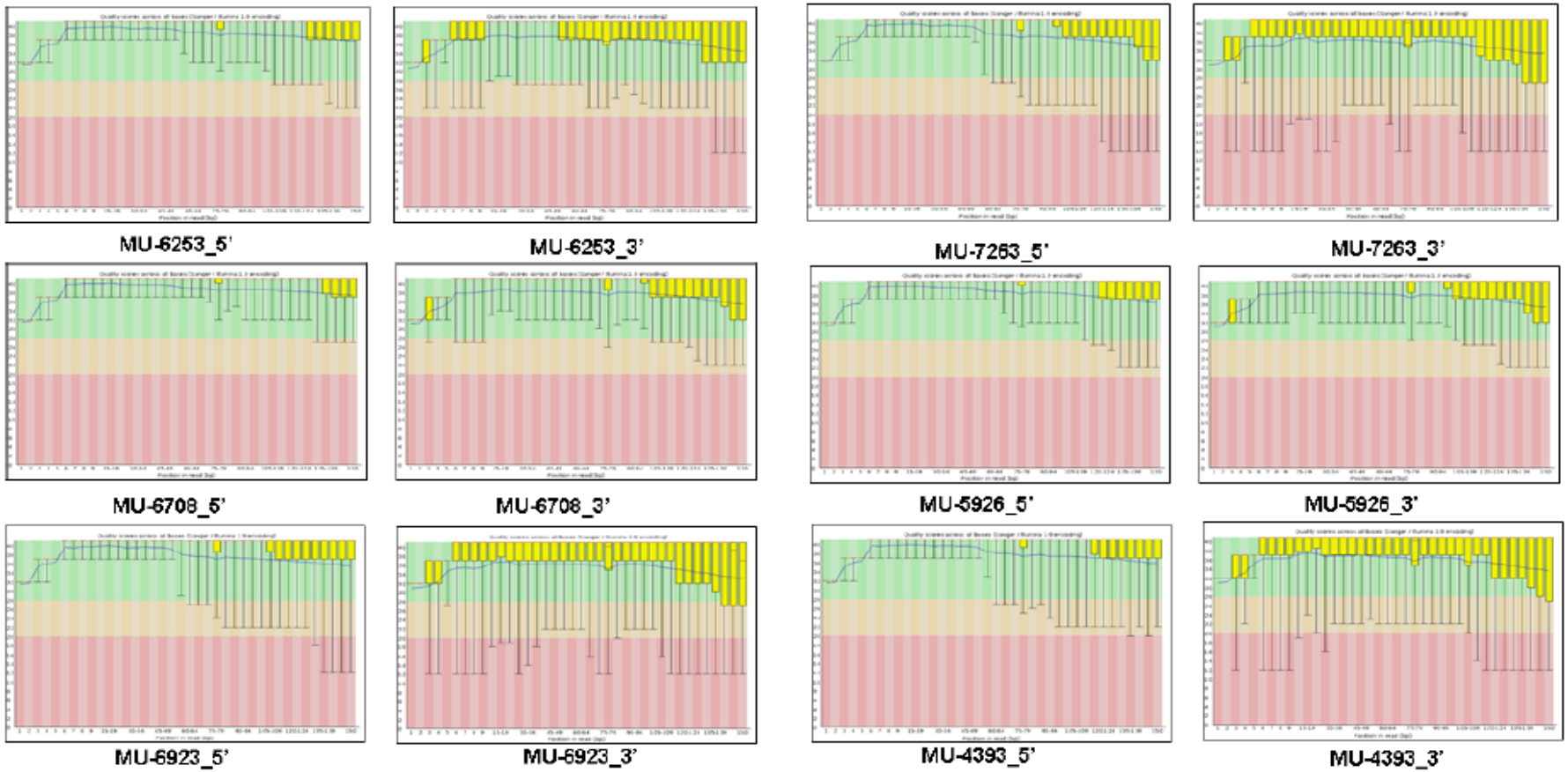


Figure 4.7: FastQC report showing quality of single end reads of cloned bulls spermatozoa (MU-6253, MU-6708 and MU-6923) and somatic cell donor bulls spermatozoa (MU-7263, MU-5926 and MU-4393). Phred score is represented on (y-axis) and probability of occurrence of a particular base at specific position is represented on (x-axis). Green, orange and red colors in the background represents very good calls, reasonable quality and poor quality calls respectively.

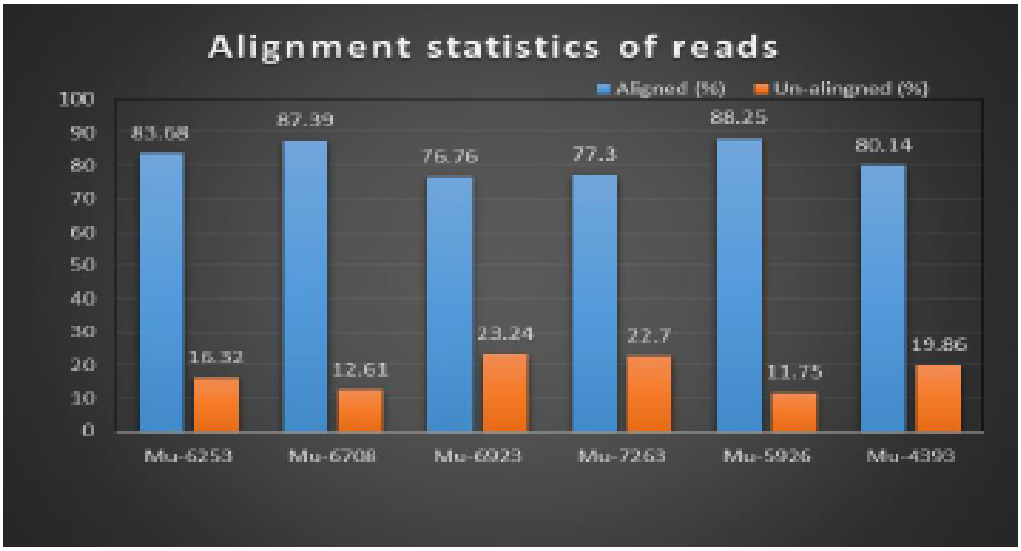


Figure 4.8: The total percentage of reads that aligned against *Bos Taurus* reference genome UMD 3.1.1.

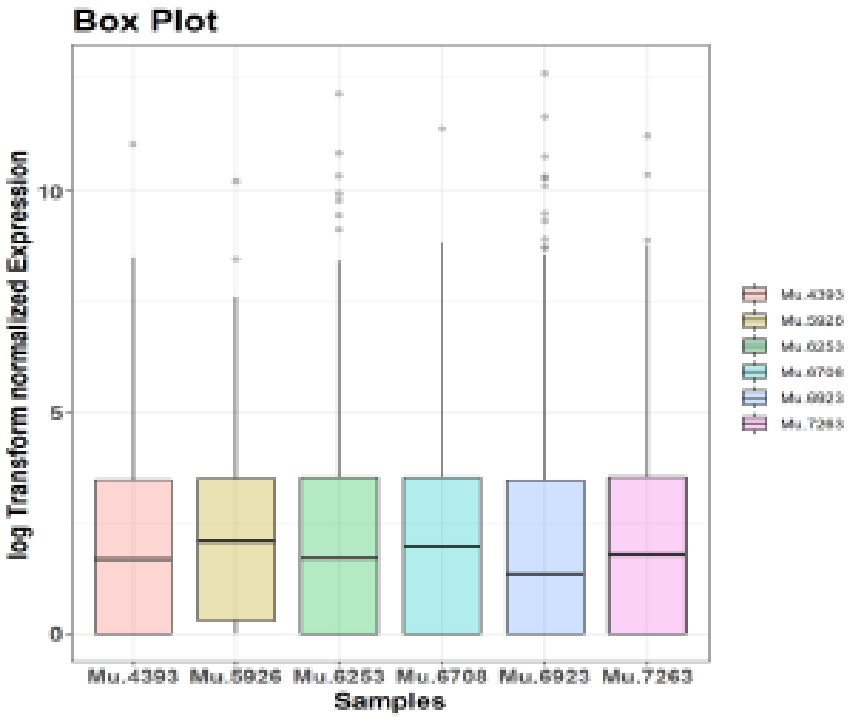


Figure 4.9: Distribution of normalized signal values in the three replicates of cloned bulls spermatozoa (MU-4393, MU-5926 and MU-7263) and somatic cell donor bull spermatozoa (MU-6253, MU-6708 and MU-6923) as shown by Box-whisker plot.

After the preliminary processing of raw data, the data was normalized using relevant parameters and the normalized expression values were used for further analysis. The Box-whisker plot showing the distribution of normalized expression values in each of the replicates of cloned as well as somatic cell donor bull groups is represented in Figure 4.9.

Principal Component Analysis (PCA) is a statistical procedure which allows the summarization of large data sets into smaller ones, which can be easily visualized and analyzed without any significant loss of information present in larger data sets. It was performed to check the quality of sequencing data generated and identification of association between samples. It was found that the samples were grouped according to their origins. The replicates of parent bulls spermatozoa grouped together and one of the replicates of cloned bulls spermatozoa (MU-6708) was grouped along with them, the other two cloned bulls spermatozoa (MU-6253,MU-6923) showed a variation of 41% from this group (Figure 4.10).

Table 4.4: Alignment statistics of cloned bulls spermatozoa and parent bulls spermatozoa.

Parameter	Total number of reads	Aligned reads (%)	Unaligned reads (%)	Maximum read length
Cloned bulls spermatozoa				
MU-6253	3,20,34,111	83.68	16.32	150
MU-6708	3,47,61,272	87.39	12.61	150
MU-6923	3,44,53,347	76.76	23.24	150
Somatic cell donor bulls spermatozoa				
MU-7263	3,37,27,420	77.30	22.7	150
MU-5926	3,60,23,396	88.25	11.75	150
MU-4393	3,52,70,945	80.14	19.86	150

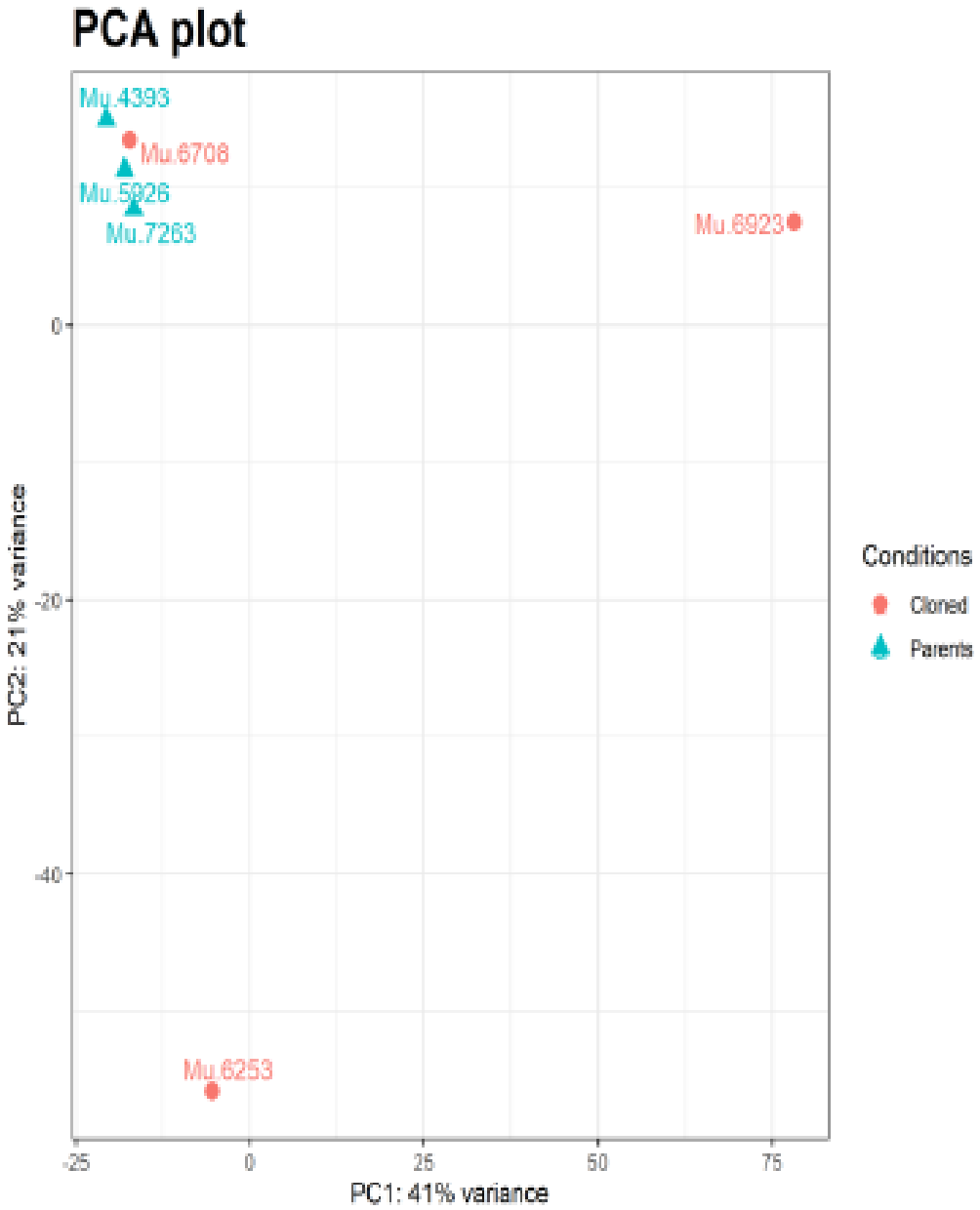


Figure 4.10: 2D scatter plot of principal component analysis (PCA) showing the quality of sequencing data generated. The replicates of cloned bull spermatozoa (Mu-6708) is grouped together with the replicates of somatic cell donor bulls spermatozoa (Mu-7263, Mu-5926 and Mu-4393) whereas the cloned bulls (MU-6923, MU-6253) are present at 41% variance to the above group.

The spermatozoal transcripts distribution on individual chromosome of cloned and somatic cell donor bull groups revealed that maximum number of reads were mapped on chromosome number 5 from all the individuals (Figure 4.11).

4.3.3.2 Volcano plot of cloned vs somatic cell donor bulls spermatozoa

Volcano plot is another type of scatter plot which allows quick visual identification of those data set that display changes of high magnitude which are statistically significant on plotting the measure of statistical significance against the magnitude of the change (FC) for identification of differential expressed data points. In this study, the scatter plot has been used to determine transcripts which are differentially expressed in cloned bull spermatozoa group and parent bull spermatozoa group based on certain specific P-value and log fold change. The most up-regulated transcripts are displayed on the right side of the plot, the most down-regulated transcripts are displayed on the left side of the plot, and the most statistically significant transcripts are displayed on the top of the plot (Figure 4.12)

4.3.3.3 Cluster analysis of transcriptome expressed differentially between cloned and and parent bulls spermatozoa

Hierarchical clustering analysis of the differentially expressed transcripts between cloned bulls spermatozoa and somatic cell donor bulls spermatozoa was done at FC≥2 (*P<0.05*). The cluster analysis showed that the replicates of each group clustered together (Figure 4.13) thus indicating similar expression pattern among replicates of same group. The transcriptome profile of top 100 differentially expressed genes in cloned MU-6923 resembled more closely to MU-5926, MU-6253 showed resemblance with MU-7263, MU-4393 transcriptome pattern resembled to Mu-6708 indicating the similarity of expression between the cloned and their somatic cell donor animal.

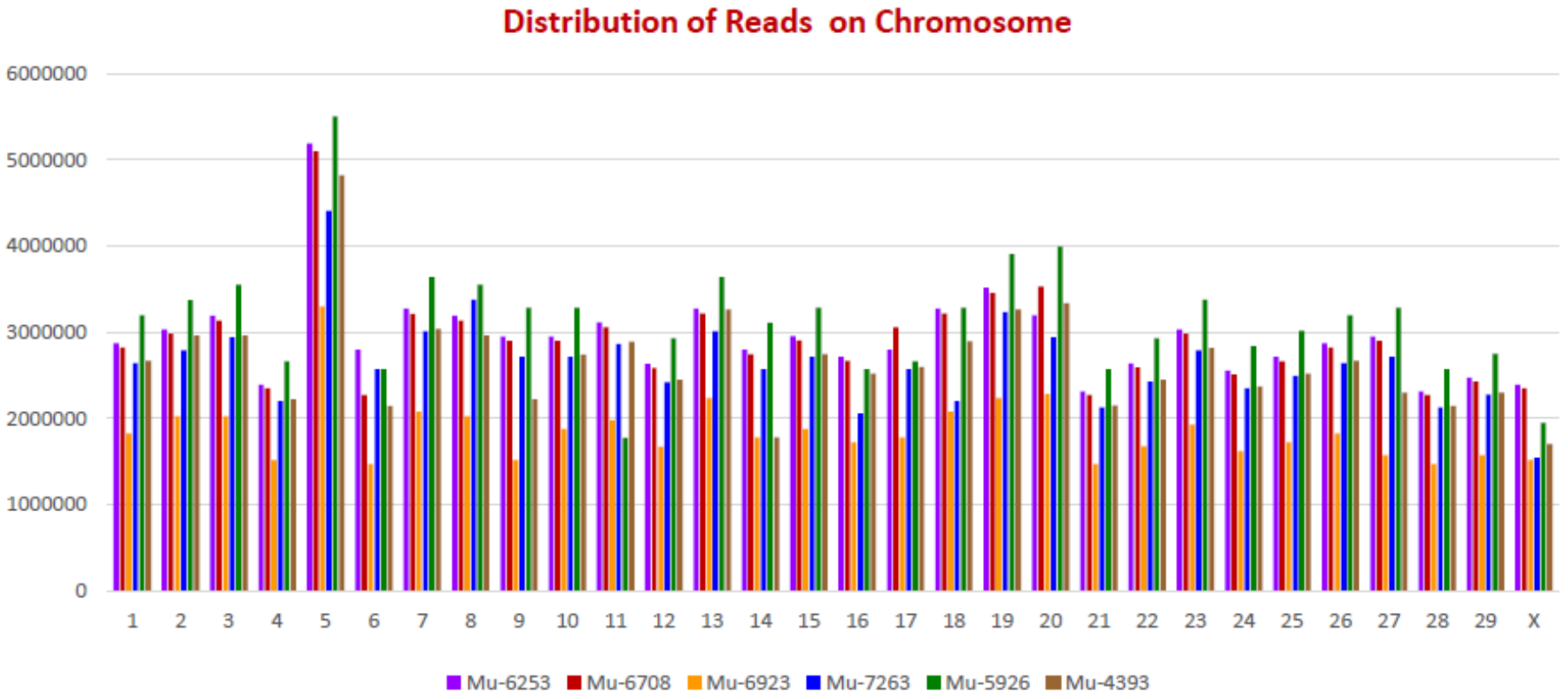


Figure 4.11: The chromosome wise distribution of reads for all the samples of cloned bulls spermatozoa (MU-6253, MU-6708 and MU-6923) and parent bulls spermatozoa (MU-7263, MU-5926 and MU-4393).

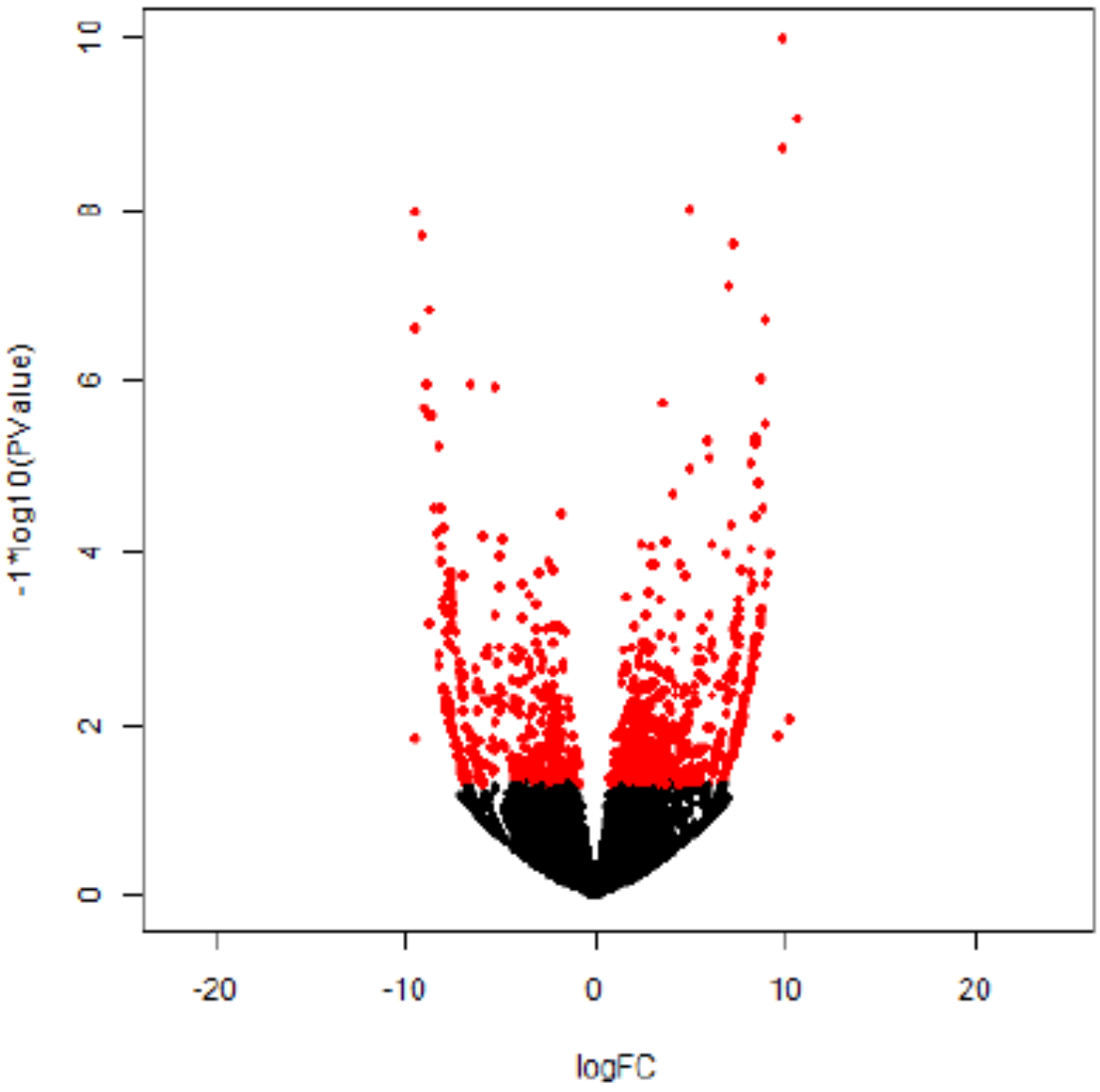


Figure 4.12: Volcano plot showing differentially expressed up-regulated and down-regulated transcripts in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa. Significantly differentially expressed transcripts are represented by red dots and non- significantly expressed transcripts are represented by black dots. The dots on the top denote most significantly expressed transcripts where as dots towards the right and left sides denote transcripts up-regulated and down-regulated respectively in cloned bulls spermatozoa as compared to somatic cell donor bulls spermatozoa .

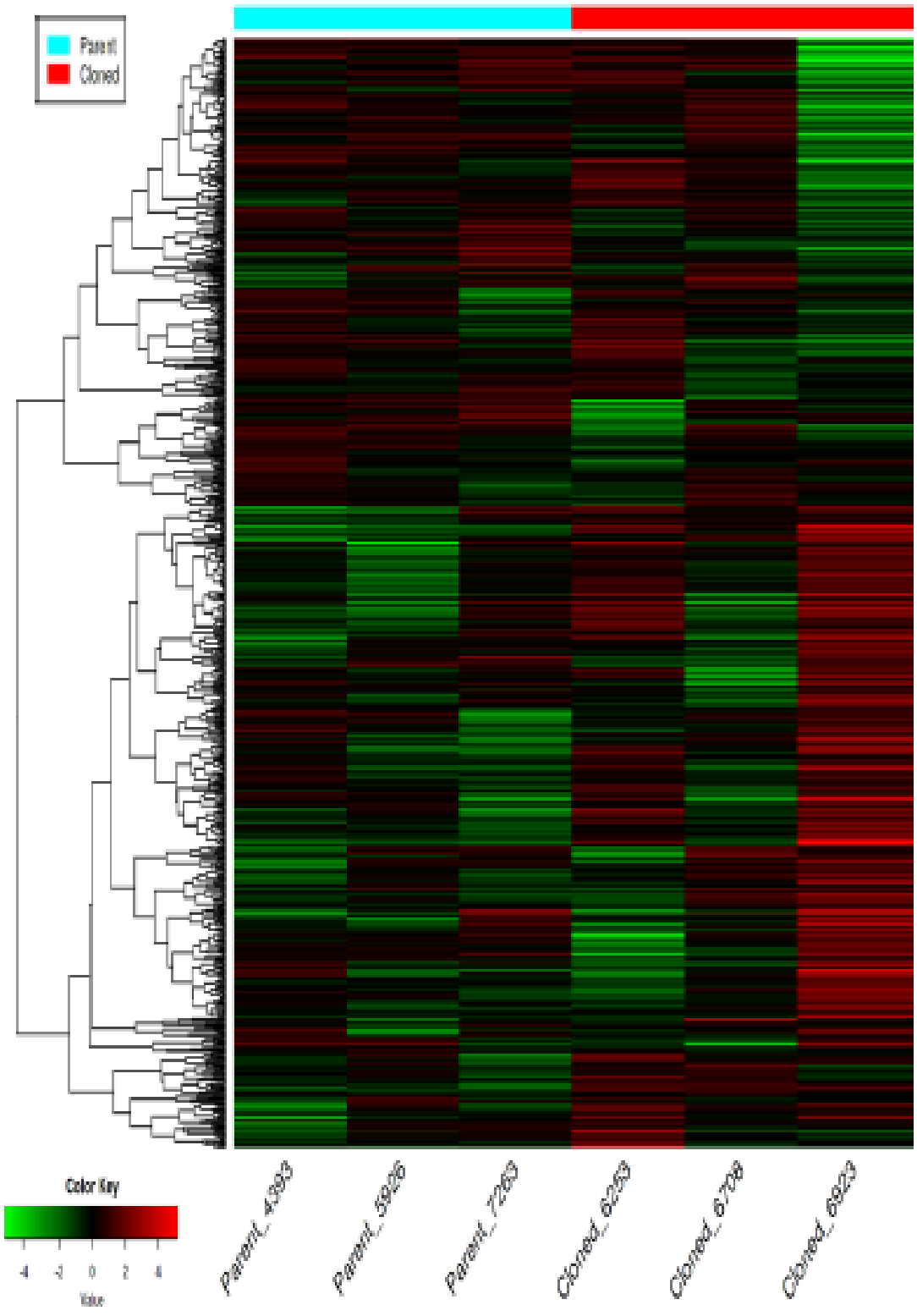


Figure 4.13: Hierarchical clustering analysis of all the transcripts expressed differentially in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa (FC ≥2, P<0.05).

4.4. Transcripts expressed differentially between cloned and somatic cell donor bulls spermatozoa

The total number of transcripts evaluated were 27,481 out of which 18,703 transcripts were expressed commonly in both cloned and somatic cell donor bulls spermatozoa, 4,120 transcripts were unique to cloned bull spermatozoa group, and 4,658 transcripts were unique to parent bull spermatozoa group (Figure 4.14). At FC≥2; *P<0.05*) 2,349 transcripts were found to express differentially in cloned bulls spermatozoa relative to parent bulls spermatozoa i.e. 51 transcripts at FC values ≥10,559 at FC values ≥5 and 1,739 at FC values ≥2-fold) (Figure 4.15). Out of which 566 transcripts were up-regulated and 410 transcripts were down-regulated significantly FC≥2 (*P<0.05*) (Figure 4.16). Table 4.5 and Table 4.6 display a list of top 20 up-regulated and top 20 down-regulated differentially expressed transcripts in cloned and somatic cell donor bulls at fold change ≥2.The respective gene IDs and FC differential expression of these are also given in these tables.

4.5. Gene ontology analysis of cloned vs somatic cell donor bull group

To understand the biological significance of differentially expressed genes, functional classification has to be performed using Gene Ontology (GO) by using Web Based Gene set analysis software. The identified differentially expressed genes are further functionally classified using gene ontology and pathway analysis. Consequently, GO has proved to be extremely valuable to know and analyze large amounts of data generated from a range of high-throughput investigative techniques to provide insights into the changes in gene expression between cloned and somatic cell donor bulls spermatozoa. The ontology covers the following domains molecular function, biological process and cellular component.

In this study gene ontology was performed to understand the functions of transcripts differentially expressed between cloned bulls spermatozoa and somatic cell donor bulls Spermatozoa. Using GO terms, NGS results can be summarized to provide insights into the changes of gene expression between cloned bulls spermatozoa and somatic cell donor bull spermatozoa. The gene ontology was done using transcripts that were expressed differentially at log FC ≥2 (*P<0.05*).

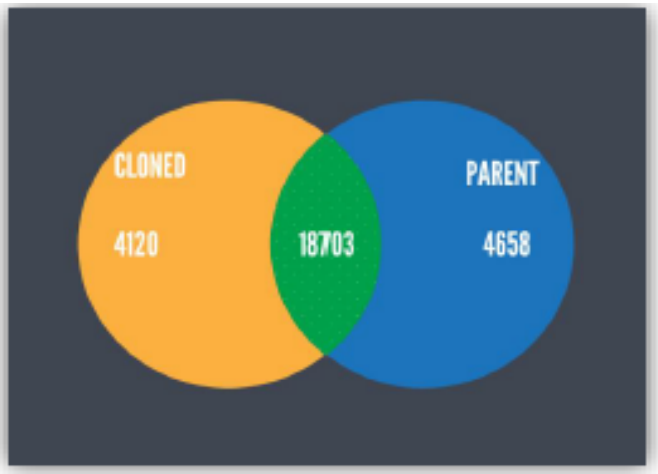


Figure 4.14: Venn diagram representing unique and commonly expressed transcripts in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

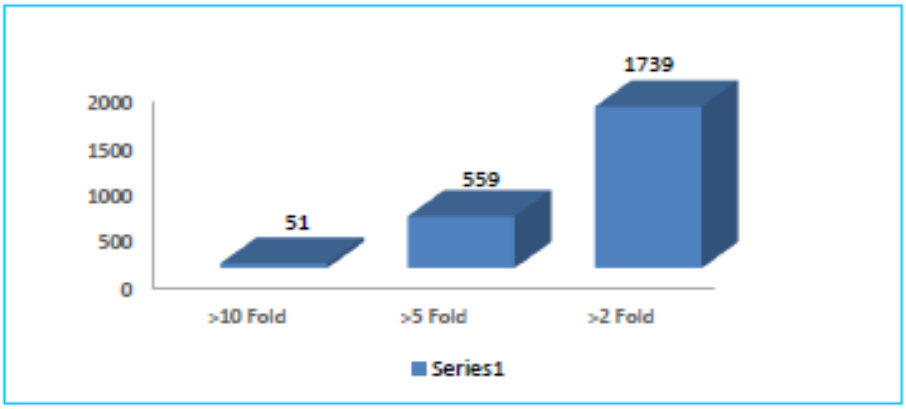


Figure 4.15: The bar graph represents the number of transcripts expressed differentially at different FC values (≥10, ≥5 to ≥2-fold).

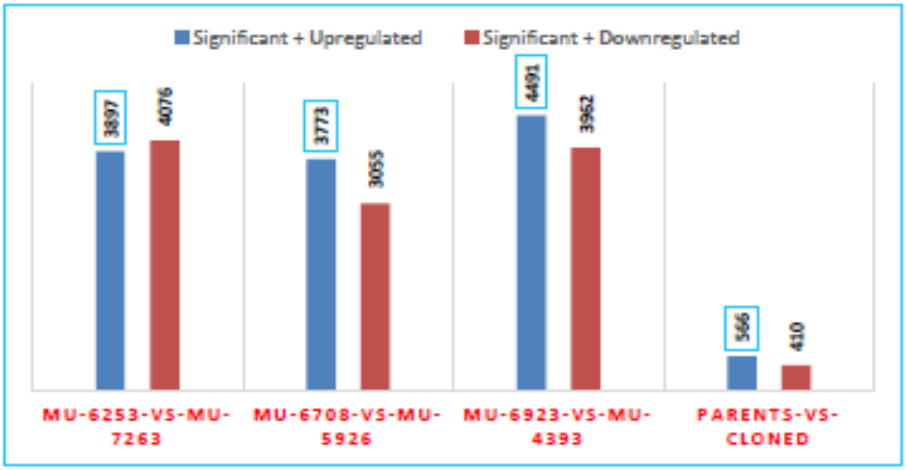


Figure 4.16: Bar graph depicting the total number of up- and down-regulated transcripts in cloned relative to somatic cell donor bulls at FC ≥2 ($p<0.05$)

Table 4.5: List of top 20 transcripts up-regulated in cloned relative to somatic cell donor bulls with FC≥2.

Sr. No.	Gene ID	Gene	Fold change	Gene description/ function
1.	ENSBTAG00000002568	GPKOW	24.31	MRNA Splicing
2.	AC_000182.1|	METRN	23.55	Meteorin regulates glial cell differentiation and promotes the formation of axonal networks during neurogenesis.
3.	AC_000182.1|	FAM234A	23.50	
4.	AC_000186.1|	SIPA1	23.17	The product of this gene is a mitogen induced GTPase activating protein (GAP).
5.	ENSBTAG00000038691	DPH7	23.16	This gene encodes a protein that contains a WD-40 domain, and is thought to be involved in diphthamide biosynthesis.
6.	AC_000182.1|	C25H16ORF71	23.15	--
7.	ENSBTAG00000000480	IFRD2	23.13	Interferon-related developmental regulator
8.	AC_000176.1|	LOC101906311	23.01	--
9.	ENSBTAG00000001830	LRRC46	23.00	Leucine Rich Repeat
10.	AC_000174.1|	GATSL3	22.97	Intracellular arginine sensor

11.	AC_000168.1|	RIC8A	22.87	Gene Ontology (GO) annotations related to this gene include *binding* and *guanyl-nucleotide exchange factor activity*.
12.	AC_000187.1|	CDR1	22.87	Cerebellar Degeneration-Related Protein
13.	AC_000175.1|	ZNF584	22.82	ZNF584 (Zinc Finger Protein 584) is a Protein Coding gene. Gene Ontology (GO) annotations related to this gene include *nucleic acid binding*.
14.	AC_000175.1|	SPIRE2	22.75	Gene Ontology (GO) annotations related to this gene include *actin binding*.
15.	AC_000164.1|	PCSK4	22.68	Member of subtilisin-like proprotein convertase family,
16.	AC_000182.1|	KCTD5	22.61	Act as a substrate adapter in some E3 ligase complex
17.	AC_000187.1|	PIM2	22.60	Serine/threonine kinase activity involved in cell survival and cell proliferation.
18.	AC_000163.1|	DANCR	22.57	This gene produces a long non-coding RNA that functions as a negative regulator of cell differentiation
19.	AC_000182.1|	AMDHD2	22.55	Amidohydrolase Domain Containing 2
20.	ENSBTAG00000004290	DDX28	22.54	DEAD box protein family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth and division.

Table 4.6: List of top 20 down-regulated transcripts in cloned relative to somatic cell donor bulls with FC≥2.

Sr. No.	Gene ID	Gene	Fold change	Gene description/ function
1.	AC_000187.1|LOC101906457	--	-21.45	--
2.	LOC104970904	--	-21.45	--
3.	ENSBTAG00000009988	SMARCB1	-21.44	Relieves repressive chromatin structures, allowing the transcriptional machinery to access its targets more effectively. The encoded nuclear protein may also bind to and enhance the DNA joining activity.
4.	AC_000182.1	NPTX2	-21.39	Encodes neuronal petraxins family proteins i.e. synaptic proteins related to C-reactive protein. This protein is involved in excitatory synapse formation. It also plays a role in clustering of AMPA-type glutamate receptors at established synapses, resulting in non-apoptotic cell death of dopaminergic nerve cells.
5.	AC_000179.1|LOC104975617	--	-21.37	--
6.	AC_000187.1|PJA1	--	-21.32	--
7.	AC_000187.1|FATE1	--	-21.23	--
8.	ENSBTAG00000020159	NKAIN1	-21.11	NKAIN1 (Sodium/Potassium Transporting ATPase Interacting 1) is a Protein

9.	AC_000171.1|MAF1	--	-21.01	--
10.	LOC101907916	--	-20.99	--
11.	LOC104969073	--	-20.98	--
12.	ENSBTAG00000019073	TRAF7	-20.97	Tumor necrosis receptor-associated factors.
13.	AC_000177.1|FOXI1	--	-20.86	--
14.	LOC614821	--	-20.86	--
15.	ENSBTAG00000010497	TRAF2	-20.83	TRAF proteins associate with and mediate the signal transduction from members of the TNF receptor superfamily.
16.	AC_000173.1|ELF3	--	-20.80	--
17.	ENSBTAG00000012644	UROD	-20.75	This gene encodes an enzyme in the heme biosynthetic pathway.
18.	ENSBTAG00000019357	ZNF688	-20.71	ZNF688 (Zinc Finger Protein 688) is a Protein Coding gene.
19.	AC_000164.1|GJC2	--	-20.69	--
20.	AC_000160.1|ATG4B	--	-20.69	--

The GO analysis revealed that the number of categories affected in biological processes, molecular function and cellular components were 12, 18 and 21, respectively. i) The most affected biological processes were metabolic process, regulation of biological process response to stimulus and cellular process. (Figure 4.17a), ii) Similarly, the categories that were the most enhanced under molecular function were binding, catalytic activity and molecular function (Figure 4.17b) and iii) The most enriched GO terms under cellular components were cellular anatomical entity, intracellular and protein containing complex (Figure 4.17c).

4.6 Pathways affected in cloned bulls spermatozoa group relative to parent bulls spermatozoa

Pathway analysis is done to determine pathways associated with differentially expressed genes, pathway analysis was performed by KEGG software. The development of RNA-Seq allows the parallel measurement of the expression of multiple genes. The information gained from high throughput sequencing offers an unprecedented chance to fully characterize biological processes. Pathway analysis is one of the promising tool which identifies the mechanism that underlie adaptive physiological compensatory responses, diseases, and new avenues for investigation. Pathways consist of collections of genes and proteins that perform a well-defined biological function. The transcripts expressed differentially above and below log FC ≥2 ($P<0.05$) in cloned animal relative to it somatic cell donor group showed involvement in 331 pathways (Figure 4.18).

4.6.1 Pathways affected by up-regulated transcripts in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa

The significantly 566 up-regulated transcripts at fold change 2 in cloned bulls spermatozoa reveals their involvement in different pathways. The top 10 most affected pathways are given in Table 4.7.

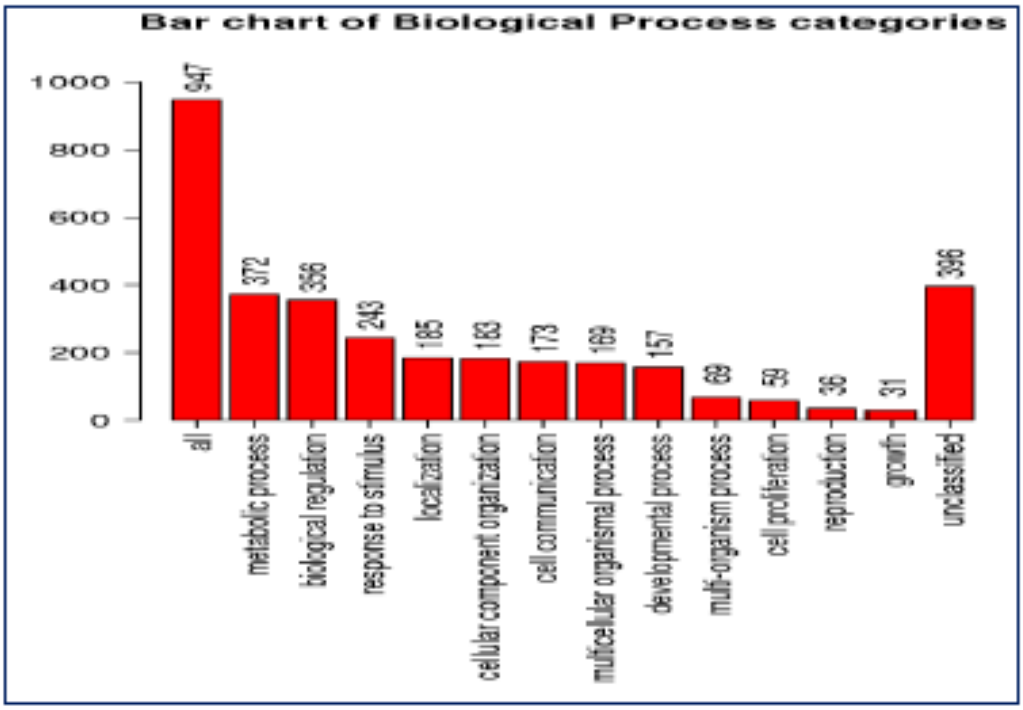


Figure 4.17a: GO study showing the biological process most enriched differentially expressed transcripts at FC ≥2 (P<0.05) in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

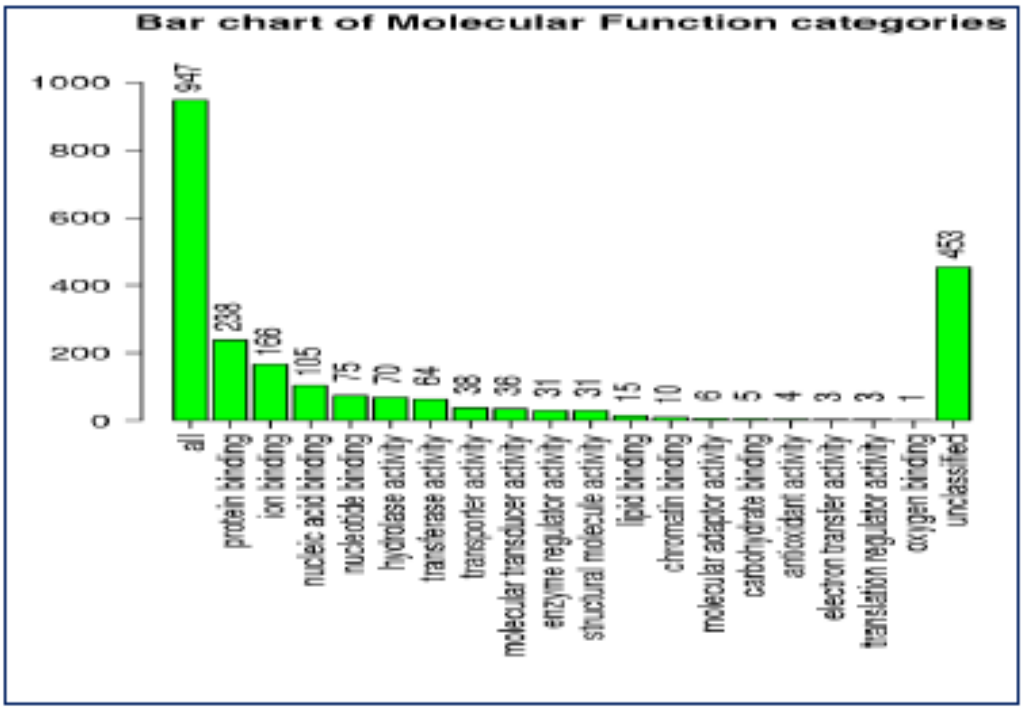


Figure 4.17b: GO study showing the molecular functions most enriched differentially expressed transcripts at FC ≥2 (P<0.05) in spermatozoa of cloned bulls relative to somatic cell donor bulls spermatozoa.

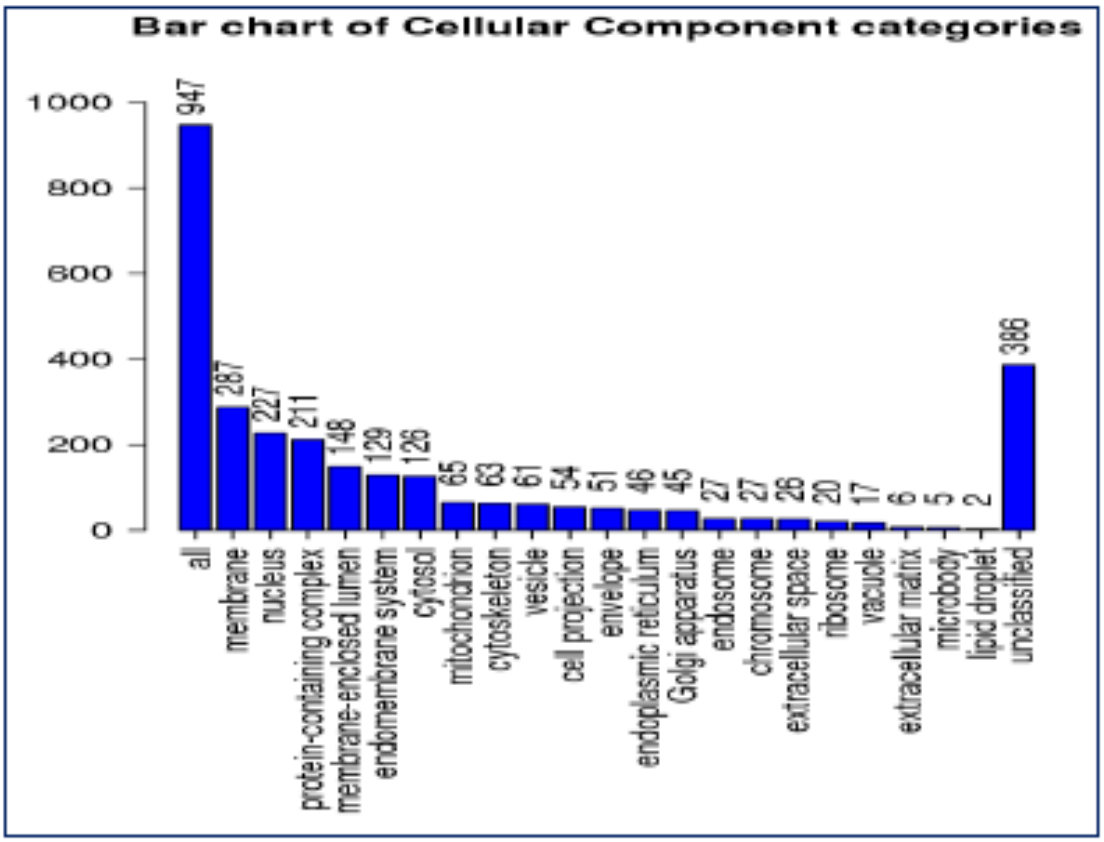


Figure 4.17c: GO study showing the cellular components of most enriched differentially expressed transcripts at FC ≥2 (P<0.05) in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

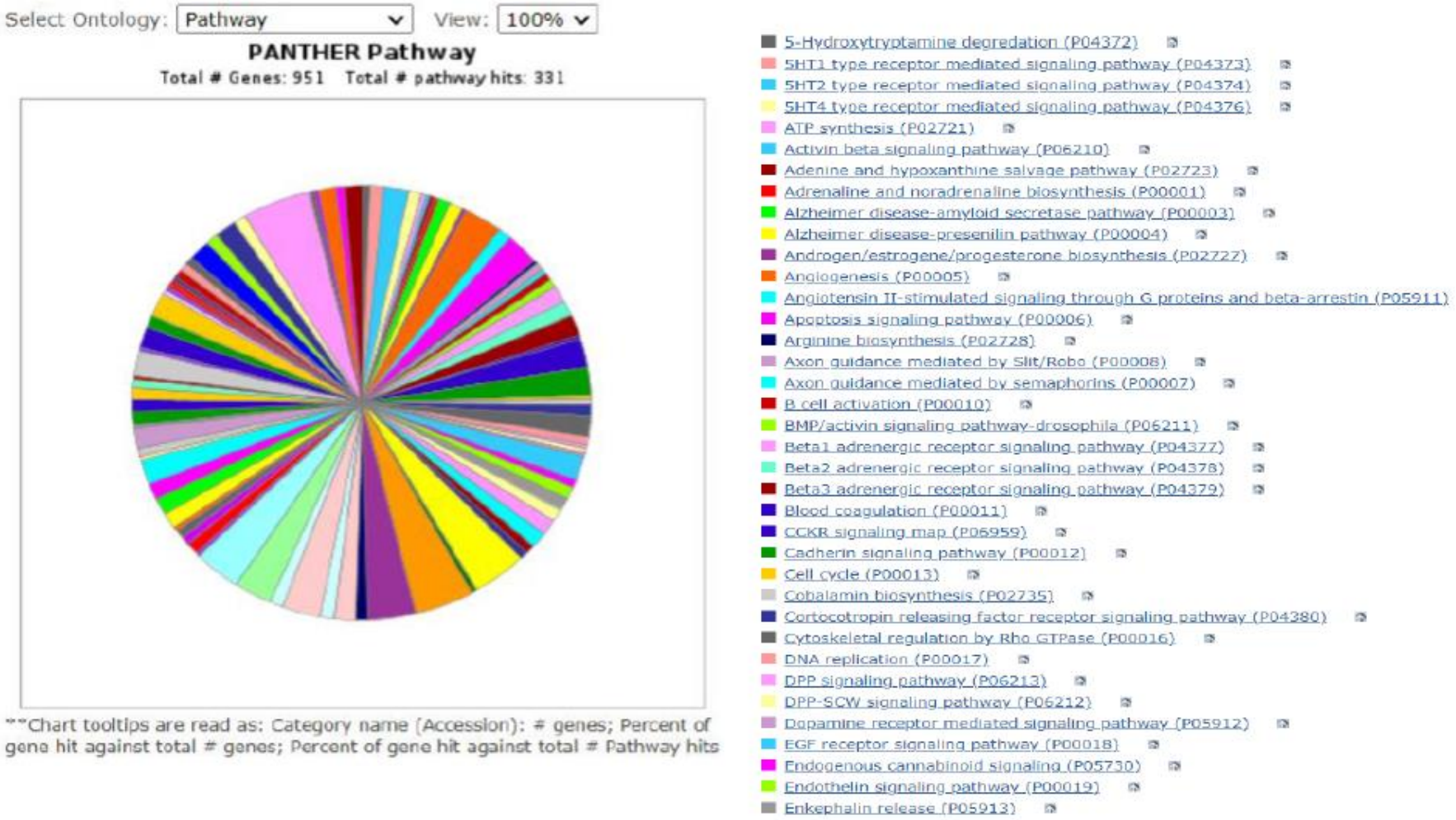


Figure 4.18: Pathways affected by the differentially expressed transcripts at FC ≥2 ($p<0.05$) in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

Table 4.7: Top 10 pathways affected in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa by up-regulated transcripts.

Sr. No.	Pathway	Pathway ID
1.	Base excision repair	bta 03410
2.	2-Oxocarboxylic acid metabolism	bta 01210
3.	Autophagy	bta 04136
4.	Ribosome	bta 03010
5.	Fructose and mannose metabolism	bta 00051
6.	Arginine and proline metabolism	bta 00330
7.	Lysine Degradation	bta 00310
8.	Mitophagy	bta 04137
9.	Arrhythmogenic right ventricular cardomyopathy	bta 05412
10.	Insulin signaling pathway	bta 04910

In our study base excision repair and autophagy pathway were found to have significant effect on cloned bulls spermatozoa (Figure 4.19 & 4.20).

4.6.2 Pathways affected by down-regulated transcripts in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa

Similarly on considering the differentially expressed transcripts below fold change 2 in cloned relative to its somatic cell donor animal spermatozoa revealed 410 transcripts to be down-regulated. The top 10 major pathways hit by these transcripts are given in the Table 4.8.

Base Excision Repair Pathway

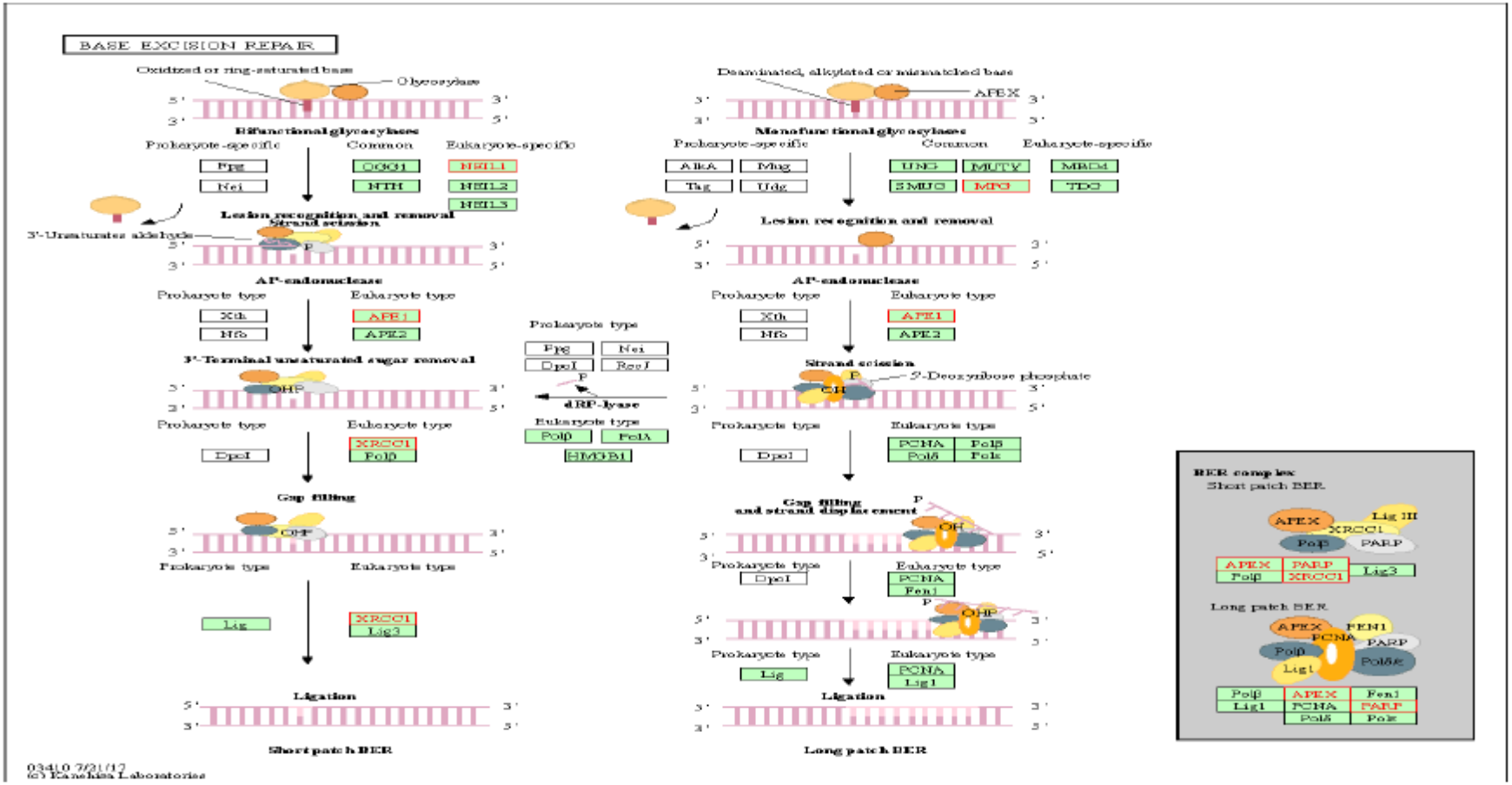


Figure 4.19: KEGG maps showing the network of genes that regulate the base excision repair pathway. The gens shown in red were up-regulated in cloned bulls spermatozoa relative to parent bulls spermatozoa in the pathway.

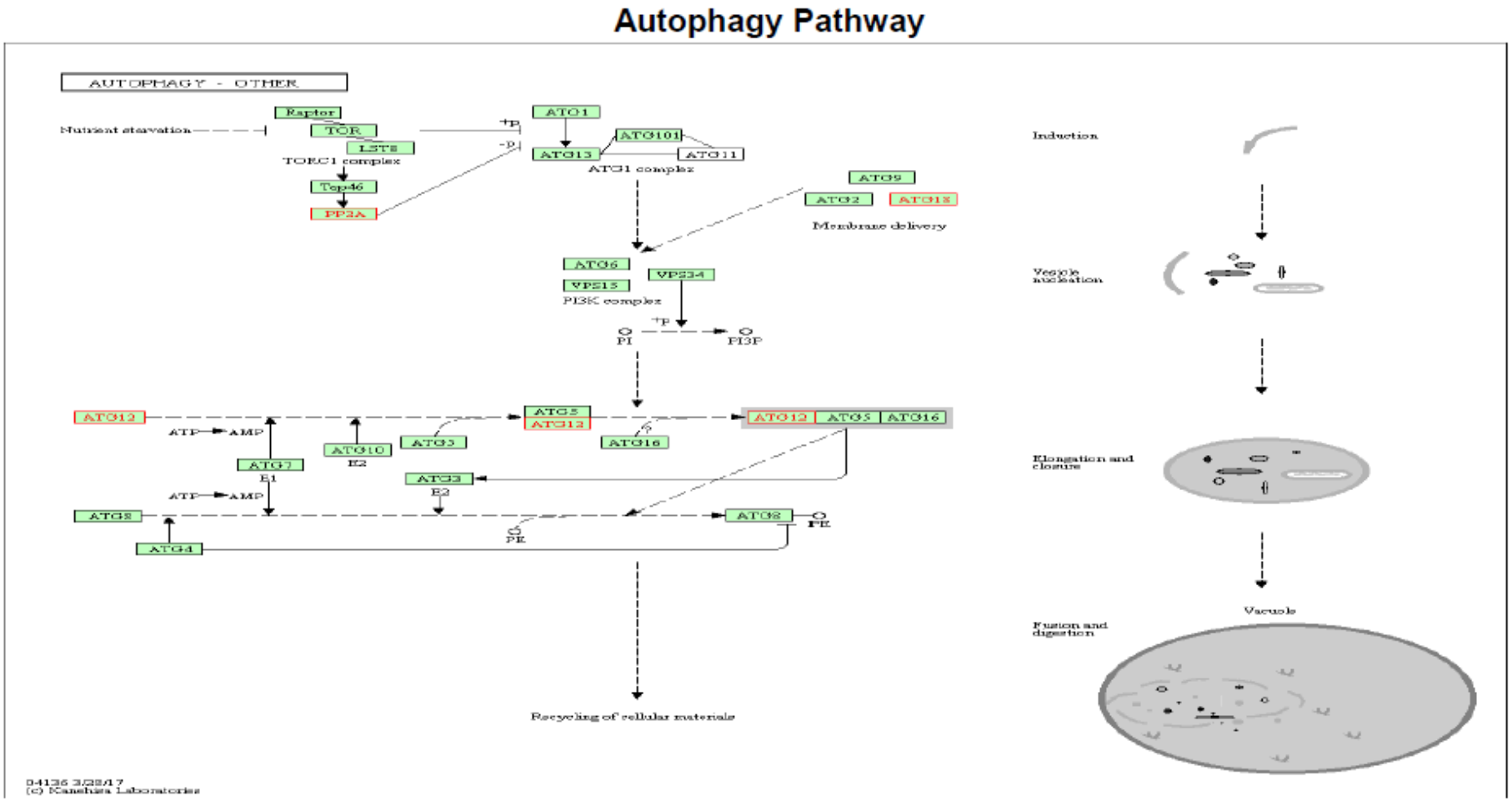


Figure 4.20: KEGG maps showing the network of genes that regulate the autophagy pathway. The gens shown in red were down-regulated in cloned bulls spermatozoa relative to parent bulls spermatozoa and significantly affected the pathway.

Table 4.8: Top 10 pathways affected in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa by down-regulated transcripts.

Sr. No.	Pathway	Pathway ID
1.	Ovarian steroidogenesis	bta 04913
2.	Regulation of lipolysis in adipocytes	bta 04923
3.	Antigen processing and presentation	bta 04612
4.	Longevity regulating pathway	bta 04211
5.	Salivary secretion	bta 04970
6.	Rasp1 signaling pathway	bta 04015
7.	Aldosterone synthesis and secretion	bta 04925
8.	AMPK signaling pathway	bta 04152
9.	Insulin signaling pathway	bta 04910
10.	Cytokine-cytokine receptor onteraction	bta 04060

In our study longevity pathway was found to have significant effect on cloned bulls spermatozoa.

Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource that provides information about the cell, organism, ecosystem, biological pathways, and molecular level information. It is used as a reference for analysis and interpretation of large datasets generated by high throughput sequencing such as next generation sequencing. KEGG is considered as “computer representation” of biological pathways presenting experimental knowledge on various functions of cell. In this study KEGG pathway maps were drawn to study the regulatory mechanism by which of differentially expressed transcripts at FC$\geq$2 in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

4.7. Expression pattern of transcripts in cloned bulls spermatozoa and somatic cell donor bulls spermatozoa regulating reproduction, bull fertility and early embryonic development

Sperm role is far beyond transporting DNA. It is now known that spermatozoa transcripts are mainly involved in regulating reproduction, sperm motility, sperm capacitation and bull fertility. The list of important transcripts obtained from

literature available and then comparing its presence and abundance in our study (*P<0.05*) is given in the Table 4.9, Table 4.10, Table 4.11 and Table 4.12.

Table 4.9: Transcripts involved in reproduction, fold change in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

Sr. No.	Transcripts	Fold change	P-value
1.	*IZUMO1*	3.74	0.34
2.	*ZPBS*	2.40	0.49
3.	*HSPA2*	1.98	0.18
4.	*BSP1*	0.70	0.45
5.	*CCT5*	0.40	0.81
6.	*YWHAZ*	0.22	0.98
7.	*SPACA1*	-0.48	0.78
8.	*SPAM1*	-0.63	0.67
9.	*CCT4*	-2.25	0.11
10.	*MFGE8*	-6.16	0.10

Table 4.10: Transcripts involved in determining bull fertility, fold change in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

Sr. No.	Transcripts	Fold change	P-value
1.	*SPADH1*	3.38	0.31
2.	*ATP8B3*	1.77	0.63
3.	*ADCY10*	1.53	0.10
4.	*NCADD3*	1.48	0.42
5.	*BSP5*	0.93	0.97
6.	*ENO1*	0.71	0.45
7.	*TNP1*	0.57	0.99
8.	*TMSB10*	0.05	0.66
9.	*PRKAR2A*	-0.12	0.93
10.	*PLCZ1*	-0.24	0.65

Table 4.11: Transcripts involved in sperm motility, capacitation, sperm-egg interaction, fold change in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

Sr. No.	Transcripts	Fold change	P-value
1.	*IZUMO1*	3.74	0.34
2.	*BSP5*	0.93	0.97
3.	*BSP1*	0.70	0.45
4.	*CUL3*	0.70	0.89
5.	*LDHC*	0.62	0.34
6.	*SORD*	0.37	0.57
7.	*ACRBP*	0.12	0.89
8.	*SMCP*	0.04	0.96
9.	*SPACA1*	-0.48	0.78
10.	*ACE*	-5.96	0.12

Table 4.12: Transcripts involved in embryonic development, fold change in cloned bulls spermatozoa relative to somatic cell donor bulls spermatozoa.

Sr. No.	Transcripts	Fold change	P-value
1.	*MEPCE*	5.91	0.13
2.	*SOX14*	0.37	0.91
3.	*TFB2M*	0.07	0.93
4.	*FOXG1*	-0.01	0.98
5.	*CYCS*	-0.41	0.83
6.	*HMGCR*	-0.48	0.67
7.	*TBX15*	-0.57	0.53
8.	*PAX3*	-1.18	0.60
9.	*SOX4*	-1.87	0.61
10.	*PRDM14*	- 2.73	0.25

After going through the literature thoroughly, we identified transcripts from our NGS data that are involved in controlling the mentioned processes. On comparing the expression pattern of these transcripts in cloned bulls spermatozoa group and somatic cell donor bulls spermatozoa group, we found that several genes up or down-regulated. But most importantly that expression of genes involved in regulating reproduction, sperm motility, sperm capacitation and bull fertility and early embryonic development are not much affected.

DISCUSSION

The genetic improvement in domestic animals has been achieved by inseminating females with the semen of desirable genotype bulls to produce higher productive calves. Therefore, availability of best genotype bulls is a fundamental requirement to boost genetic gains to achieve higher production. In India, due to a severe shortage of elite bulls, semen available from progeny-tested bulls is not sufficient to cover even 1% of the breedable population of livestock. Recent studies indicated that genomics, transcriptomic and proteomic approaches have the potential to be used in the efficient assessment of semen quality and bull fertility (Somashekar *et al.* 2015; Selvaraju *et al.* 2017).

ICAR-National Dairy Research Institute, Karnal has produced several cloned bull calves out of which three cloned bulls are currently producing semen. During the present study cryopreserved semen of cloned bulls and their somatic cell donor bulls was washed with bovipure reagent by density gradient centrifugation and then treated with somatic cell lysis buffer (SCLB) for removal of somatic cells. The use of bovipure reagent for removal of somatic cells from bull semen was done by Vijayalakshmy *et al.* (2018) who also reported that separation of sperm with bovipure reagent significantly improved sperm recovery and sperm motility as compared to other methods used for removal of somatic cells. Along with this, SCLB, which can successfully remove most of the somatic cell contaminants form semen has been used for lysis of somatic cells present in semen for preparation of human spermatozoal RNA (Goodrich *et al.,* 2007). As there was no expression of somatic cell specific genes in isolated RNA from semen samples, it confirmed the removal of most of the somatic cells.

As per our information, there is no report on the comparative global transcriptome profile of cloned and its somatic cell donor spermatozoa in any species. In present study Patman software was used to align the reads obtained against *Bos taurus* reference genome UMD 3.1.1. For all the semen sample the more than 32 million reads were obtained and total reads that got aligned were 76.76% to 88.25%, in different replicates. We evaluated 27,481 transcripts out of which 18,703 transcripts were expressed commonly in both cloned and somatic cell donor bulls spermatozoa, 4120 transcripts were unique to cloned

bull spermatozoa group, and 4658 transcripts were unique to parent bull spermatozoa group. At FC≥2; *P<0.05*) 2349 transcripts were found to express differentially in cloned bulls spermatozoa relative to parent bulls spermatozoa i..e. 51 transcripts at FC values ≥10, 559 at FC values ≥5 and 1739 at FC values ≥2-fold). Out of which 566 transcripts were up-regulated and 410 transcripts were down-regulated significantly FC ≥2 (*P<0.05*). There are some reports covering the presence and importance of the transcripts present in *Bos taurus* spermatozoa by Sellappan (2017) who reported the occurrence of 13,838 transcripts associated with various stages of spermatogenesis, fertilization, embryo development and pregnancy associated glycoproteins (PAGs) having a possible influence beyond early embryonic development. The most abundant transcripts in his study were *CHMP5, YWHAZ* and were involved in male gamete generation, sperm function and acrosome reaction. In our present study these transcripts were found and they did not show any significant difference (*P<0.05*) in cloned and somatic cell donor bulls spermatozoa. PAG 5 which was pregnancy associated glycoprotein found in their study on comparing with our study did not show any significant difference in abundance in both the groups. Additional to this there were some tRNAs which were reported to affect embryonic gene expression such as *TRNAE-UUC, TRNAL-AAG, TRNAG-GCC* which were also reported in our study with no significant difference (*P<0.05*) in both the major groups. Genes such as *PLCZ1, PLCB1* involved in calcium signaling and spermatozoon-induced activation of oocyte were identified in their as well as our study. Our results, compared with Sellappan (2017) studies showed that there was no significant difference (*P<0.05*) in transcripts related to spermatogenesis and pregnancy associated transcripts in both the groups.

Nidhi *et al.* (2018) worked on whole transcriptome profile of *Bos indicus* where they identified the presence of *GJA5* gene involved in formation of intercellular channel required for gap junctions. On comparing its presence in our present study, *GJA5* was not identified somatic cell donor parent but was present in cloned group*. ZBTB20* another transcription factor studied by the team depicted role in activation of transcription particularly in sertoli cells was also present in our study and found no significant difference in cloned bulls spermatozoa compared to somatic cell donor bulls spermatozoa.

In order to find the transcripts related to sperm motility, Bissonnette *et al.* (2009) evaluated the potential of semen quality and observed that transcripts encoding a serine/threonine testis-specific protein kinase (*TSSK6*) which are usually associated with high motility status. On comparing its presence with our present study, it did not showed any significant difference (*P<0.05*) in these two groups.

Peddinti *et al.* (2008) identified *ATP5B*, involved in oxidative respiration in high fertile spermatozoa. *ATP5B* is found to catalyze the production of ATP from ADP in presence of proton gradient across the mitochondrial membrane and this ATP is used for the motility of sperm and capacitation. In our study too, expression of *ATP5B* was seen higher in cloned animal as compared to the somatic cell donor parent. Another transcript *AKAP4* expression was significantly higher in high fertility spermatozoa. *AKAP4* is a scaffold protein involved in signaling and metabolism. Its high expression in spermatozoa could result in higher motility. In our study the expression of *AKAP4* showed on significant difference in both the groups. AKAP4 transcripts were more expressed (*P<0.05*) in cloned bulls spermatozoa in comparison to somatic cell donor bulls spermatozoa.

Identification of biomarkers related to fertility in spermatozoa of crossbred bulls through comparative proteomics revealed that proteins MDH2, NCAPD3, USP12 and ENO1 were over expressed in high fertile group, whereas TMEM43 and BSP1 were over expressed in low fertile bulls (Aslam *et al.,* 2018). Among these they selected ENO1 and BSP1 for further validation by immuno-blotting. ENO1 is mostly present in tail of motile sperm. ENO1 is also involved in protection of male gamete against oxidative stress. Some reports also suggest the positive association between bull fertility and concentration of *ENO1* in spermatozoa of taurine bulls. We also observed similar expression level of *ENO1* in cloned and somatic cell donor bulls spermatozoa, while expression of *TMEM43* was 4 fold more in cloned compared to somatic cell donor bulls spermatozoa.

Calcium play a crucial role in sperm motility, capacitation, acrosome reaction and fusion with egg plasma membrane has been reported for many decades. Leclerc *et al*. (2020) reported calmodulin in bovine ejaculated sperm and identified certain transcripts involved in various biological processes in sperm

via CALM binding proteins like CCT5, PLCZ1. CCT 5 is involved in interaction with zona pellucida whereas PLCZ1 was involved to activate egg upon fertilization. Esposito *et al.* (2004) revealed that ADCY10 knock out males were infertile as their sperms were immotile. Our results revealed that on comparing the transcripts with both the groups of spermatozoa, no significant difference (*P<0.05*) was obtained *CCT5* and *PLCZ1* transcripts level. Although *ADCY10* transcript showed 1.5 fold more in cloned bulls spermatozoa as compared to somatic cell donor bulls.

Byrne *et al.* (2012) identified markers like fibronectin-domain containing proteins (BSP1, BSP3, BSP5), sperm adhesins (SPADH1, SPADH2) which showed major involvement in sperm egg interaction. Our study also supports that these transcripts are normally present in both the groups of spermatozoa but their expression level did not show any significant difference (*P<0.05*) in either of the group.

Ozbek (2021) identified some of the energy related enzymes i.e. outer dense fiber protein(ODF) which were involved in sperm motility and also *ODF2* bind and maintain acetylated levels of α-tubulin (Zhao *et al.,* 2018). On considering the importance of *ODF2* transcript in our study, it was found that the abundance of it was significantly high (*P<0.05*) in spermatozoa of cloned animal group compared to somatic cell donor group. Expression of integrin subunit beta 5 (*ITGb5*) in germ cells and consequential oocyte is vital for fertilization and embryonic development in bovine (Velho *et al.,* 2019). In our study *ITGb5* transcript did not show any significant difference in both the groups.

Byrne *et al.* (2012) analyzed the involvement of immunoglobulin superfamily members (*IZUMO*) in interaction between the sperm and zona pellucida. They confirmed its importance by performing its gene-knockout study. Similar observation was made by Park *et al*. (2012) where they also pointed the role of *IZUMO* in acrosome reaction. In our study *IZUMO1* showed significant difference (*P<0.05*) in both groups. They also reported ATP synthase H+ transporting mitochondrial F1 copmlex beta subunit (ATP5B), alpha-2-HS-glycoprotein 2 (AHSG), enolase 1 (*ENO1*) more in high fertile bulls. These

results when compared to the present study show the presence of all the three with no significant difference (*P<0.05*) in both the groups.

Binder of sperm (BSP) are found to regulate efflux of phospholipids and cholesterol from sperm membrane to promote capacitation (Desnoyers *et al.,* 1992), but studies on BSP has reported variable results i.e. BSP protein expression in semen was negatively correlated with bull fertility (Gwathmey *et al*., 2006; D'Amours *et al.,* 2010; Aslam *et al.,* 2018) while Kasimanickam *et al.* (2019) reported a positive correlation between *BSP* expression and bull fertility. In our study *BSP1* showed significant difference (*P<0.05*) in both the groups whereas *BSP5* did not show any difference.

DNA methylation along with histone modification play a fundamental role in modulating gene expression in spermatozoa. *DNMT1, DNMT3L* genes encode an enzyme that transfers methyl groups to cytosine nucleotides of genomic DNA and affect the gene expression (Kato *et al.,* 2007; Seinsenberger *et al.,* 2013). In our study most of these transcripts did not show any significant difference in both the groups. Fertility associated genes i.e. *CRISP2* and *ZFP36L1* has important role in spermatogenesis, variation off lagellar motility, acrosome reaction, and gamete fusion (Liu *et al.,* 2019), *HGF* expressed in the vas deferens and epididymis and involved in sperm motility (Naz *et al.,* 1994). In our study the abundance of *HGF* and *ZFP36L1* did not show any significant difference (*P<0.05*) between the two groups, however *CRISP2* showed significant difference in both the groups.

The basic information about the similarity or dissimilarity between the semen obtained from cloned animals and non-cloned animals or donor animals can be gained from Comparative analysis of preliminary semen parameters of cloned animal and non-cloned animal semen (including donor animal semen) (Shi *et al.,* 2020). Therefore, we conducted a study to compare the fresh semen parameters of cloned bulls and non-cloned bulls of similar age and generated their global transcrptome profile. A total of eight parameters of fresh semen were compared between the cloned bulls and non-cloned bulls and based upon statistical analysis we concluded that there was no significant difference between the semen parameters of cloned bulls and non-cloned bulls. Similar

study was conducted by Selokar *et al.* (2019) who compared the fresh semen parameters of cloned bulls with non-cloned bulls and donor bulls. A number of semen parameters were recorded by them such as ejaculate volume, sperm concentration, sperm mass motility etc. During their study no difference was found in the semen parameters of non-cloned bulls, including the donor bulls. These studies provide significant evidence to say that semen of cloned bulls and non-cloned bulls is similar.

To the best our knowledge this is the first study which elucidates the global transcriptome profile of spermatozoa of buffalo cloned bulls and their respective somatic cell donor bulls. Semen obtained the two different sources i.e. cloned bulls and their respective somatic cell donor bulls were compared on molecular basis through their transcriptome profiles. In the present study the transcripts associated with important traits in spermatozoa showed nearly similar expression in cloned bulls spermatozoa and parent bulls spermatozoa. We have also used semen of cloned bulls (Mu-6708; Swarn and Mu-6923; Rajat) for artificial insemination of 20 buffalo heifers. The fertility rate after AI was found to be 65%, which is comparable to that of donor bull (Selokar *et al.,* 2019). When NGS data of semen parameters of fresh semen of cloned bulls and non-cloned bulls/donor bulls along with the pregnancy rates was compared, it supported the fact that the offspring produced through animal cloning technique (SCNT) are normal and the semen of cloned bulls can be employed in livestock breeding programmes and various ART purposes.

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CHAPTER – 5

Summary and Conclusions

5. SUMMARY AND CONCLUSIONS

The contribution of bulls to breeding programs for improvement of milk production is about 64% which is significantly higher than that of females (only 36%). In India, the conventional progeny testing program used to determine bull's genetic merit is time consuming and resulting population of elite bulls is not sufficient to cover even 1% of the breedable population of livestock. Therefore, availability of best genotype bulls is a fundamental requirement to boost genetic gains to achieve higher production. Presently buffalo bull cloning by somatic cell nuclear transfer (SCNT) is considered as the best available option to reproduce elite bulls in shortest time and their semen would contribute to achieve the demand of ever-growing frozen semen, which is the prime requirement of conventional breeding programme (Selokar *et al.,* 2019). Study of spermatozoal transcriptome provides a molecular basis to determine the quality of semen as they are associated with spermatogenesis, bull fertility and early embryonic development. The present study was done to generate global transcriptome profile of spermatozoa of cloned bulls and their respective somatic cell donor bulls using Next Generation Sequencing and for the identification of differentially expressed spermatozoa transcripts in both groups.

Fresh semen of cloned bulls and non-cloned bulls was used for comparative analysis of preliminary semen parameters. Total eight parameters were used for comparison and the data obtained showed that there was no significant difference ($P<0.05$) in these semen parameters between cloned bulls and non-cloned bulls.

The spermatozoa of both cloned bulls (MU-6253, MU-6708 and MU-6923) as well as parent bulls (MU-7263 from population, MU-5926 and MU-4393) treated with somatic cell lysis buffer (SCLB; 0.5% Triton X-100 and 0.1% SDS). Treated samples showed a highly reduced or no somatic cells in semen samples.

Then sperm pellet was homogenized by vigorous crushing in liquid nitrogen then treated with lysis buffer, pre-heated Trizol and β-mercaptoethanol (BME)

and incubating the content for minimum 30 min at 65°C. An increase in sperm lysis was observed on vortexing the contents during the incubation period.

The concentration of RNA isolated from spermatozoa of cloned buffalo bulls was 102.19±4.49 ng/µl and somatic cell donor bull was 109.73±8.05 ng/µl. The 260/280 ratio of RNA isolated from sperm of cloned buffalo bull was 1.59±0.03 while somatic cell donor bull was 1.70±0.08. The synthesis of cDNA from RNA of cloned bulls and parent bulls was confirmed by amplification of *GAPDH* gene (176 bp). The removal of somatic cells from sperm population was confirmed by expression of *SRY* and *DAZL* gene and no expression of *V-KIT, CD4 and CDH1* gene.

For transcriptome analysis RNA isolation from $30x10^6$ spermatozoa. The yield of RNA varied from 116 ng/µl to 204 ng/µl. The RNA Integrity Number (RIN) values for all the samples was between 7.0 to 7.5 and electropherograms showing bands of 28S rRNA and 18S rRNA, that clearly reflected the isolated RNA was intact and of good quality.

After examining the quality of RNA, cDNA libraries were prepared for each biological replicate using the TruSeq Small RNA Library Preparation Kits in single end format. Then sequencing of these cDNA libraries was done using Illumina sequencer. Quality control of raw reads obtained was done using FastQC tool. The FastQC reports give Phred scores on Y axis and tells about the probability of occurance of a particular base at a specific position in the reads on X-axis. The Phred score below 30 shows bad quality data and requires filtering. The good quality reads obtained after QC were then aligned to *Bos taurus* reference genome UMD3.1.1 for further analysis.

For cloned bulls MU-6253 (3,20,34,111 reads), MU-6708 (3,47,61,272 reads) and MU-6923 (3,44,53,347 reads) the total reads that got aligned were 83.68%, 87.39% and 76.76% respectively. Simultaneously, for paents bulls MU-7263 (3,37,27,420 reads), MU-5926 (3,60,23,396 reads) and MU-4393 (3,52,70,945 reads) the total reads that got aligned were 77.3%, 88.25%, 80.14% respectively.

The Box-whisker plot showing the distribution of normalized expression values in each of the replicates of cloned as well as somatic cell donor bull groups. PCA showed that replicates of parent bulls spermatozoa grouped together and one of the replicates of cloned bull spermatozoa (MU-6708) was grouped among with them, the other two cloned bulls spermatozoa (MU-6253, MU-6923) showed a variation of 41%.

In volcano plot the most up-regulated transcripts are displayed on the right side of the plot, the most down-regulated transcripts are displayed on the left side of the plot, and the most statistically significant transcripts are displayed on the top of the plot. The spermatozoal transcripts distribution on individual chromosome of cloned and somatic cell donor bull groups revealed that maximum number of reads were mapped on chromosome number 5 from all the individuals.

Hierarchical clustering analysis indicating transcriptome profile of top 100 differentially expressed genes in cloned MU-6923 resembled more closely to MU-5926, MU-6253 showed resemblance with MU-7263, MU-4393 transcriptome pattern resembled to Mu-6708 indicating the similarity of expression between the cloned and their somatic cell donor animals.

The total number of transcripts evaluated were 27,481 out of which 18,703 transcripts were expressed commonly in both cloned and somatic cell donor bulls spermatozoa, 4120 transcripts were unique to cloned bull spermatozoa group, and 4658 transcripts were unique to parent bull spermatozoa group. At FC≥2; $P<0.05$) 2349 transcripts were found to express differentially in cloned bulls spermatozoa relative to parent bulls spermatozoa i.e. 51 transcripts at FC values ≥10, 559 at FC values ≥5 and 1739 at FC values ≥2-fold). Out of which 566 transcripts were up-regulated and 410 transcripts were down-regulated significantly FC ≥2 ($P<0.05$).

The GO analysis revealed that the number of categories affected in biological processes, molecular function and cellular components were 12, 18 and 21, respectively. i) The most affected biological processes were metabolic process, regulation of biological process response to stimulus and cellular

process. ii) Similarly, the categories that were the most enhanced under molecular function were binding, catalytic activity and molecular function and iii) The most enriched GO terms under cellular components were cellular anatomical entity, intracellular and protein containing complex.

The significantly 566 up-regulated transcripts at FC>2 in cloned bulls spermatozoa reveals their involvement in different pathways. In our study base excision repair and autophagy pathway were found to have significant effect on cloned bulls spermatozoa. Similarly 410 down-regulated transcripts affects the regulation of lipolysis in adipocytes, AMPK signaling pathway, insulin signaling pathway etc non-significantly (*P<0.05*) while longevity pathway was found to have significant effect on cloned bulls spermatozoa.

KEGG enrichment analysis detected genes that were enriched in base excision repair and autophagy pathway. The transcripts mainly associated with regulating reproduction, sperm motility, sperm capacitation, bull fertility, and early embryonic development was almost similar in cloned bulls spermatozoa and parent bulls spermatozoa.

In conclusion, results of the present study suggest that:

- Pure spermatozoa population obtained after successful removal of somatic cells from semen samples. Removal of somatic cells from sperm population was confirmed by expression of *SRY* and *DAZL* gene and no expression of *V-KIT, CD4 and CDH1* gene.

- For NGS, the RNA yield varied from 116 ng/µl to 204 ng/µl from spermatozoa and the RIN values for all the samples were between 7.0 to 7.5. cDNA libraries were prepared and sequencing was done using Illumina sequencer. The quality reads were aligned to *Bos taurus* reference genome UMD3.1.1 for further analysis.

- For all the semen sample the more than 32 million reads obtained and total reads that got aligned were 76.76% to 88.25%, in different replicates. The maximum number of reads were mapped on chromosome number 5 from all the individuals.

- Total number of transcripts evaluated were 27,481 out of which 18,703 transcripts were expressed commonly in both cloned and somatic cell donor bulls spermatozoa, 4120 transcripts were unique to cloned bulls spermatozoa and 4658 transcripts were unique to somatic cell donor bulls spermatozoa.

- 2349 transcripts were found to express differentially (FC≥2; P<0.05) in cloned bulls spermatozoa relative to parent bulls spermatozoa i.e. 51 transcripts at FC values ≥10, 559 at FC values ≥5 and 1739 at FC values ≥2-fold).

- The significantly 566 up-regulated and 410 down-regulated transcripts at FC>2 in cloned bulls spermatozoa reveals their involvement in different pathways. KEGG enrichment analysis revealed that in present study base excision repair, autophagy and longevity related pathway were found to have significant effect in cloned bulls spermatozoa.

- The transcripts mainly associated with regulating reproduction, sperm motility, sperm capacitation, bull fertility, and early embryonic development was almost similar in cloned bulls spermatozoa and somatic cell donor bulls spermatozoa.

- The results of comparative analysis of fresh semen parameters of cloned bulls and non-cloned bulls semen as well as NGS transcriptome data supports the fact that the cloned bull semen may be used for various ART purposes.

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RESUME

ACADEMIC QUALIFICATIONS

- Passed high school in 2013 from ICSE board with 76.5% marks.
- Passed intermediate in 2015 from CBSE board with 76.4% marks.
- Completed B.Sc. in Biotechnology from GNDU with 75.14% marks.
- M.Sc. in Animal Biotechnology from ICAR-National Dairy Research Institute, Karnal (2018-2021) with 7.77 CGPA.

Key Skills

- Semen processing
- RNA isolation
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- Microbiological Techniques

Additional Information

Computer proficiency in MS Office (Word, Excel, Power Point etc.)

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