RNA isolation from crossbred bull spermatozoa for analysing differential abundance of sperm specific gene transcripts

Indrajit Ganguly*[1], Sushil Kumar, G K Gaur, Umesh Singh, D K Mandal, Mahesh Kumar, Indranil Bagchi, Bimlendu Roy, Sunil Kumar, Sandeep Mann and Rani Singh
ICAR-Central Institute for Research on Cattle, Meerut-250001, Uttar Pradesh, India

ABSTRACT

Although mature spermatozoa seem to be transcriptionally inert, however, thousands of mRNA transcripts have been reported to be present inside the mature sperm, being trapped during the course of spermatogenesis. These remnants RNAs may perhaps serve as a potential "markers" of spermatogenesis by virtue of their involvement, directly or indirectly, in the process of fertilization, early embryonic cleavage, and fertility. Gene expression profiling of mammalian sperm is a novel non-invasive tool to evaluate male fertility. RNA isolation from sperm is little tricky in contrast to RNA isolation from testis, and required stringent RNA quality control. The present study demonstrates a comprehensive RNA isolation protocol from crossbred Frieswal (HF X Sahiwal) bull semen, with stringent quality/purity checking, critically required for studying differential abundance of sperm transcripts. Initially, semen samples were subjected to discontinuous (45:90) Percoll gradient centrifugation, explicitly eliminating damaged spermatozoa and contaminating somatic cells. Total RNA was extracted from sperm pellets using heated Tri reagent and an on-column DNase treatment was carried out. The cDNA was synthesized using RT-PCR. The cDNA samples were then amplified by PCR using specific intron spanning primers to rule out contamination, if any, within isolated sperm RNA by g-DNA (PRM1 and DAZL), testicular germ cells like spermatocytes (KIT), epithelial cells (E-cadherin- CDH1) and leucocyte (CD4 and CD45). Further, the presence of transcripts like DazL, PRM1, PRM2, PRM3, TNP1, and TNP2 were also demonstrated in the ejaculated spermatozoa by PCR amplification. This method together with rigorous quality assurance mentioned here are minimum requirement for bias free analysis of differential abundance of sperm transcripts as well as high throughput transcriptomics research.

Keywords: Frieswal, sperm RNA, Percoll, spermatogenesis, HF X Sahiwal
Present address: [1]ICAR-NBAGR, Karnal, Haryana
***Corresponding author**: drindrajit@gmail.com.

INTRODUCTION

Mature spermatozoa are generally transcriptionally inactive; however, they harbour a variety of mRNA molecules, assumed to be originated from the trapped cytoplasmic content remaining after spermiogenesis (Gilbert et al. 2007). Recent studies proposed that the composition and quantity of sperm RNA transcripts might have a valuable diagnostic significance for male fertility as well as putative role in chromatin repackaging, genomic imprinting and early embryonic development (Miller et al. 2005). It has been estimated that each spermatozoon, haploid spermatid and diploid somatic cell contains approximately 10–20 fg, 450 fg and 10–20 pg of total RNA, respectively (Krawetz, 2005; Gilbert et al. 2007; Goodrich et al. 2007). It shows that each somatic cell holds approximately 1000 times more RNA than a mature spermatozoon. Because of the above fact, the spermatozoal transcript profile could easily be distorted by somatic RNA contaminant (WBC, epithelial cells, and immature diploid spermatocytes). Therefore, stringent protocol for sperm RNA isolation with proper quality control for excluding probable contamination arising from genomic DNA and somatic cell RNA need to ascertained before proceeding to work with sperm RNA. Here, we are presenting a method for isolation of high-quality RNA from Crossbred (Frieswal: HF X Sahiwal) bull sperm with stringent quality control as well as amplification of few sperm transcripts to show its reliability towards characterizing RNA abundance in the sperm cells and utilization of such RNA for searching suitable biomarkers specific to fertility or embryonic development.

MATERIALS AND METHODS

Experimental Samples

Semen samples were obtained from crossbred Frieswal bulls with the aid of an AV (42-45ºC) at early morning by the semen collector. Immediately after

collection it was assessed for normal semen quality parameters. Ejaculates were quickly placed in a water bath at 34°C, after collection, and evaluated for volume, sperm concentration and progressive motility per cent. Sperm concentration was estimated by using a photometer (Accucell, IMV-France) after appropriate calibration for bovine semen. Semen was diluted in tris-egg yolk-citric acid-fructose-glycerol extender and progressive motility was assessed subjectively (in scale of 0–100% at nearest 5% intervals), by placing a drop of diluted semen on a glass slide covered with a cover slip and viewing at least 5 microscopic fields at 200x magnification under a phase-contrast microscope fitted warm stage (37 ^{0}C). Testicular tissues of buffalo (*Bubalus bubalis*) were collected from a local abattoir in RNA later, transported to the laboratory and stored at -80 ^{0}C before use.

Evaluation of viability of spermatozoa

Both eosin nigrosin (EN) vital stain and hypo-osmotic swelling test (HOST) were used for the evaluation of the sperm plasmalemma integrity. For the EN test, smears were prepared by mixing 20 µl of sperm fraction and an equal volume of EN solution (1 g eosin, 5g nigrosin, 2.9 g sodium citrate in 100ml of distilled water). The percentage of membrane-intact sperm identified by EN was determined by counting 200 sperm under magnification (×400) with bright-field microscopy. Unstained sperm were viable whereas sperm showing partial or complete purple staining were considered as dead cells.

The response of bovine spermatozoa to the HOS test was assessed with fructose and sodium citrate solution (150 mOsm/L). After semen collection, 100µL of raw semen was added to 1.0 mL of pre-warmed (37°C) HOS solution, mixed gently and incubated for 1 hour at 37°C. Similarly, for each ejaculate a control sample was incubated in 2.9% sodium citrate solution, isotonic to seminal plasma. After incubation, a small drop of thoroughly mixed sample was placed on a clean grease free glass slide, covered with a cover slip and observed under a phase contrast microscope at x400 magnification. From each smear a total of 100 spermatozoa were evaluated viewing at least in five different fields.

Sperm Separation Procedure

Sperm purification was carried out on a discontinuous gradient of 45 and 90% (v/v) Percoll (Fig.1). Generally the preparation of 90% Percoll solution using a 9: 1 mixture of Percoll and a 10x stock of Sp-TALP (Parrish et al. 1988) was avoided because it may give rise a hyperosmotic solution due to the presence of considerable solid Percoll beads in the solution (Parrish et al. 1995). A modification was therefore used here (as proposed by Parrish et al. 1995) to produce a medium that was iso-osmotic with Sp-TALP. Briefly, to prepare 90% Percoll solution as per modification, Percoll was mixed 9: 1 with a concentrated solution containing 31 mM KCl, 800 mM NaCl, 3 mM $NaH2PO_4$ and 100 mM HEPES. The pH of the concentrated solution was previously adjusted to pH 7.3 with 1 N NaOH. The following chemicals were then added (mM final concentrations): $CaC1_2$ (2.0), $MgC1_2$ (0.4), lactic acid (21.6) and $NaHC0_3$ (25.0).To prepare the 45% Percoll solution, the 90% Percoll medium was mixed 1: 1 with Sp-TALP. The gradient consisted of 0.5 ml of fresh semen sample layered over 2 ml of 45% Percoll and 2 ml of 90% Percoll in a 15-ml conical plastic tube. The tube was centrifuged at 700 g for 30 min. The pellet from the bottom was carefully collected after discarding above layers, washed and centrifuged twice at 250 g for 5 min in Sp-TALP solution. The pellet containing the motile spermatozoa was kept at -80 ^{0}C in RNAlater (Ambion, Austin, TX, USA) until RNA extraction.

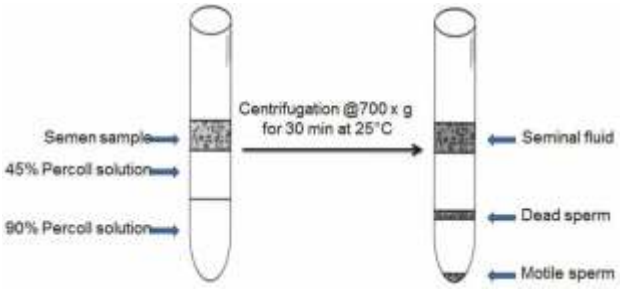

Figure 1. Percoll gradient centrifugation

RNA extraction from spermatozoa

Extraction of total RNA from bovine spermatozoa, and buffalo (*bubalus bubalis)* testicular tissues was carried out using Tri Reagent (Sigma). The protocol

was followed according to the manufacturer's recommendation with minor modifications. For extraction, the TRI reagent was heated at 60^{0}C and the samples were incubated for half an hour. The most important part to remember here is to vortex the sample vigorously and pipetting several times through a sterile syringe fitted with a 24 gaze needle after every 10 min of incubation to completely dissociate sperm membranes. In order to prevent any bias potentially arising from the RNA extraction procedure, the same method was used for RNA isolation from the buffalo testicular tissue samples. Equal number of spermatozoa (100 million) was used for RNA isolation from each sample. The subsequent steps of the protocol were performed as recommended by the manufacturer. Total RNA sample (aqueous phase) were then passed through a RNA extraction column (GenElute Binding column, Sigma) upon which an RNAse-free, DNAse I treatment (Ambion) was performed in order to eliminate contaminating genomic DNA from the samples. RNA was quantified with a Nanodrop ND-1000 spectrophotometer (Nanodrop, Thermo Fisher Scientific, Wilmington, Delaware USA).

Sperm RNA Quality assessment

Total RNA (~60 ng) extracted from crossbred bull spermatozoa and buffalo (*Bubalus bubalis)* testicular tissues were subjected to reverse transcription using Protoscript First Strand cDNA Synthesis Kit (New England Biolabs, Beverly, MA) as per manufacturer's recommendations. The complementary DNA were then amplified by PCR using specific primers to check contamination, within isolated Sperm RNA, by g-DNA (PRM1, DAZL), testicular germ cells like spermatocytes (KIT), epithelial cells (E-cadherin-CDH1) and leucocyte (CD4 and CD45) (Table 1). The reverse transcription PCR (RT-PCR) products were resolved in 1x Tris-acetate EDTA, 2 % agarose gel stained with ethidium bromide. Primers were designed using Primer 3 Software (http://frodo.wi.mit.edu/primer3/input.htm).

Genomic DNA contamination of the RNA samples was tested by RT-PCR using intron spanning primers specific to bovine protamine 1 (PRM1) and deleted azoospermia-like (DAZL) genes. The positive control was a purified bovine genomic DNA extracted from blood using GenElute™ Blood Genomic DNA Kit (Sigma–Aldrich). Similarly, absence of epithelial and germ cell contaminant in spermatozoal RNA was tested using primers specific to molecular markers like CDH1 and KIT, respectively, where buffalo testicular cDNA was used as positive control. Leukocyte contamination was evaluated by using PCR primers targeting the CD4 and CD45 antigen. The positive control for leukocyte contamination were (1) cDNA of WBC obtained from reverse transcribed total RNA extracted from a blood sample using Histopaque 1077 and TRI reagent (Sigma–Aldrich) as per manufacturer's instruction and (2) Buffalo testicular cDNA.

Table 1. Checking of contaminations in the RNA isolated from spermatozoa

Contamination	Gene/ marker	Primer name/ sequences	GenBank ID	Product size (bp)
Genomic DNA	PRM1	PRM1F- 5'AGATACCGATGCTGCCTCAC3' PRM1R-5'GTGGCATGTTCAAGATGTGG3'	NM_174156	234 (334 with intron)
Genomic DNA	DAZL	DAZL-F 5' CAC CAG CCA AGG CTA TGT TT 3' DAZL-R 5' CAC CAG TTC GAT CCG TGA TT 3'	NM_001081725	158 (573 with intron)
Somatic cell	B. Taurus E-cadherin (CDH1)	CDH1-F 5'CCG TGA GAG TTT TCC CAC AT 3' CDH1-R 5'CAT TGG TGA CTG GGT CTG TG 3'	NM_001002763.1	296
Somatic cell	v-kit oncogene homolog	KIT-F 5'GAC CTG GAG GAC TTG CTG AG3' KIT-R 5'AGG GGC TGC TTC CTA AAG AG3'	XM_612028	316
Leucocytes	Cd4	CD4-F 5' CAA TGG CAA AGT CCT GTT GG 3' CD4-R 5' GAT CTG AGA CAT CCG TTC TGC 3'	AJ535319	184
Leucocytes	CD45	CD45-F 5' GACATCGCAGTGTTTGTTGC 3' CD45-R 5' GGAGGTTCACATTCCTCTCC 3'	NM_001206523.1	239

Table 2. Primers used to amplify specific sperm transcripts

Sl. No.	Gene/ marker	Primer name/ sequences	GenBank ID	Product size (bp)
1	PRM2	PRM2F 5' CCACGTGAAGAGTCCAACTG 3' PRM2R 5' TGTGAGTCCTCCCGTAGACC 3'	NM_174157.3	128
2	PRM3	PRM3F 5'GCGTGAGCCAGGATAACTTC 3' PRM3R 5'ACCTCTGAGTCGGCGTCTT 3'	NM_001078053.1	169
3	TPN1	TNP1F 5'GACCAGCCGCAAATTAAAGA 3' TNP1R 5' TTTGCTGCCACTTCTTTTGA 3'	NM_174199.2	85
4	TPN2	TNP2 F- 5'ACAGACACACCATGCACTCC 3' TNP2 R- 5' CTTGATCACCTTTCCCTCCA 3'	NM_174200.1	100

Amplification of PRM2, PRM3 TPN1and TPN2

After a thorough quality check of isolated RNA, few additional sperm transcripts like protamine 2 (PRM2), protamine 3 (PRM3), transition protein 1 (TNP1) and transition protein 2 (TNP2) were amplified by RT-PCR utilizing specific primer pairs (Table 2). This was further to confirm the isolated RNA quality and suitability of RNA isolation procedure in the downstream experiments of studying transcript abundance in the spermatozoa of different category of bulls.

RESULTS AND DISCUSSION

Synthesis and translation of testis-specific mRNAs are governed by stringent physiological and genetically control mechanisms. The mRNAs found in mature spermatozoa appear to be untranslated remnants and may be used as a fingerprint of spermatogenesis (Ostermeier et al 2002). Understanding the differential transcripts abundance in the sperm of normal vs impaired individual is very useful to associate the altered amounts of specific transcripts with the problems of spermatogenesis. Around 5,000 different mRNA transcripts have been reported in sperm and 25% of them encode for proteins involved in transcription and regulation of transcription (Miller and Ostermeier, 2006). Most of the mRNAs in sperm are found to be involved in the functions of cell proliferation, signal transduction, and oncogenesis, all of which are related to spermatogenesis (Ostermeier et al. 2002). These surviving mRNA transcripts may therefore be a useful indicator of gene activity during the critical steps of spermatogenesis, and could give information about mechanisms of testicular problems, fertility and embryonic development. Although it seems very straightforward to target sperm transcripts as an alternative noninvasive procedure for understanding male gonadal problems, however, it may leads to erroneous results until one standardized RNA isolation procedure critically taking all sorts of precaution to restrict contamination from other sources is employed.

Here we tried to isolate quality RNA from mature spermatozoa of Crossbred Frieswal (HF X Sahiwal) bull semen with stringent purity/quality verification as well as amplification of few additional sperm specific transcripts. The discontinuous Percoll gradient (Fig.1) used to purify the semen samples eliminated the damaged spermatozoa, dead cells and cell debris, ensuring the downstream experiments with RNA from intact cells. Microscopic examination as well as PCR based quality checking confirmed the results of absolutely pure spermatozoa obtained after Percoll density gradient centrifugation. Routine semen quality parameters like volume, concentration, initial progressive motility, etc were measured for each sample. EN vital staining as well as HOS test was also performed. Unstained sperm were viable whereas sperm showing partial or complete purple staining were considered as dead cells (Fig.2A). Under HOS test, sperm without swollen or coiled tails were considered as non-viable (Fig.2B). HOS test showed significant ($P< 0.01$) positive correlation with progressive motility($r= 0.91$) and sperm viability ($r= 0.93$) (data not shown).

Because of the membrane sturdiness of the bovine spermatozoa, total RNA was extracted by incubating

Figure 2. EN vital staining and Hypo-osmotic swelling test (HOST) for crossbred Frieswal sperm. A. Unstained sperm are viable and sperm showing partial or complete purple are dead. B. HOST-responded sperm showing typical coiling of tail and non-responded with no coiling.

the spermatozoa in TRI reagent for half an hour at (60^{0}C). To avoid any potential bias arising from the extraction procedure, spermatozoa as well as buffalo testicular tissue samples were extracted with the same method. Spectrophotometer values of sperm RNA samples were observed between1.70–1.75 for OD260/OD280. In contrast, RNA isolated from buffalo testicular tissues had an OD260/OD280 ratio between1.8–2.0, indicating that they were pure and clean. Samples with desired OD ratios were subjected to further quality check to assure utmost sample purity.

In order to overcome genomic DNA contamination (gDNA) in the isolated sperm RNA an on-column DNase treatment was carried out. Further to check complete elimination of gDNA from the samples intron spanning primers specific to DAZL and PRM1 genes were targeted. In the presence of genomic DNA, the intron-spanning primers produced an amplicon of 334 bp and 573 bp for DAZL and PRM1 genes, respectively (Fig. 3). Sperm cDNA samples without genomic DNA contamination were used for further works.

The absence of contamination by somatic and germ cells was always confirmed by visual examination of the semen fractions under microscope. Furthermore, the absence of contamination of germ cells and epithelial cells in all the RNA extractions was tested by RT-PCR targeting specific molecular markers like KIT and CDH1, respectively (Table 1, Fig. 4). Contamination free RNA sample was chosen for further processing.

An assessment of contamination by cells of

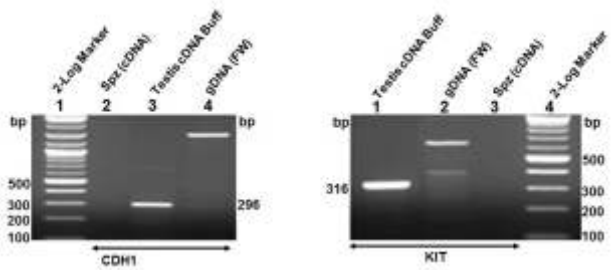

Figure. 4 Epithelial and germ cell contaminant in the isolated RNA was tested by PCR targeting specific markers like CDH1 and KIT using an equivalent of 100 ng of sperm cDNA, buffalo testis cDNA, and gDNA samples. The absence of PCR products specific to CDH1 (Fig 4A. lane 2) and KIT (Fig 4B. lane 3) reveal sample purity. M: molecular ladder (2-log DNA ladder-0.1 -10.0 kb, NEB, Beverly, MA, USA)

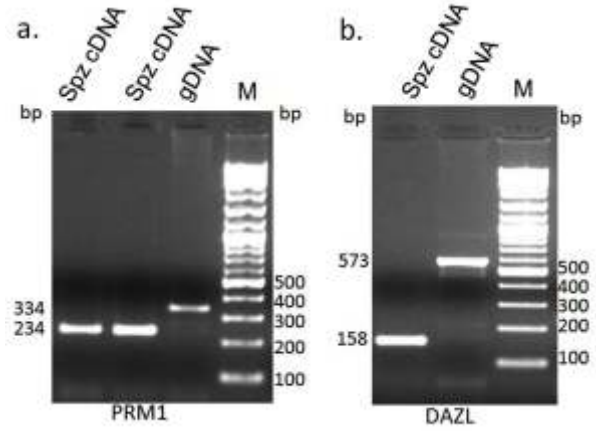

Figure 3. Checking of isolated sperm RNA for genomic DNA contamination. a. Intron-spanning PRM1primers produce an amplicon of 234 bp from pure spermatozoa cDNA, without gDNA contamination. An amplicon of 334 bp is obtained from gDNA with PRM1primers. b. Spermatozoa cDNA sample giving rise an amplicon of 158 bp corresponding to DazL cDNA without gDNA contamination. An amplicon of 573 bp is obtained from gDNA with DazL primers. M: molecular ladder (2-log DNA ladder-0.1 -10.0 kb, NEB, Beverly, MA, USA)

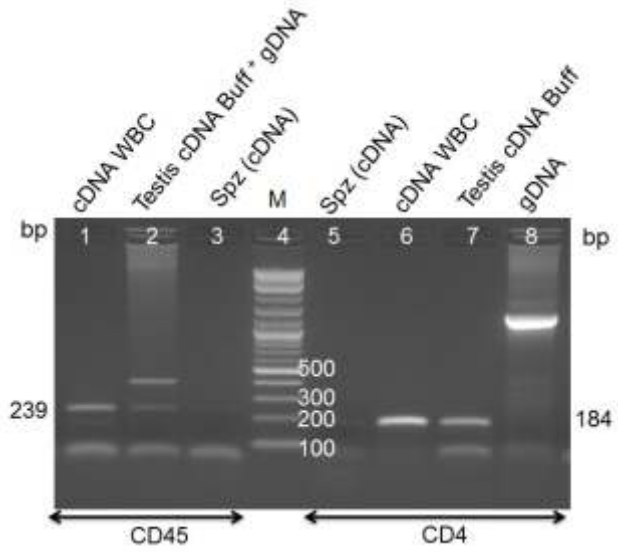

Figure 5. Assessment of contamination by cells of hematopoietic origin (leucocytes) using PCR targeting the marker CD4 and CD45. The absence of contamination (lane 3 and 5) shows purity of sperm RNA. M: molecular ladder (2-log DNA ladder-0.1 -10.0 kb, NEB, Beverly, MA, USA)

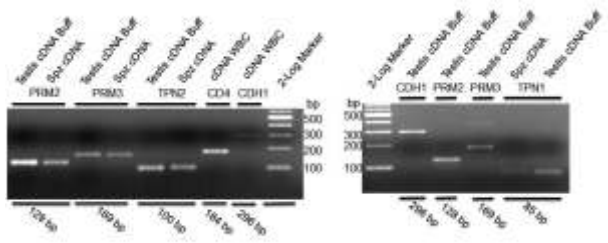

Figure 6. Showing PCR amplifications of different transcripts. Sample used as template for PCR is mentioned in the top, whereas, amplicon length has been indicated at the bottom of the figure.

hematopoietic origin (leucocytes) was carried out by RT-PCR using the CD4 and CD45 marker. The presence of contamination as observed in WBC (Fig. 5: lane1 and 6), buffalo testicular tissue (Fig. 5: lane 2, 7) and gDNA (Fig. 5: lane 2 and 8) remained undetected in the sperm samples (Fig. 5: lane 3 and 5) used for further analysis.

After a thorough quality checking of isolated RNA, additional sperm transcripts like protamine 2 (PRM2), protamine 3 (PRM3), transition protein 1 (TNP1) and transition protein 2 (TNP2) were amplified by RT-PCR utilizing specific primer pairs (Table 2, Fig 6). Amplification of desired products further confirmed the isolated sperm RNA quality and suitability of this procedure for further downstream experiments of studying transcript abundance in different category of bulls (Ganguly et al. 2013).

In conclusion, the present study demonstrated a comprehensive RNA isolation protocol from crossbred bull semen with stringent quality/purity checking mandatory for researchers entering in this field for studying differential abundance of sperm transcript. It is always to remember that very negligible somatic RNA contaminant (WBC, epithelial cells, and immature diploid spermatocytes) could easily distort any spermatozoal transcript profile.

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