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## Detection of osteopontin gene transcripts in bull spermatozoa

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**Abstract**

The present experiment was aimed to detect osteopontin gene transcript from bull spermatozoa. Fresh semen samples from 12 Jersey cross breed bulls were collected using artificial vagina. The volume and concentration of the individual samples were recorded. The spermatozoa were separated from bull semen samples by swim up protocol using sperm TALP. The initial concentration of semen sample was checked immediately before proceeding for RNA isolation and normalization of initial concentration was done. So, initial amount of every sample was made equal. Total RNA from the bull spermatozoa were extracted by the RNeasy® Mini Kit, Qiagen as per manufacturer's protocol. The purity and concentration of the samples were measured at A280/260 by using Nano Drop™ 1000 spectrophotometer. The first strand cDNA was synthesized from 1µg total RNA using M-Mu LV Reverse Transcriptase Revert Aid™ H minus first strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's protocol. PCR product of about 267 bp fragment length of OPN gene was confirmed by conventional PCR. The bulk PCR product of about 100µl was purified by Gen Elute™ PCR Clean-Up Kit Sigma-Aldrich as per manufacturer's protocol. The concentration and purity of PCR product were 64ng/µl and 1.6 respectively. The cloning and expression of OPN gene was carried out by using TA cloning kit. The ligated product was then transformed into *E. coli* DH5α cells. The plate was incubated for overnight at 37 °C for selection of the colonies and stock cultures were maintained in LB media at -80 °C. The clones which showed the amplification of 400 bp DNA fragment were considered as positive clone carrying desired insert. Plasmid isolation was done as per the protocol followed in Hi Yield™ Plasmid Mini Kit (RBC Cat. NO. YPD100) and the eluted DNA were stored at -20 °C. The concentration and purity of PCR purified product were 67ng/µl and 1.6 respectively. The eluted plasmid DNA was sequenced commercially (Amnion, Bangalore) using M13 primers. The cloned partial sequence of OPN gene isolated from bovine spermatozoa was submitted at DDBJ (Accession No. AB983656). On blast analysis using NCBI nucleotide blast (Online tool), 99 per cent identity was found with reported consensus sequences except single nucleotide variation in DNA sequence at 234<sup>th</sup> nucleotide. There was substitution of adenine instead of cytosine.

**Keywords:** Osteopontin, bull spermatozoa, cloning, sequencing

**Introduction**

OPN has been shown to be a sperm surface molecule in rats [1]. OPN is secreted from ampulla and the vesicular gland and it was detected in accessory sex glands fluid in bull [2,3]. OPN interacts with cell surface integrins, CD44, fibronectin, collagen and several integrin types which are present in the membranes of peritubular cells, sertoli cells and basement membranes of the tubules, acrosome of spermatids and spermatogonial stem cells [4]. Seminal plasma OPN binds to ejaculated sperm through integrin receptors. This complex binds with oocyte integrins. This is a proposed model for OPN action in sperm-egg interaction [5].

OPN gene transcripts were only associated with certain stages of the seminiferous cycle and their synthesis is regulated by the major events that control spermatogenesis and germ cell development during spermatogenesis [6]. OPN appears at the cell surface and endosomes of the epithelial cells in the efferent ducts and epididymis of rat. It mainly serves to bind with calcium crystals, thus preventing luminal calcium containing crystal aggregation that would reduce the sperm fertilizing capability. Sperm acquires additional OPN from the accessory sex glands [7]. Integrins ( $\alpha_v$  and  $\alpha_5$ ) have been identified in the bovine sperm membrane and can bind to oviductal OPN [8]. OPN is found in ejaculated and cauda epididymal sperm and presence of OPN in bovine sperm membranes was confirmed along with the presence of

multiple isoforms which may have other functions [9]. OPN in canine seminal plasma and sperm membrane extracts was demonstrated by anti-OPN antibody [10, 11]. In boars, the abundance of the proteins AWN-1 and OPN related to fertility was characterized in three distinct fractions of the ejaculates. Although there were no major differences in the relative abundance of AWN-1 and OPN in the ejaculates collected. Differences in the concentration of these specific proteins in seminal plasma from the highest and lowest fertility boars may largely reflect differences in total protein concentration and the sperm concentration of each ejaculate, which ultimately determines the amount of seminal plasma present after dilution of the ejaculate [12]. OPN transcript was detected in mithun (*Bos frontalis*) seminal plasma and it is positively correlated with post-freeze-thaw quality of cryopreserved spermatozoa [13]. In earlier studies, OPN was primarily demonstrated from seminal plasma of different species. Hence, the present study was designed to detect OPN gene transcript from bull spermatozoa.

## Materials and Methods

### Materials

#### Plasticware and glassware

All the plasticware used for this study viz., centrifuge tubes, microcentrifuge tubes, microtips (different graduation) and PCR tubes were purchased from Thermo Scientific. Real time PCR strips and master clear cap strips were purchased from Biorad, USA.

All the glassware viz., microscopic cover slip, microscopic slide, conical flasks and beakers were purchased from Borosil, India.

#### Commercial kits

The following commercial kits were procured for cloning and sequencing studies.

- RNeasy® mini kit (Qiagen Cat. No.74104)
- Revert Aid™ H minus first strand cDNA synthesis kit (Thermo Scientific Cat. No. #k1631)
- GenElute™ PCR clean-up kit (Sigma-Aldrich, Cat. No. NA1020)
- Hi Yield™ plasmid mini kit (RBC Cat. NO. YPD100)

### Chemicals

The following chemicals were procured for gel electrophoresis

- Tris acetate gel running buffer (TAE) (50X) (Medox®, India)
- Gel loading dye (6X) (Thermo Scientific, USA)
- Gel red nucleic acid stain (Biotium, Canada)
- Agarose (Sigma-Aldrich, USA)
- DNA marker 100bp plus ladder (Thermo Scientific, USA)

### Methods

#### Total RNA extraction, cDNA synthesis

Semen samples from 12 Jersey cross breed bulls were obtained using an artificial vagina. Sperm volume was determined by collecting the semen in graduated centrifuge tube and sperm concentration was evaluated by standard Neuburger hem cytometer method. Motile sperms were isolated by the swim-up procedure. 250 µl of semen was layered under 1 ml of aliquots of the sperm TALP medium in each of 15-ml centrifuge tubes. After incubation for one hour at 37 °C in an atmosphere with 5 per cent CO<sub>2</sub>, 850 µl was removed from the top of each tube [14]. The swim-up separated

spermatozoa were then diluted with sperm TALP to a final volume of 5 ml, centrifuged at 1200 rpm for 10 minutes. The supernatant was discarded, the sperm pellet was diluted to 5 ml with sperm TALP, centrifuged again at 1200 rpm for 10 minutes and the supernatant was discarded. Total RNA from the bull spermatozoa were extracted by the RNeasy® mini kit (Qiagen Cat. No.74104) as per manufacturer's protocol. Reverse transcription was carried out in a 20µl reaction volume by using Revert Aid™ H minus first strand cDNA synthesis kit (Thermo Scientific Cat. No. #k1631). The reaction was carried out in a thermal cycler (Eppendorf Mastercycler®, Germany) with the following incubation conditions

42 °C for 60 minutes

70 °C for 5 minutes

The synthesized cDNA was stored at -20 °C until further use.

#### Polymerase chain reaction

Primers for OPN were designed from the sequence of the cattle (Genebank accession NO: AY878328) by using ExpASY tool.

**Table 1:** Primer sequences for OPN gene

Primers	Primer sequence	Product length
OPN forward	5' ATGCATGACGCACCTAAGAAG 3'	267bp
OPN reverse	5' TCAATTGACCTCAGAAGAGGC 3'	

The polymerase chain reaction for a portion of OPN gene was carried out in a 20 µl volume. PCR programme for amplification of OPN gene in (Eppendorf Mastercycler®, Germany) was carried out with an initiation of 94 °C for 2 minutes followed by

94°C for 15 seconds	} 30 cycles
52°C for 15 seconds	
72°C for 10 seconds	

Final extension was completed at 72 °C for 1 minute. The PCR products were run on a 2 per cent agarose gel in a tris acetate EDTA (TAE) buffer containing gel red at a concentration of 0.35ml/ml of the gel. 10 µl of the product and 2 µl of the DNA marker (100 bp plus ladder) were loaded in the gel. Gel electrophoresis was carried out in TAE buffer at 150V for 45 minutes and visualized under UV transilluminator for the presence of DNA bands of specified size and the image was stored in gel documentation system (Bio Rad, USA). The bulk PCR product of 100 µl was purified using Gen Elute™ PCR clean-up kit (sigma-Aldrich, USA, Cat. No.NA1020) as per manufacturer's protocol. The OD value of the PCR purified DNA standard concentration was measured at 260 nm/280 nm on Thermo Scientific Nano Drop™ 1000 spectrophotometer (Nano Drop Technologies, LLC, Wilimington, DE, USA).The purified PCR product was stored at-20 °C until further use.

#### Cloning of OPN gene

PCR purified product was then ligated into the PTZ57R/T vector using TA cloning kit in 1:3 ratio of vector to DNA as per manufacturer's protocol. The ligated product was incubated at 4 °C overnight. The ligated product was then transformed into *E. coli* DH5α cells. Stock cultures were

maintained in LB media containing 15 per cent glycerol in microcentrifuge tubes and stored in -80 °C.

### Confirmation of OPN gene clones by colony PCR

The clones were picked from the LB ampicillin plate and streaked onto another LB ampicillin plates and grown overnight at 37 °C. The next day colony PCR was performed to confirm the presence of insert. Few cells from the LB ampicillin plate were mixed with the PCR components and PCR was carried out with M13 primers. The clones which showed the amplification of 400 bp DNA fragment were considered as positive clone carrying desired insert. Plasmid isolation was done as per the protocol followed in Hi Yield™ plasmid mini kit (RBC Cat. NO. YPD100). The eluted DNA was stored at -20 °C until further use.

### Sequencing and confirmation of cloned OPN sequence

The eluted plasmid DNA was sequenced commercially (Amnion, Bangalore) using M13 primers. Unwanted sequence was removed by VecScreen. OPN sequence was subjected to BLAST analysis and sequence was submitted at DDBJ.

## Results

### Collection of semen sample and separation of spermatozoa from semen

Fresh semen samples of Jersey cross breed bulls were collected from organized breeding center by using artificial vagina. The volume and concentration of the individual samples were recorded (Table 1). The spermatozoa were

isolated from bull neat semen samples by swim up protocol using sperm TALP.

The initial concentration of semen sample was checked immediately before proceeding for RNA isolation. Thus normalization of initial concentration was done so that initial amount of every sample was made equal.

**Table 1:** Volume and concentration of bull semen samples (n=12)

Bull number	Volume (ml)	Concentration (millions/ml)
1	4.0	870
2	4.5	940
3	3.5	740
4	3.0	690
5	3.5	820
6	3.5	780
7	3.0	660
8	4.0	860
9	4.0	910
10	4.5	980
11	3.5	730
12	4.5	960

### Total RNA isolation and quantification

Total RNA from the bull spermatozoa were extracted by the RNeasy® Mini Kit, Qiagen as per manufacturer's protocol (Plate 1). The purity and concentration of the samples were measured at A280/260 by using Nano Drop™ 1000 spectrophotometer (Table 2).

**Table 2:** Concentration and purity of total RNA isolated from bull semen samples (n=12)

Bull number	Concentration (ng/μl)	Purity
1	34.0	1.5
2	42.5	1.7
3	21.0	1.3
4	14.1	1.2
5	25.0	1.3
6	22.6	1.4
7	12.8	1.0
8	28.0	1.4
9	39.0	1.6
10	50.8	2.0
11	19.2	1.2
12	47.0	2.1

### Confirmation of OPN and GAPDH gene by conventional PCR

The first strand cDNA was synthesized from 1μg total RNA using M-MuLV Reverse Transcriptase Revert Aid™ H minus first strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's protocol. PCR product of about 267 bp fragment length of OPN gene for cloning and expression was confirmed by conventional PCR (Plate 2).

### Purified PCR product

The bulk PCR product of about 100μl was purified by GenElute™ PCR Clean-Up Kit Sigma-Aldrich as per manufacturers protocol (Plate 3). The concentration and purity of PCR product were 64ng/μl and 1.6 respectively.

### Cloning and expression of OPN gene

The cloning and expression of OPN gene was carried out by using TA cloning kit. The ligated product was then transformed into *E. coli* DH5α cells. The plate was incubated

for overnight at 37 °C for selection of the colonies and stock cultures were maintained in LB media at -80 °C.

### Confirmation of clones by colony PCR

The clones which showed the amplification of 400 bp DNA fragment were considered as positive clone carrying desired insert (Plate 4).

### Plasmid isolation

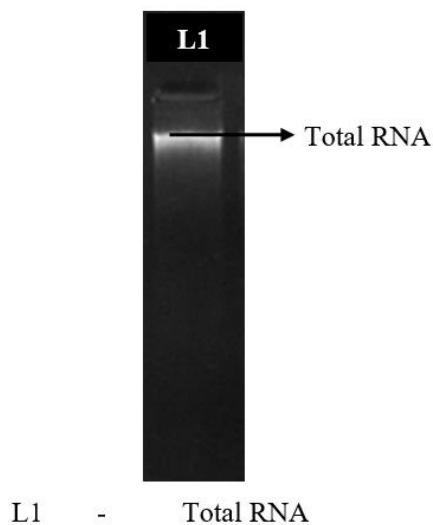
Plasmid isolation was done as per the protocol followed in HiYield™ Plasmid Mini Kit (RBC Cat. NO. YPD100) and the eluted DNA were stored at -20 °C until further use for real time PCR assay (Plate 5). The concentration and purity of PCR purified product were 67ng/μl and 1.6 respectively.

### Sequencing

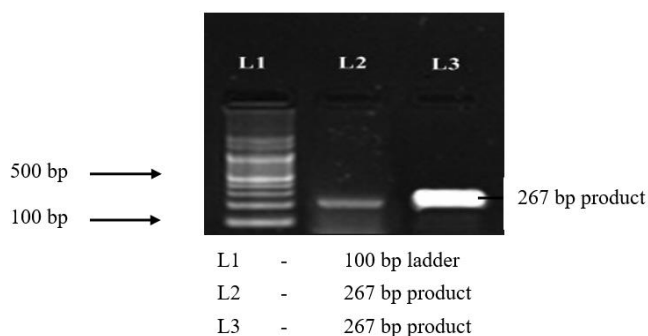
The eluted plasmid DNA was sequenced commercially (Amnion, Bangalore) using M13 primers (Sequence of cloned OPN gene in Figure 1). The cloned sequence of OPN isolated from bovine semen was submitted at DDBJ (Accession

No.AB983656). On blast analysis using NCBI nucleotide blast (Online tool), 99 per cent identity was found with reported consensus sequences except single nucleotide

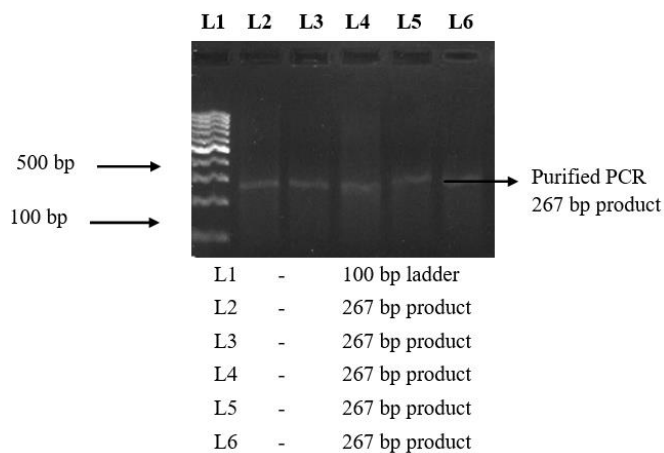
variation in DNA sequence at 234<sup>th</sup> nucleotide. There was substitution of adenine instead of cytosine.



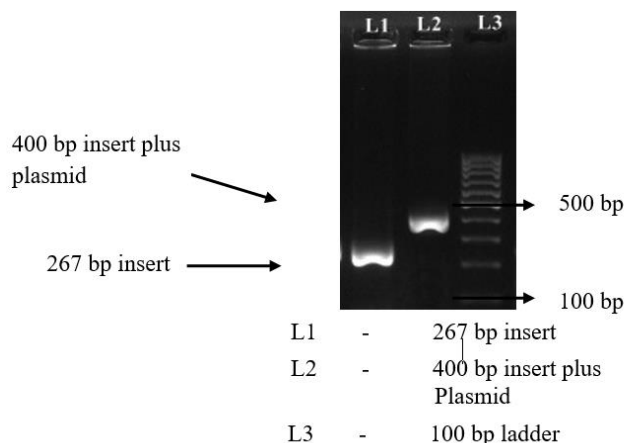
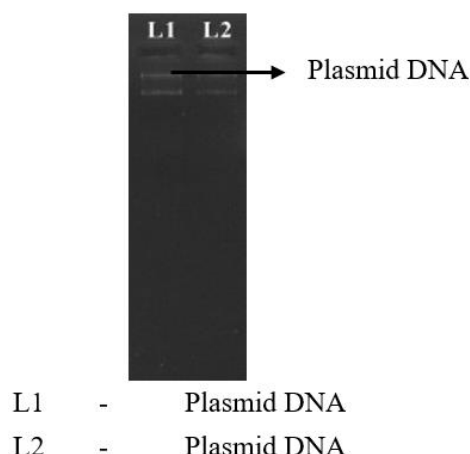
**Plate 1:** Total RNA isolated from bovine spermatozoa



**Plate 2:** Confirmation of OPN gene of product size 267 bp by conventional PCR



**Plate 3:** Purified PCR product of 267 bp

**Plate 4:** Confirmation of positive clones by colony PCR**Plate 5:** Confirmation of plasmid DNA

ATGCATGACGCACCTAAGAAGACGAGTCAGCTGACTGACCACAGCAAGGAAACCAACAGTAGCGAGCTTTCCAAAGAAC  
 TCACGCCAAAGGCCAAGGATAAGAACAAGCATTCGAATCTGATTGAGAGTCAGGAAAATTCCAAACTCAGCCAAGAATTC  
 CATAGCCTTGAAGACAAGCTAGACCTAGATCATAAGAGTGAAGAAGACAAAACACATGAAAATTCGTATTTCTCATGAATT  
 AGATAGTGCCTCTTCTGAGGTCAATTGA

**Fig 1:** OPN cloned sequence from bull semen

## Discussion

In case of spermatozoal RNA isolation technique, the major limitation is the presence of low amount of cytoplasm in sperm cells, low concentration of total RNA and the resistance of the cellular membrane to lysis. The most widely used protocol for RNA extraction from ejaculated sperm cells by using acid guanidium thiocyanate phenol-chloroform extraction<sup>[15]</sup> and the extraction with TRIzol RNA isolation reagent<sup>[16]</sup>. The analysis of spermatozoa RNA in absorption spectrum at 260/280nm and 260/230 nm ratios had very low values when measured using the Nanodrop spectrophotometer indicating the presence of salt, protein or polysaccharide contamination in semen.

Some protein contamination could remain in the aqueous/organic phase during the RNA extraction resulting in low 260/280 values. Other possibility of low value was addition of glycogen to support the precipitation of small amounts of total RNA and polysaccharides usually decrease this ratio. Even after precipitating sperm RNA without glycogen, the low value of 260/230 ratio remained the same. Although the contamination interfered with RNA quantification, but there was no observation of inhibitory effects on subsequent enzymatic reactions as indicated by cDNA synthesis and RNA amplification<sup>[17]</sup>.

Experimental trial was conducted by comparing five different methods for RNA extraction from bull sperm and reported that ejaculated bull sperm contained 0.0018 pg of RNA per spermatozoon. Therefore, the maximum expected yield of RNA was less than 2 µg and the amount was too low for accurate measurement by UV absorbance at 260 nm<sup>[16]</sup>. RNA isolation from porcine spermatozoa using Trizol resulted in 4 to 10 ng of total RNA from 1x10<sup>6</sup> spermatozoa<sup>[18]</sup>.

RNA isolation from sperm still presents several challenges. RNA quantity in a sperm cell is considered low as one haploid spermatozoon contains about 10–20 fg of RNA when compared to 450 fg of RNA in a haploid spermatid and 10–20 pg of RNA in one diploid somatic cell. Removal of somatic cells like white blood cells, erythrocytes and immature diploid spermatocytes from ejaculates should be done to ensure RNA from sperm alone to be available for isolation. Because sperm are highly condensed cells, isolating cellular contents is difficult and there are differences among species in sperm attributes and chromatin packaging<sup>[19]</sup>.

Experiments conducted with semen samples from fresh, flash frozen, extended frozen, extended cooled and centrifuged extended cooled in stallion and concluded that total sperm recovery rate following purification, as measured by sperm concentration was highest for fresh, similar among extended frozen, extended cooled, centrifuged extended and lowest for



flash frozen. Fresh semen produced the best results as it contained the most live sperm, whereas extended samples prolonged sperm life to a degree, but sperm in flash-frozen samples were dead and thus their RNA got degraded [20].

Micro RNAs (miRNAs) play an essential part in animal gene regulation and these short transcripts are essential for normal development and cellular homeostasis. When two groups of bulls having different fertility were examined and found that there was abundance of miRNAs in bovine spermatozoa. miRNAs detected in low fertility bull were expressed at higher levels than those in the high fertility bull [21]. The higher expression levels of the miRNAs in the low fertility bulls means that miRNAs might be down regulating expression of genes whose products play an important role in fertilization and early embryonic development.

In testis and sperm, mir-16 expression was higher when compared to the other miRNAs and mir-191 was highly expressed in testis [22]. In gene regulation studies related to spermatogenesis, there are many miRNAs which have to be detected but little is known about their expression level or patterns and they may have important roles in sperm physiology including fertilization, egg activation and embryo development.

### Cloning and expression of OPN

Isolation and characterization of full length open reading frame (ORF) of OPN cDNA from kidney of Korean native cows (Hanwoo) demonstrated that the isolated cDNA was 1190 bp in length containing a complete ORF of 837 bp. It also encoded a precursor protein of Hanwoo's OPN consisting of 278 amino acids with a signal peptide of 16 amino acids. When compared to the corresponding sequences of Holstein bone marrow OPN, the amino acid homology of Hanwoo's kidney OPN was found to be 99.3%. Hanwoo's kidney OPN and Holstein bone marrow OPN was different only in two amino acid residues (at 42 and 56), amino acid residue 42 is Thr (T)  $\leftrightarrow$  Ile (I), and amino acid residue 56 is Ala (A)  $\leftrightarrow$  Thr (T) respectively [23].

In this study, partial OPN transcript (266 nucleotide sequence) was demonstrated from bull spermatozoa and it showed 99 per cent identity with reported consensus sequences except single nucleotide variation in DNA sequence at 234<sup>th</sup> nucleotide. There was substitution of adenine instead of cytosine.

### Conclusions

From this experiment, it is evident that OPN is localized on spermatozoa and it may participate in many physiological events in sperm-oocyte interaction.

### Acknowledgement

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