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Characterization and in-silico analysis of *Osteopontin* gene in vechur cattle of Kerala

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Abstract
Characterization of the genes pertaining to immune system in Vechur cattle might provide an insight into the mechanisms involved in disease resistance in general and may aid in breeding animals for disease resistance. Osteopontin (OPN), a phosphoprotein plays various roles in immune response, such as activating macrophages, acting as chemokine, attracting neutrophils, delaying cell apoptosis, opsonizing bacteria, acting as pro-inflammatory, aiding in angiogenesis and tissue remodelling. The present study was framed to characterize the *OPN* genes in Vechur cattle. Characterization revealed 909 bp length *OPN* mRNA contained protein coding open reading frame of 834 bp which codes for 278 amino acids. Pair blasting with *Bos taurus* sequence (NM_174187.2) displayed 99 per cent homology and other related ruminant species in close proximity. Sequence analysis for presence of any nucleotide variation disclosed three novel nucleotide transitions in coding region and two non-synonymous transitions at 188th and 229th position of nucleotide sequence. Serine amino acid was found to be highest frequency of 15.11 per cent of total protein constituting the primary OPN protein. In-silico analysis of predicted OPN protein sequence showed presence of signal peptide and nature of protein suggesting secretory type protein. The observed variation in nucleotide sequence of mRNA sequences and it predicted protein of *OPN* gene in Vechur cattle may pave way for further study on association between existing polymorphisms and host disease resistance nature in those breeds.

Keywords: Characterization, osteopontin, in-silico analysis, vechur cattle

Introduction
Osteopontin (OPN), a phosphoprotein plays various roles in immune response, such as activating macrophages, acting as chemokine, attracting neutrophils, delaying cell apoptosis, opsonizing bacteria, acting as pro-inflammatory, aiding in angiogenesis and tissue remodelling. OPN, a multifunctional protein isolated form bone matrix in bovine [5], also known as secreted phosphoprotein (SPP 1) or early T cell activation (Eta-1). OPN is known to be secreted in most of the luminal organs and glands by epithelial cells and therefore found in most of body fluids such as bile, saliva and milk [3]. It was found to contain a glycine-arginine-glycine-aspartate-serine (GRGDS) cell binding domain corresponding to $\alpha_v\beta_3$ integrin explaining cell adhesion nature of this protein [8]. Research findings opined that OPN was essential in activation of dendritic cells to initiate T-cell mediated immunity in promoting Th_1 type immunity [9]. In a study conducted on OPN suggested it was involved in *Mycobacterium avium subsp. paratuberculosis* (MAP) infection in dairy cows. Study indicated that *OPN* expression was increased in ileum and ileocecal lymph node, suggestive of *OPN* playing a major role in MAP infection [6]. Recent research has demonstrated *Toll Like Receptors* (*TLR*) cross talk in *OPN* induction [10]. They showed *TLR2* was strong agonists (*S. aureus*) in inducing *OPN* production when compared to *TLR4* agonists (*E. coli*). This revealed that *OPN* regulation by *TLR* signalling may modulate immune response specific to pathogens. Nucleotide sequence on OPN gene of Vechur cattle was not available and hence the present study was aimed characterization of *OPN* gene in Vechur cattle of Kerala.

Materials and Methods
University Livestock Farm, Mannuthy formed the venue for this study. Six healthy Vechur cows were selected as the experimental animals for this study. Milk samples were collected in 10 mL sterile container under aseptic conditions. Total RNA was isolated from milk somatic cells using TRIzol® LS reagent (Life Technologies, USA) with little modification in procedure. The RNA isolated was treated with Deoxyribonuclease (DNase) enzyme amplification grade (Sigma-Aldrich) to remove DNA contamination in total RNA, if any.

First strand cDNA was synthesized using Revert Aid First strand cDNA synthesis kit (Thermo scientific). About 500 ng of total RNA from blood samples were taken for cDNA synthesis. Primers used for amplification were designed by using online software Primer 3 (v.0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) utilizing Genbank accession no. NM_174187.2 for amplification of *OPN* gene. (Table 1).

Table 1: Primer sequence for full gene amplification of *OPN* gene

Names	Sequences (5'→3')	Tm (ºC)	Product size
OPN-a Forward	CATCACAGGGGACTGGACT	63.89	697 bp
OPN-a Reverse	GGAAAGCTCGCTACTGTTGG	63.52	
OPN-b Forward	AGAGTCCAGATGCCACAGAG	63.87	396 bp
OPN-b Reverse	TATTGTCTCCCACCCTGCTT	63.51	

PCR conditions were optimized for annealing temperature and the reaction was carried out in Bio-Rad thermal cycler (T®-100). Separate reaction mix were kept to amplify *OPN* genes in couple of fragments, with *OPN*-a fragment with 697 bp and with *OPN*-b fragment with 396 bp length product. The resulted product was gel eluted and outsourced for sequencing by Sanger's method. In both cases, forward and reverse sequences were sequenced and final complete sequences were obtained in FASTA format along with respective ABI files containing chromatogram. Complete sequence of each gene was obtained by multiple sequence alignment technique using Clustal W method in MegAlign DNASTAR. Various insilico analysis was done using various bioinformatics tools. Such as SignalP (http://www.cbs.dtu.dk/services/SignalP/) used to predict signal peptide and its cleavage sites in amino acid sequence. TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) used to predict transmembrane helices based on Hidden Markov Model. DNASTAR Protean- used for protein primary structure prediction. PSI PRED (http://bioinf.cs.ucl.ac.uk/psipred/) used for protein secondary structure prediction. SWISS MODEL (http://swissmodel.expasy.org/) used for tertiary structure prediction.

Results and Discussion

Gene specific primers were used to amplify *OPN* amplified both PCR products of *OPN-a* (Fig. 1) and *OPN-b* (Fig.1) expected size of amplified products were visualized in 0.8 per cent agarose gel under gel documentation system. PCR products were sequenced by Sanger sequencing (Scigenome, Kochi) and sequences obtained were manually checked with corresponding chromatogram. Sequence alignment was done using Clustal W method to obtain complete sequence of *OPN* transcript. The full length cDNA sequence of Vechur OPN has been submitted to NCBI Genbank (Accession No. KU985440.1).

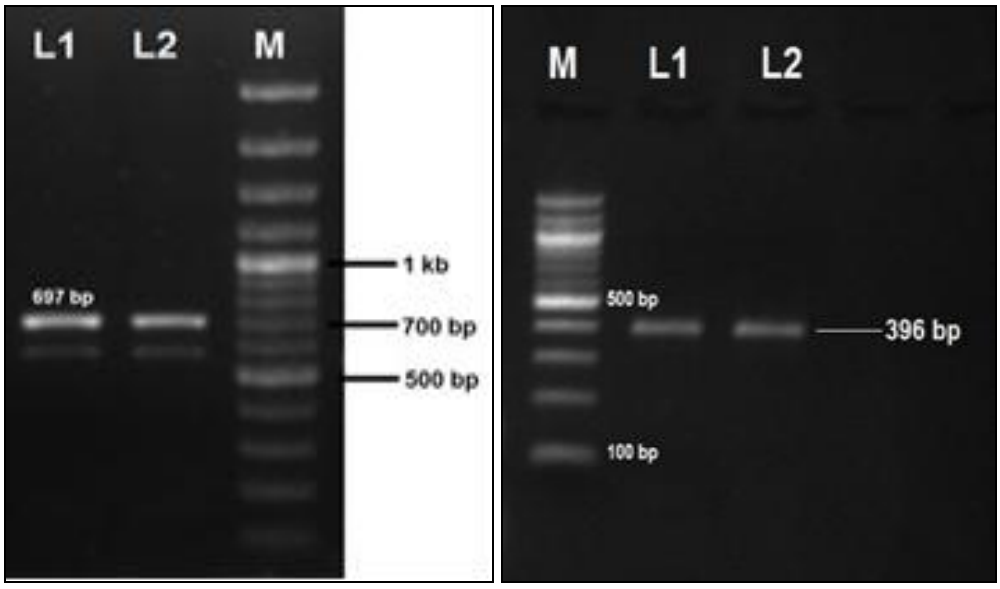

Fig 1: Gel demonstrating *OPN*-a (697 bp) and *OPN*-b (396 bp) with 100 bp DNA ladder (M)

Further sequence analysis was done using bioinformatics tools. Pair blasting of Vechur *OPN* sequence with reference sequence (NM_174187.2) showed 904 bp to be identical out of 909 bp subjected for blasting. Result of blasting uncovered five nucleotide transitions at coding region. Out of five, three novel transitions caused synonymous substitution and other two transitions caused non-synonymous substitutions. Sequence analysis of Vechur *OPN* gene revealed five nucleotide variations in coding region. Out of five (Table 2), two nucleotide variations caused non-synonymous substitutions and other three novel transitions caused synonymous substitutions.

Table 2. Nucleotide variation in Vechur *OPN* gene

Location	Position at reference sequence (NM_174187.2)	Nucleotide change	Consequences	Amino acid variation
cds	108	C > T	Synonymous substitution	
cds	188	T > C	Non-synonymous substitution	I > T 42
cds	229	A > G	Non-synonymous substitution	T > A 56
cds	378	C > T	Synonymous substitution	
cds	486	G > A	Synonymous substitution	

Similar results were reported on sequenced *OPN* from cDNA isolated from kidney of Korean native cow (*Bos taurus* x *Bos indicus*). Sequencing of *OPN* revealed 837 bp ORF in Vechur cattle, which was in accordance with similar studies [7]. OPN sequenced using cDNA from lactating mammary tissue in yak and found that *OPN* sequence contained an ORF of 837 bp coding for 278 amino acids [2]. OPN transcript characterized from buffalo mammary gland and reported an ORF of 842 bp coding for 280 amino acids [4]. Vechur *OPN* sequence showed 99 per cent homology with predicted *Osteopontin* transcript variant X1 (*Bos taurus*) and least similarity with *Capra hircus* sequence among ruminants.

Amino acid sequence and ORF was predicted using EditSeq DNASTAR, which exposed an ORF of 837 bp coding for 278 amino acids. Predicted protein sequence was blasted with available sequences in NCBI database, showed 100 per cent homology with predicted *Osteopontin* isoform X1(*Bos taurus*). Change in amino acid from isoleucine (non-polar) to threonine (polar) at 42nd position and threonine (polar) to alanine (non-polar) at 56th position of predicted amino acid sequence could impart variation in secondary and tertiary structure of Vechur OPN protein. Primary structures of the protein determine the nature of secondary structure and subsequent folding based on their charge and polarity. Primary structure of Vechur *OPN* protein was analysed using Protean DNASTAR software and presented in Tables 3 and 4.

Table 3: Predicted structural class of Vechur *OPN* protein

Parameter	Values
Molecular weight	30903.98 Dalton
Length	278 amino acids
Isoelectric point	4.35
Charge at pH 7	-31.26

Table 4: Vechur *OPN* whole protein composition analysis

Amino acid(s)	Number in count	% by weight	% by frequency
Charged (RKHYCDE)	106	43.49	38.13
Acidic (DE)	62	24.31	22.30
Basic (KR)	29	12.57	10.43
Polar (NCQSTY)	91	29.91	32.73
Hydrophobic (AJLFVW)	62	21.46	22.30

Predicted molecular weight and isoelectric point would be helpful in isolating this protein for proteomics study. Nature of OPN protein at normal animal body pH was found to be highly acidic in nature (-31.26). Studies stated that acidic nature of *OPN* protein played beneficial role in udder innate immune and adaptive immune mechanism [1]. Research finding suggested that OPN in milk acted as transporter molecule for lactoferrin (basic nature) by binding electrostatically and extend the antimicrobial activity of lactoferrin in milk [12]. Primary structure of protein showed highest per cent frequency of serine (15.11 per cent) amino acid. Studies found serine residues in *OPN* protein undergo post-translational phosphorylation which imparts the functional activity to this protein [11]. Aspartic (negative charge) stood next to serine in terms frequency of occurrence (12.59 per cent) thus contributing to acidic nature of protein. In terms of individual amino acid serine found to be with highest frequency of 15.11 per cent of total protein, followed by aspartic, glutamine and lysine which were 12.59, 9.71 and 8.27 per cent, respectively.

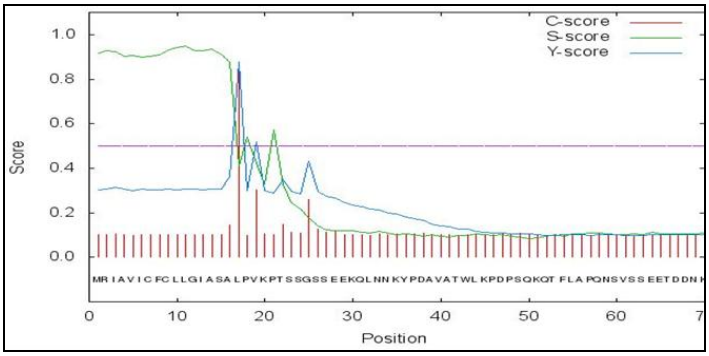

Fig 2: Signal peptide and cleavage site for predicted OPN protein

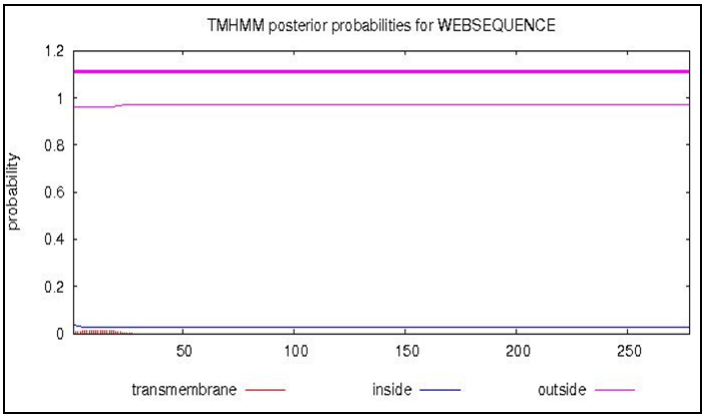

Fig 3: Transmembrane region in OPN protein

Signal peptide prediction exposed presence of signal peptide (green colour line) along with cleavage site (red colour bar) with probability of prediction in Y- axis and position of amino acid sequence in X-axis (Fig. 2). Length of predicted signal peptide was found to be 16 amino acids with cleavage site between 16th and 17th amino acid, consequently only 262 out of 278 amino acids formed mature protein. TMHMM prediction (Fig. 3) showed that OPN protein was seen completely outside the cell membrane, suggesting secretory nature of protein. Transmembrane prediction showed that complete sequence belonged to extra cellular region indicative of secretory nature of protein.

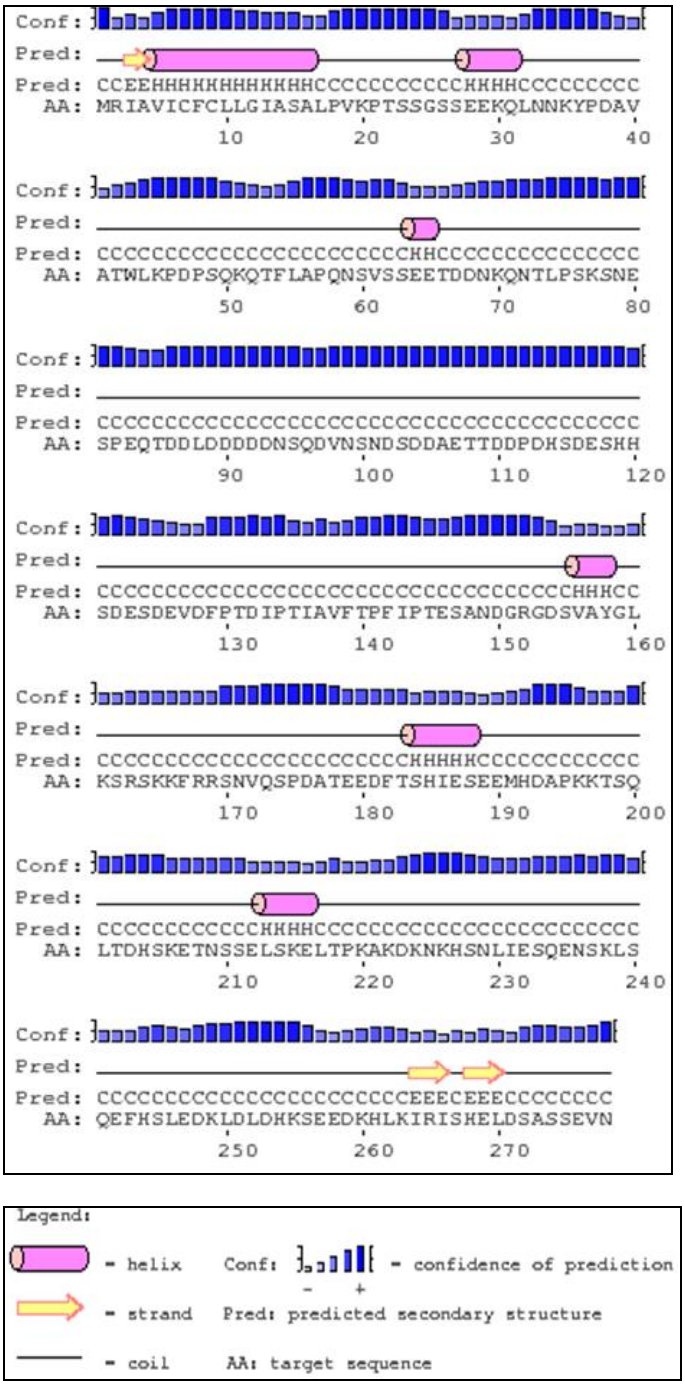

Fig 4: Secondary structure of predicted OPN protein

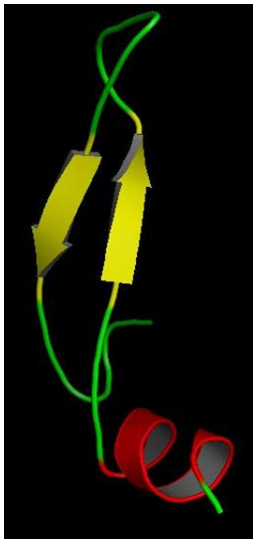

Fig 5: Tertiary structure of predicted OPN protein

Secondary structure was viewed using PSI PRED (Fig. 4) presented majority of protein belonging to random coil. Tertiary structure of protein was viewed in SWISS MODEL (Fig. 5) where red colour helix represented alpha helix, yellow colour arrow showed beta sheet and green colour indicated random coil of tertiary structure of OPN protein. Tertiary structure showed major portion comprised of random coil followed by beta sheet.

Conclusion

To our knowledge, this is the first study to sequence and analyze structural variations of *OPN* in Vechur cattle breed. The observed structural variants in the mRNA sequence of *OPN* gene in Vechur cattle breed will provide an important basis for further study on the relationship between polymorphisms and host disease resistance.

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