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Molecular characterization and in-silico analysis of the cDNA encoding conglutinin from sheep liver

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ABSTRACT

Conglutinin, known to be the high molecular weight serum lectin, appears to play an important role in bovine immune defense mechanisms, by binding to microbial surface carbohydrates thereby inducing aggregation and preventing the spread of pathogens. Though there is very limited information regarding the presence of this gene in small ruminant like sheep, also till date no study has revealed the sequence information of conglutinin in sheep. However the present study depicts the cloning and characterization of the cDNA sequence of 497bp encoding conglutinin (neck and carbohydrate recognition domain) from sheep for the first time and edited sequence of 488bp was submitted to NCBI with accession number JQ692170. Moreover sequence information revealed that nucleotide sequence of sheep conglutinin was 93.4% homology to the bovine conglutinin, 93.9% to buffalo conglutinin, 93.4% to nilgai conglutinin, 81.6% to CL–43 and 90.2% to CL-46 respectively; also liver is known to be the organ for the presence of this novel gene in this small ruminant for first time. In addition the present study also revealed the sequence analysis of the sheep conglutinin using DNA star, phylogenetic analysis using Mega 4.0.2 and secondary structure prediction by using online software like SWISS MODEL ProtParam.

Key words: Carbohydrate recognition domain, cDNA, Cloning, Conglutinin, Liver, Sheep

Conglutinin is a high molecular-weight lectin originally detected in bovine serum. It belongs to the family of collectins that binds to sugar residues in a Ca^{2+}-dependent manner and is effector molecules in innate immunity. Conglutinin appears to play an important role in ruminant immune defense mechanisms, by binding to microbial surface carbohydrates thereby inducing aggregation and preventing the spread of pathogens. Moreover, conglutinins are secretory pattern recognition receptors that can act as opsonins and recent studies have shown that it causes the destruction of microorganisms by stimulating phagocytic cells. Limited or scanty information is available regarding the presence of the conglutinin in other bovidae species like small ruminants and in other non-ruminants till date. Extensive studies are going on world wide for better understanding of the ruminant immune system in recent days and similar kind of studies to understand the immune system of the small ruminant like sheep are warranted. The present study aimed towards characterization of the cDNA encoding high molecular-weight lectin conglutinin (neck and carbohydrate recognition domain) from sheep liver and its sequence analysis.

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MATERIALS AND METHODS

Total RNA isolation and cDNA synthesis

Fresh sheep liver sample was obtained from the slaughter house; cold chain was maintained while collecting and transporting the tissue till it has to be further processed in the laboratory. About 50–100 mg of liver tissue sample was taken and sliced using a sterile scalpel blade and transferred to 1.5 ml microfuge tubes to that 1 ml of Trizol reagent was added. Tissue was homogenised using Potter-Eivehjem homogenizer in Trizol, followed by the total RNA was isolated as per the standard protocol (Sambrook and Russell 2000). RNA absorbance was determined at 260 and 280 nm to determine quantity and ensure integrity of extracted RNA. cDNA was synthesized using MuMLV reverse transcriptase in total volume 20 µl consisting of 5 µl respective total RNA (1–5µg/ µl), 1 µl of 12–15 mer oligo dT primer (0.5mg/ml),1 µl of RNase inhibitor (40U/ml), 2 µl of 10mM dNTPmix, 2 µl of DTT, 2 µl of 10X reverse transcriptase buffer, 1 µl of

reverse transcriptase enzyme (5U/ µl) and 6 µl of nuclease free water and incubated for 1 h at 37°C.

Polymerase chain reaction (PCR) to amplify sheep conglutinin (SCGN) gene from cDNA

PCR reaction was carried out using proof reading enzyme kit. PCR reaction was carried out using forward and reverse primers consisting of 5'-GGCTCGAGGGGGAGAGTGGGCTTGCAGA-3' and 5'-GGGAATTCTCAAAACTCGCAGATCACAA-3' respectively, which were established for bovine conglutinin (Wakamiya, United States Patent Dec. 27, 2005). The PCR reaction mixture of final volume 25 µl was made which consisted of forward and reverse primers (50pmol) 0.5 µl each; Template cDNA 1.0 µl; dNTP mix (10 mM) 1.0 µl; 5X Taq buffer (without $MgCl_2$) 5.0 µl, and KAPA polymerase 1µl. The final volume of 25 µl was made with nuclease free PCR grade water. The PCR was done as per the kit.

The PCR conditions employed initial denaturation at 95°C for 5 min, followed by 35 repeated cycles of 95°C for 45 sec, 60°C for 1 min, 72°C for 1 min. Final extention at 72°C for 5 min. The amplicon (492bp) along with the 100 bp DNA ladder were resolved by 1% agarose gel electrophoresis and the ethidium bromide stained gel was visualized in gel documentation system.

Cloning of IFN-α amplicon

The PCR amplicon (497bp) along with the 100 bp DNA ladder were resolved by 1% agarose gel electrophoresis and the ethidium bromide stained gel was visualized under long UV range light. The specific band corresponding to exactly PCR amplicons were cut and purified using gel extraction kit as per the manufacturer's instructions. PCR product so obtained was ligated to the pJET cloning vector using 2X ligation buffer (10.0 µl), insert (purified PCR DNA) 3–5 µl depending upon the concentration; (insert: vector molar ratio 3:1 gave optimum ligation efficiency), pJET-blunt end cloning vector (1µl), T4 DNA ligase [3U/µl (1.0µl). The volume was adjusted to 20 µl with nuclease- free water. The ligation reaction was carried out at 22°C for 30 min. The ligation mixture was transformed into *E. coli* DH5α competent cells using TSS protocol for screening recombinant colony containing the insert. Plasmids were isolated from recombinant colonies by using commercially available plasmid isolation kit as per manufacturer's guidelines.

Screening of recombinant plasmids pJET

To characterize the recombinant plasmid containing insert, the restriction enzyme like *Pst*1 was employed to release the insert fragments of 2600bp, 566 bp and 256 bp from the recombinant plasmid. The digestion mixture consisted of plasmid DNA (2–5 µl), 10 X RE buffer (2µl), *Pst1* 1µl (10U/ µl) and volume made to 20 µl with nuclease-free water, subjected to digestion for 4 h at 37°C. The insert release was analysed by 1.5% agarose gel containing ethidium bromide resolved along with the 100 bp plus ladder.

Sequence analysis

The recombinant plasmid was isolated from the culture with MDI plasmid miniprep kit as per manufacturers guidelines and was sequenced using T7 universal primer. After obtaining the sequence of sheep conglutinin, the neck and CRD of SCGN sequence BLAST analysis was done to confirm the gene of interest further it was aligned with the other CGN sequences available in NCBI public database using MegAlign (DNA star). Phylogenetic tree based on evolutionary distances, nucleotide substitution and identity with respect to other species constructed using (Mega 4.0.2). Secondary structure of SCGN was predicted from the amino acid sequences using SWISS MODEL ProtParam (Arnold *et al.* 2006).

RESULTS AND DISCUSSION

The PCR product of sheep conglutinin (neck and carbohydrate recognition domain) consists of 497 bp was well resolved using 1.5% agarose gel containing ethidium bromide along with the 100 bp plus ladder along with the insert release of 497bp (Fig.1. lane1 and lane 2). The restriction digestion of recombinant plasmid with enzyme Pst I which is having two sites in insert and one site in pJET frame releases the 2600bp, 256bp and 566bp fragments but non-recombinant will be linearised (Fig. 2). The recombinant

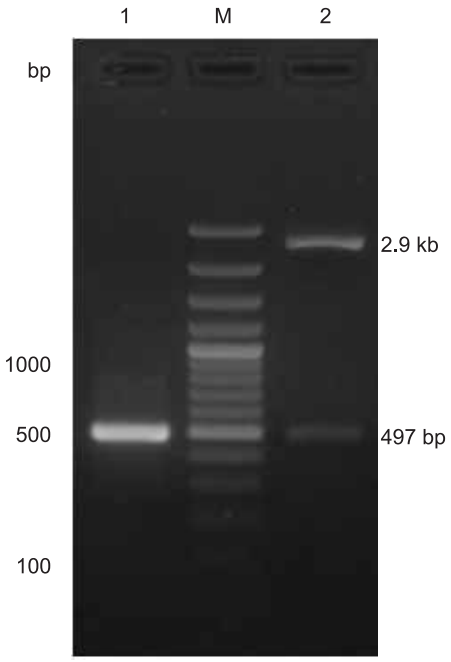

Fig. 1. PCR and restriction analysis of SCGN. Lane M : 100 bp DNA ladder; lane 1 : PCR amplicon of 497 bp; lane 2: insert release of 497 bp and pJET vector of 2.9 kb.

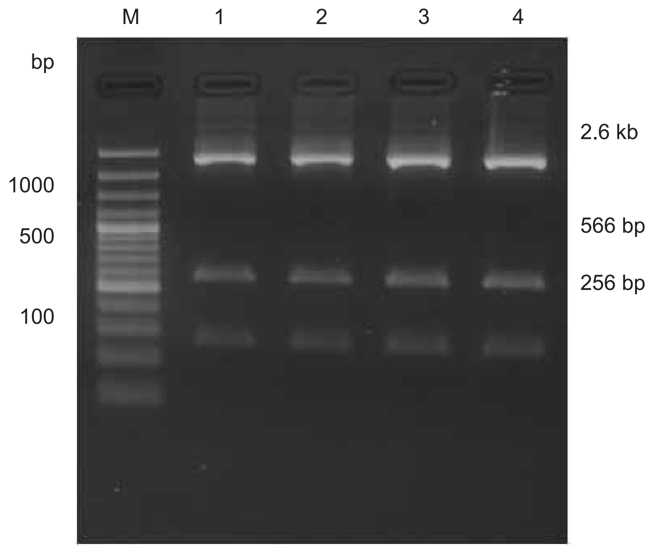

Fig. 2. Characterization of recombinant plasmid by RE Pst1. Lane M : 100 bp DNA ladder; lane 1–4 : Insert release of 566 bp and 256 bp from recombinant plasmid.

plasmids thus characterized were sequenced, upon sequence analysis; the obtained 488bp of (NCRD) of conglutinin partial sequence of sheep was submitted to NCBI Genbank with accession number JQ692170.

Though there are very limited information available on the sequence information regarding SCGN recent studies revealed that conglutinin is not only confined to the bovines but also exists in the other non ruminants (Dec *et al.* 2011) however in the present study the nucleotide alignment report of the sheep conglutinin with other lectins in *Bos taurus* and conglutinin in other bovidae species were depicted in Fig. 3. The sequence analysis revealed that the nucleotide sequence of sheep conglutinin was found to be 93.4% similar to the bovine conglutinin, 93.9% to buffalo conglutinin, 93.4% to nilgai conglutinin, 81.6% to CL-43 and 90.2% to CL-46 respectively (Fig .4) based on the inter-species homology determined by percent identity. This identity of the sheep conglutinin with the other ruminants is mainly attributed to the functionally conservedness of this novel protein in ruminant immune system. The phylogenetic information

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Alignment Report of Untitled ClustalW (Slow/Accurate, IUB)
29 May 2012 17:07
Majority            AGGGGGAGAGTGGGCTTGCAGAGGTCAATGCTCTCAAGCAGCGGGTGACAATCTTAGAGGGACATCTGCGACGCTTCCAGAATGCCTTCAGTCAGTATAA
                             10        20        30        40        50        60        70        80        90       100
O.aries CGN         ..........................................A..............................................TC......... 100
B.tragocamelus CGN ..........................................................T....................A.................... 100
B.bubalis CGN      ..........................................................T....................A.................... 100
B.taurus CGN       ..........................................................C....................A.................... 100
B.taurus CL-46      ......................C..........................C..............A..A.A....C..............TC..G...... 100
B.taurus CL-43     .........C.TCAG.CCT......TG..A....A.G.......A...G..A..........G.AG.T.A....C.......CATAG...C......C.G 100

Majority            GAAAGCGGTGCTCTTCCCTGATGGCCAGGCTGTCGGGGAGAAGATCTTCAAGACGGCAGGTGCTGTAAAATCATATTCAGATGCAGAGCAGCTCTGCAGA
                            110       120       130       140       150       160       170       180       190       200
O.aries CGN         ...C..........................................................TG..C.................CC............C. 200
B.tragocamelus CGN .......................................................A............................................ 200
B.bubalis CGN      .......................................................A............................................ 200
B.taurus CGN       ......................................................A..............G.............................. 200
B.taurus CL-46     .................................T...A........................................G.....CC.............. 200
B.taurus CL-43     .....................................................................G........G..................... 200

Majority            GAGGCTAAGGGACAGCTGGCCTCCCCACGCTCTGCAGCCGAGAACGAGGCCGTGACACAGATGGTCAGAGCCCAGAAAAAGAATGCTTACCTGAGCATGA
                            210       220       230       240       250       260       270       280       290       300
O.aries CGN         .............................................A..............T.............C...G.A................... 300
B.tragocamelus CGN ..................................A......A........T................................................. 300
B.bubalis CGN      ..................................A...............T................................................. 300
B.taurus CGN       .................................T.........................................G........................ 300
B.taurus CL-46     ......................................T...............G.....C...........A....C..TG......T........... 300
B.taurus CL-43     .................................T..........................C...........A....C...C.................. 300

Majority            ATGACATCTCCACGGAGGGGAGGTTCACTTACCCCACTGGGGAAATACTGGTCTATTCCAACTGGGCCAATGGGGAGCCCAACAACAGCG---ATGATGG
                            310       320       330       340       350       360       370       380       390       400
O.aries CGN         ............................C..............................T.........C....................---....... 397
B.tragocamelus CGN ......................................................................A...................---....A.. 397
B.bubalis CGN      ......................................................................A...................---....A.. 397
B.taurus CGN       ....................................................................G...................T.---..G.... 397
B.taurus CL-46     ...................C.A......C........G.....GTC.......................G.................A.A---...C... 397
B.taurus CL-43     ............AA.....C.A......C.....A..G....GGTC.....A................CCC..............T..G.CAA.A..C.A 400

Alignment Report of Untitled ClustalW (Slow/Accurate, IUB)
29 May 2012 17:07
Majority            ACAACCAGAGAACTGTGTGGAAATCTATCCTGAGGGCAAGTGGAATGACGTACCCTGCAGTAAGCAACTCCTTGTGATCTGCGAGTTTTGA
                            410       420       430       440       450       460       470       480       490
O.aries CGN         ..........................C..........C...........A......................C..................        488
B.tragocamelus CGN .........................C......................T..........................................        488
B.bubalis CGN      .........................C......................T.......................C..................        488
B.taurus CGN       ..........................T......T.........................................................        488
B.taurus CL-46     ...................C.G.......GG..............................G...C.........................        488
B.taurus CL-43     .GGC............C.T..G......T.C..T.....T.........A..GAA.....GG..G.G.G...C........T.....C...        491

Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

Decoration 'Decoration #2': Hide (as '.') residues that match the Consensus exactly.
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Fig. 3. Alignment report of SCGN nucleic acid sequence by clustalW.

Percent Identity

	1	2	3	4	5	6	
1		93.4	93.9	93.4	90.2	81.6	1
2	6.9		99.6	96.5	90.0	82.2	2
3	6.4	0.4		96.5	90.0	82.6	3
4	6.9	3.6	3.6		89.8	83.4	4
5	10.6	10.8	10.8	11.0		83.6	5
6	21.2	20.4	19.9	18.8	18.5		6
	1	2	3	4	5	6	

1 O. aries CGN
2 B. tragocamelus CGN
3 B. bubalis CGN
4 B. taurus CGN
5 B. taurus CL-46
6 B. taurus CL-43

Fig. 4. Nucleotide homology of SCGN gene with other species.

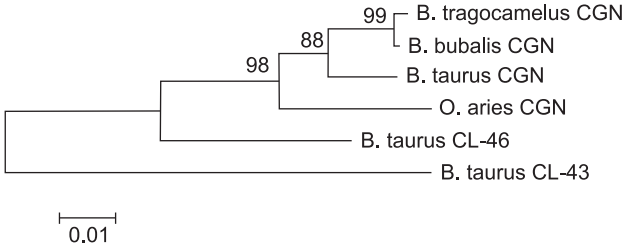

Fig. 5. Phylogenetic tree constructed upon the alignment of SCGN nucleotide sequence.

derived on the basis of nucleic acid sequences revealed that independent cluster for large ruminants like buffalo and nilgai have formed also separate cluster of SCGN between bovine conglutinin and CL-46 indicating early divergence of SCGN in ruminant species (Fig.5). Predicted protein sequence of sheep conglutinin (partial) using DNA star revealed approximate molecular weight of 17372.91 daltons consists of 162 amino acids (25 strongly basic (+) (K,R), 5 strongly acidic (-)(D,E), 35 hydrophobic A,I,L,F,W,V) and 56 polar (N,C,Q,S,T,Y) amino acids). The functional activity of the conglutinin in recent days in ruminant immune system also reveals the production of the reactive oxygen species which is known to be one of the major antimicrobial properties existing with it (Dec *et al.* 2012). However predicted three-dimensional structure of SCGN using nucleotide sequences by online software like SWISS MODEL ProtParam depicts that structure of SCGN consists of 6 beta sheets (Arnold *et al.* 2006) (Fig.6). Moreover the present study is going to be the first report on the presence of this versatile novel gene in the small ruminant like sheep, also though the liver was known to be the organ for the presence of the conglutinin gene in *Bos taurus* (Hansen *et al.* 2002). It is the for the first time similar findings have been noticed in the present study for the presence of the conglutinin gene in the liver of the sheep. However present study also dictates the sequence information regarding the sheep conglutinin and its comparison with existing conglutinin and other collectins

Fig. 6. SWISS MODEL- predicted structure of sheep conglutinin (Arnold *et al*. 2006).

confined to bovines, will pave way to carry out in detail study on the structural, functional and other immunomodulatory property of this versatile protein in small ruminants like sheep, in order to have better understanding of the immune system of this small ruminant.

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